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(Acta Zhengzhou University Oversea Version)

Life Science Journal, the Acta Zhengzhou University Oversea Version, is an international journal with the purpose to enhance our natural and scientific knowledge dissemination in the world under the free publication principle. The journal is calling for papers from all who are associated with Zhengzhou University – home and abroad. Any valuable papers or reports that are related to life science—in their broadest sense—are welcome. Other academic articles that are less relevant but are of high quality will also be considered and published. Papers submitted could be reviews, objective descriptions, research reports, opinions/debates, news, letters, and other types of writings. Let's work together to disseminate our research results and our opinions.

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Preface

Changyu Shen

President, Zhengzhou University, Zhengzhou, Henan 450001, China

Through the joint efforts of Zhengzhou University and the North American Alumni Association of the University, the Acta Zhengzhou University Overseas Version ***Life Science Journal*** is being made available to all readers. This journal not only offers an interface between all scientists in the life sciences fields, but also opens an academic window to the world for Zhengzhou University. Furthermore, the journal will become a linkage between alumni of Zhengzhou University abroad and their alma mater.

In reviewing social history it becomes evident that human beings have always been involved in exploring the origins both of material substance and of life. These explorations have been in the forefront of scientific progress, and technological innovations that bring about revolutionary changes in human society stem from principal breakthroughs in the basic sciences. While in the 20th century developments in the physical sciences took leading and dominant roles in scientific development, in the 21st century the core of scientific progress may gradually shift to the life sciences. The exploration of life includes the discovery of genetic material and the birth of molecular biology, which advances provide humans with opportunities for understanding the origin and mystery of life. Such work also gives rise to promising developments in biological engineering technology. The atlas of the human genome is absolutely an important milestone in the history of the life sciences. Within the context of these developments, the establishment of ***Life Science Journal*** will provide an additional arena for life science research.

Life Science Journal is an interdisciplinary and international journal, and will publish academic theses, research reports, academic critiques and reviews. The journal will report on the newest tendencies, academic trends, and research achievements in the life sciences both in China and abroad, and will serve as a medium of communication between Zhengzhou University researchers and those in other countries of the world.

There are no limitations and no borders for science. Moreover, as the Chinese saying has it, "The mountain, only by not rejecting any dirt becomes so high, and the ocean, only by not refusing any water, becomes so deep." In comparable fashion, we strongly believe that through our hard work, the ***Life Science Journal***, by promoting the progress of the life sciences, can play its role in revealing the mysteries of life, and in contributing to the health of human beings and the welfare of society.

Life and Immortality: A Comparison of Scientific, Christian, and Hindu Concepts

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Abstract: In this paper conceptions of immortality provided by two of the world's leading religions, Christianity and Hinduism, are critically compared with each other, as well as with the possibilities of atomic immortality implied by modern physical science, with the aim of ascertaining how these religious and scientific views may or may not be compatible or mutually supportive. [Life Science Journal. 2005;2(1):2-6] (ISSN: 1097-8135).

Keywords: atomic science; Christianity; Hinduism; immortality; life

Everything in earthly existence, including human life in all of its facets, is involved in a process of ongoing change. Hence, permanence seems unattainable, and thereby especially desirable. The wish for immortality thus becomes one of the most important original reasons for the appearance of religions, and the motives of many scientific research fields can also be traced to this motive. Since very ancient times humans have wondered if after their deaths in this world they might continue to exist forever in some next and unchanging condition. My purpose in this paper is to contrast a modern scientific conception of the possibility of human immortality with some more traditional religious views of the same.

Scientific discoveries made during the last two centuries have determined that the fundamental building blocks of the physical universe as we now find it consist of submicroscopic "atoms", each of which in turn has a complex inner structure of elementary "particles" and "forces" (Brennan, 1992). A further solidly established scientific determination is that since the establishment of atoms during one stage of cosmic evolution they have been involved in a universal process of rearrangement. Thus individual humans in their physical aspect are aggregates of atoms that formerly and variously constituted elements of other creatures or objects, and upon the death and decomposition of humans' bodies, their atoms, rather than simply dissipating into nothingness, become parts of subsequent creatures or objects, and so forth indefinitely. In this sense deceased humans achieve an "atomic immortality".

However, atomic immortality is theoretically problematic in view of alternative scientific anticipations of future cosmic evolution. On the one hand, astronomers anticipate that if the average density of matter in the universe should prove to be less than a certain critical value, the expansion of the physical universe that began some fifteen billion years ago with the explosion of a point of infinite heat and density (the Big Bang) will continue to infinity ("open universe") (Barrow, 1991; Gribbin, 2000; Morris, 1993). Science anticipates that within an "open universe" the stars (including the earth's sun), of which receding galaxies are composed, will eventually burn themselves out, the universe will lapse into a cold darkness, and life on the planet earth will cease. In such case, the atoms of former human bodies could presumably continue as components of the dead earth, and human physical immortality might thus in a sense be sustained. However, the expectation that our dead earth will continue to be involved in an infinite cosmic expansion, thereby preserving that immortality, can for living persons never be more than an unverifiable anticipation, for the one reason that no persons would remain alive during the later (dead earth) phases of that expansion in order to verify it, as well as for the more general reason that any infinite expansion *ipso facto* denies the possibility of its own confirmation (that is, since infinity can never exceed itself, by the same token it can never be exceeded by a confirming observer "beyond" itself).

Scientific anticipation holds, as another possibility, that if the density of matter in the universe should prove to be greater than the critical value, gravitational forces will eventually cause the expansion of the universe to reverse itself and come to an end in an awesome implosion (Big Crunch) (Greene, 2003). If the Crunch occurs it would

mean that the universe is finite ("closed universe"). In the case of the Crunch the atoms involved in the expansion of the universe would presumably be transformed and compressed back into a pre-atomic point of infinite density of the sort that led tothe Big Bang. The radical transmutation of matter-energy involved in the Crunch would presumably disallow retention of any identifiable remnants of given human bodies; hence it would be extravagant in this case to maintain that the atoms that had provided persons with an extension of their existence beyond their deaths would provide true immortality. What would transpire after the Crunch is unknown, although some astronomers have conceived the possibility of a series of Big Bangs and Big Crunches, and therewith the evolution and devolution of a series of universes, or of one "oscillating universe" (Jastrow, 1992). But in the case of transformations of universes back into points of infinite heat and density, it is difficult to imagine that any atoms that had once been parts of human bodies within a given universe could survive the changes from one universe to the next; thus in the scenario of an oscillating universe human immortality would be denied.

Nonetheless, anticipations of atomic immortality are presumably meaningful to some people. Yet it seems probable that such concepts are insufficiently satisfying to most people, who need to anticipate an immortality involving not only, in one or another fashion, their post-mortem physical survival, but also the survival in some sense of the consciousness of self which they experience while alive. Such survival is absent from atomic immortality, which may involve some of the atoms of a deceased person's body being recycled as components of other living persons' bodies, yet without involving any synchronous transmigration of the first person's consciousness. Understandably, then, humans throughout their history have developed religious beliefs that accommodate the anticipation of a continuing post-mortem consciousness. To exemplify the multitude of religious beliefs, past and present, the present discussion will focus on the doctrines of two of the world's major living religions, Christianity and Hinduism.

Christianity holds that one eternal God (Jehovah) created the universe, including all of its forms of life. Although regarded as a spiritual being, this God is nonetheless conceived of as anthropomorphic, typically as Caucasian, and as having male gender (as the Christian scripture, the *Bible*, puts it: "God created man in his own image.") (The New English Bible, 1971). Christian doctrine is strikingly different in a number of ways from scientific conceptions of being. Scientific knowledge, however imposing it may be, remains teleologically neutral, affording no insight into any ultimate purpose, or lack thereof, of the universe. But Christian doctrine holds that God had a purpose in creating the universe, whereby it is concluded that the universe itself, including the lives of humans, also serve an ultimate purpose. With respect to God's own purpose, Christians maintain that he freely created the universe, not from any necessity of self-completion, but out of love for the humans who are parts of that creation, while the fundamental purpose of human lives is to love God in return, out of gratitude for their existence.

Christianity maintains that God, as part of his continuing creative activity, adds to each new human body an immaterial "soul", inclusive of "mind", which latter is the seat of a person's consciousness. After the first two humans were created, the subsequent chain of human sexual reproduction has been less a matter of direct causation by God than simply a result of humans acting in accordance with the biological "natural law" established by God; however, the addition of souls to new persons (at their moments of conception in their mothers' wombs) remains a direct and purely divine action. Whereas a strictly materialist conception of existence does not deny individual human consciousness as an attribute of a living person's "mind", it holds that mind is no more than an exceptionally complex manifestation of a person's physiology; "mind" reduces, as it were, to physiology, and physiology reduces to atoms, and so there is no mind or consciousness as a reality in addition to body. One might suppose that if consciousness is thus merely an effect of a person's bodily atoms, then the particular atoms of a given person's body that subsequently become components of other persons' bodies would transfer at least some part of the first person's consciousness to those other persons. However, the consciousness-qualities of such transferred atoms become newly and differently operative when incorporated into a new and different whole person, else the collective effect of any given person's accumulated atoms could only be that of an incoherent melange. That is, on strictly materialist terms any given person's consciousness is the result only of a particular, coherent, and unique assemblage of atoms that are melded into a nontransferable consciousness of "self". This situation is reminiscent of the fact that atoms of themselves, in separation from living organisms, have no "life". Whether or not life constitutes (in accordance with the biological philosophy of "vitalism") a stratum of being in excess of the

sums of physical atoms comprising bodies, it remains that atoms manifest life only as constituents of such bodies. As for Christian doctrine, even if it may grant that persons' bodies of themselves are composed of temporary combinations of universally similar atoms, it maintains that each person's soul is an extra-atomic and unique element, which provides each person with a self-consciousness-retentive immortality (Attwater, 1961).

Christians anticipate that humans will experience that immortality either in a paradisiacal realm—a place or state of being—for those persons having led lives acceptable to God, or in a realm of suffering for those who have not. Leading lives acceptable to God requires beliefs and moral behavior in line with certain injunctions, which, it is maintained, God has revealed to humans and which are recorded in the *Bible*. However, it is further asserted that God endows humans with free will, whereby they can choose to obey or disobey those injunctions. According to the Christian historical scenario, in the past many humans indeed disobeyed the injunctions, whereupon God sent his incarnated son Jesus to earth to undergo a sacrificial death by torture in atonement for humans' sins of disobedience. It is genuine belief in the reality and purpose of this sacrifice, plus true repentance for one's sins, that qualifies a person to receive God's saving "grace" (mercy) and thereby gain access to the preferable kind of immortality (Chamberlin & Feldman, 1961).

According to Christian doctrine it will be at the time of a "Last Judgment" that God, in the aspect of his resurrected son Jesus, will effect the consignment of individuals either to heaven or hell. In conjunction with the Judgment God will reanimate the material bodies of deceased persons as those bodies were at their point of physical prime while on earth, to be joined with their souls before entry into one of the supernatural realms. At the close of the Judgment God will bring to an end the physical universe of human earthly experience. And just as God existed eternally before his creation of the realm of human earthly experience, so he will continue to exist eternally after its annihilation, while the conjoined and individually identifiable bodies and souls of humans will also endure forever in one or the other of the supernatural realms (Smart & Hecht, 1982).

If God in his omnipotence intentionally created humans with the capacity freely to obey or disobey his injunctions, one can only assume he realized that some humans would disobey them, and thus have their reconstituted bodies consigned to punitive torture in hell. Relevant to this consideration, and to paraphrase a famous statement made (in another context) by Albert Einstein, "God (by which Einstein meant simply a supreme order in nature) does not play dice with the universe". However, it seems the anthropomorphic Christian God does indeed play dice in the case of humans with their free will, as he has endowed them with even odds for morally ruining themselves with devastating effect. In a related consideration, human earthly experience often involves suffering, and if asked why God, who is regarded by Christians as both all-powerful and loving, allows this to happen, Christians contend that without the challenge of retaining, through tests of suffering, their reciprocal love for God, that love would be merely trivial and vacuous.

Referring again to atomic immortality, science may someday achieve the great success of satisfying human curiosity concerning the components and mechanisms of the physical universe, and therewith better clarify the possibilities of atomic immortality. But, again, there is no reason to expect that science would thereby afford any clue as to a final purpose of the universe – or that it would yield any rationalizations wherewith to justify the sufferings of living humans – or that it would provide people with the dramatic stimulus of being engaged in a moral struggle to achieve a full-body and consciousness-retentive immortality – whereas Christian belief provides all of these benefits.

It is instructive to consider a contrasting religious perspective provided by Hinduism. The latter includes an exceptionally rich abundance of diverse and often conflicting beliefs, and its discussion here must be restricted principally to the doctrines of one of its many schools, that of *Advaita Vedanta* (Isayeva, 1993). The fundamental concept of this school is that of *brahman* as the uncreated and eternal ground of all being. *Brahman*, like the Christian God, is the spiritual and generative source of the universe, including all of its forms of life. But unlike the anthropomorphic, Caucasian, and male Christian God, *brahman* is regarded as amorphous, racially indistinct, and gender neutral. While undetectable as such to human sensory perception, *brahman* nonetheless thoroughly pervades all things; the individual objects and creatures of the world as humans empirically perceive these are actually only transient manifestations of the intangible and unifying *brahman* itself. Moreover, *brahman* encompasses not only the things and creatures of this world, but also an extensive roster of gods. Yet the *Advaita* viewpoint ("*Advaita*" means "non-duality") is conceived of as monistic, as the gods are regarded merely as personalized re-

flections of various aspects and activities of the one supreme and unifying reality of *Brahman* (Menon & Allen, 1960).

The concept of *brahman* as all pervasive involves the corollary that *brahman* is the fundamental reality of each individual person's "soul", or "self", erson, the *atman*, like the Christian soul, never dies. However, unlike the Christian soul, the *atman* becomes joined with a succession of temporary bodies in the course of an indefinitely long series of bodily births and deaths. Thus, another feature of *Advaita* (which it shares with other branches of Hinduism) is the belief in reincarnation, the cycles of which are known as *samsara*. It might appear that the Hindu concept of reincarnation is congruent with scientifically conceived atomism, insofar as the latter envisages the cosmic recycling of atoms, some of which pass from living body to living body. However, not only is the reincarnation of the Hindu *atman* a metaphysical process, but unlike the fortuitous destinations of recycled material atoms, any one transmigration of a given *atman* proceeds only from one specific person to another.

Moreover, totally unlike mere physical atomic recycling, Hindu reincarnation is intimately bound up with morality. As the *atman* is joined time and time again with a new body, the qualitative experience of any one of its incarnations is determined by *karma*, which is the cumulative record of the thoughts and actions, morally considered, of the individual *atman's* series of lives. Hinduism embraces a conception of the right way of living (*dharma*), and the ways in which a person's behavior in his or her past incarnations adhered to or deviated from the precepts of *dharma* generated good or bad *karma*. The moral quality of a living individual's own actions supplement his or her received *karma*, and the new total is embodied in the person's next incarnation and determines the initial quality of that next life. At the end of any one of an *atman's* incarnations, the body of that incarnation reverts to a nonspecific, unmanifested aspect of *brahma* (Deutsch, 1968).

If, over a (usually extensive) number of reincarnations a person's accumulation of good *karma* is sufficient, the cycles of *samsara* can be brought to an end. Desires motivate human actions, which in turn produce *karma*, but *karma*-producing desires result at bottom from a person's sense that he or she is ultimately a separate entity left alone to pursue egocentric cravings. But if a person gradually and profoundly comes to grasp the identity of *atman* with *brahman*, then the person's *karma*-producing desires are stilled, whereupon the person's *atman* is released from *samsara*, an achievement that constitutes liberation, or *moksha*. Following the attainment of *moksha*, and the subsequent death of a person's last physical body, the person's *atman* continues to exist forever as a supramundane component of *brahman*. By contrast with human experience within *samsara*, *moksha* provides a new state of continuous serenity, within which individuals enjoy immortality. In Hinduism there is no state or condition of hell as a counterpart to *moksha*. In the Hindu view enlightened people achieve *moksha*, while unenlightened people, whose bad actions produce bad *karma*, simply continue to undergo repeated, and sometimes qualitatively worse reincarnations, until such time as they may reverse the process and eventually become themselves enlightened (Klostermaier, 1989).

The Hindu concept of immortality is different in a number of other ways from the Christian concept. *Moksha* is a purely spiritual liberation that entails no equivalent of Christian reconstituted bodies. In addition, while Hindu immortality involves, as in the Christian case, the retention of consciousness, in the Hindu conception that consciousness expands from a personal to a universalized consciousness. The self-consciousness of the *atman* at the level of bodily involvement is elevated to identity with the universal consciousness of *brahman*. The Christian belief is that persons who achieve immortality retain their personal identities while coming directly to see and know God. Yet such persons even in their new supernaturally enlightened apprehension of God do not become *identical with* God, nor do they ever comprehend *exhaustively* the ultimate mystery of God. In a sense, then, the achievement of *moksha* may be the more profound and comprehensive immortality, since persons who attain *moksha* are thoroughly at one with *brahman*, with ultimate reality beyond any distinction or disjunction between divine and human.

Within the compass of the foregoing discussion, what can be said, in sum, about anticipations of human immortality? All persons can expect as inevitable the attainment of "atomic immortality," which, however, cannot be expected to provide people with an immortality that is consciousness-retentive. The promise of the latter is characteristically an aspect of religious belief. But this recognition immediately poses the major problem of there being no objective basis for asserting the validity of any one religious anticipation of immortality rather than any other. Devotees of given religions—Christianity, Hinduism, and many others—typically regard their mutual confirmation of the beliefs they hold in common as evidence that those beliefs are true. But this is not the universal interpersonal validity of many objectively established scientific findings, and the world's religious groups remain splintered among them-

selves and parochial in their doctrines. From which circumstance it follows that the only objective fact revealed by the long human historical record of conflicting religious beliefs is that of the psychological occurrence of those beliefs.

To focus these issues from a somewhat different angle, today there are scientifically educated Christians, Hindus, and devotees of other religions, who recognize the validity of atoms as fundamental constituents of the physical world (who can deny, in the face, for example, of atomic power plants and atomic bombs, the validity of atomic science?). But while the reality of atoms—and therewith atomic immortality, such as it may be—appears to be a necessary part of any explanation of existence, the question remains whether it is a sufficient explanation of reality. For persons who believe in the validity of *samsara* and *karma*, or in the reality of Christian indestructible souls, or in any of a further extensive assortment of religious concepts, atomism of itself is obviously not a sufficient explanation of the full fabric of being (actually many prominent physicists have believed, on a personal mystical basis, that existence in its broadest terms is determined by forces that are transcendental or spiritual in nature) (Wilber, 1985). Of course, comprehensive but mutually contradictory versions of the fundamental attributes of universal existence – including the eschatological scenarios of various religions, as well as the stance of a rigorous materialism that excludes such scenarios—cannot alike be true, although if any one comprehensive version if indeed true, it is *ipso facto* true for all persons, as we all share one and the same universe.

I realize that numerous persons endure the frequent and sometimes tragic frustrations of their life experience with courage and poise as a consequence of their religious faith of whatever variety. Surely such faith typically—and understandably—receives its greatest impetus from anticipations of immortality, since these provide a sense of reassurance that the adversities which people experience in their earthly lives amount to more than a random absurdity, as these adversities will be justified and compensated for in a future and better existence. Having these realizations in mind, I submit merely that **on the basis alone of objective, nonsectarian comprehension**, perhaps the best any person can do is to hope that humans are destined to experience a (preferably benign) immortality, although the exact cosmic status and specific characteristics of that immortality remain, for persons still in this life, totally unsearchable. Simple hope may emotionally be a poor substitute for some firm and elaborately conceived religious-doctrinal conviction, but such hope does not necessarily imply its own futility.

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Nature of Life

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Abstract: Life is a physical and chemical process. From ontology aspect, the world is timeless and the life exists forever as any other body in the nature. The nature of life is that life is a process of negative entropy, evolution, autopoiesis (auto-organizing), adaptation, emergence and living hierarchy. Up to now, there is no scientific evidence to show that life body and non-life body obey the same natural laws. But, all the researches are made by the methods of biology, biochemistry and molecular biology, etc. It is very possible that the life and non-life are essential different in the biophysics, i.e. the quantum level. In the future, it is possible to make artificial life by either biological method or electronic technique. [Life Science Journal. 2005;2(1):7-15] (ISSN: 1097-8135).

Keywords: entropy; evolution; existence; life; nature

Contents

1 Introduction

Life is unique in the known universe, which is in a diversity of forms ranging from bacteria to human. The life organisms exist in everywhere of the earth. The first forms of life on earth spontaneously arose out of a preexisting prebiotic chemical soup. Individual living organisms maintain their self-identity and their self-organization while continually exchanging materials and energy and information with their environment. It is really different between the life and non-life bodies, but nobody knows what the exact difference it is, even this is one of the most important issues that attracted people in the whole human history. There are millions of people working in life science researches, many with Ph. D. degree. More money has been spent in the life science studies than that spent in any other fields. Nature, Science, and other big journals published more papers in life science than the papers in any other topic. But, there are very few people thinking about the nature of life. This topic has attracted thinkers since the beginning of human history, but ignored by the modern society. Most philosophers ignore the issue today, perhaps because it seems too scientific. At the same time, most scientists also ignore the issue, perhaps because it seems too philosophical. The nature of life is not clear for the current intelligence. It is a topic of philosophy, and also of biology (Bedau, 2005). However, it is very difficult to get financial support for the study of nature of life.

2 Definition of Life

It is difficult to give an exact definition for the life, as the nature of life is not clear. As the references, here I give the definition from some dictionaries:

(1) Spiritual existence transcending physical death; the period from birth to death; the quality that makes living animals and plants different from dead organisms and inorganic matter. Its functions include the ability to take in food, adapt to the environment, grow, and reproduce (Encarta® World English Dictionary, 2005).

(2) The condition that distinguishes animals and plants from inorganic matter, including the capacity for growth and functional activity (Compact Oxford English Dictionary, 2005).

(3) The property or quality that distinguishes living organisms from dead organisms and inanimate matter, manifested in functions such as metabolism, growth, reproduction, and response to stimuli or adaptation to the environment originating from within the organism (Dictionary.com, 2005).

3 Essential Conceptions of Life

The biological world is viewed as a hierarch of levels. These levels include chemicals, organelles, cells, organs, organisms, and ecologies. There are three conceptions for life: as a loose cluster of properties, a specific set of properties, and metabolization. There are many other opinions of life, such as that life is something of autopoiesis and self-replication, etc. Several hundred years ago, people thought that there was a vitalism inside life bodies that keep the body to be a life. The scientific results absolutely denied the existence of vitalism. The demise of vitalism told us that no super physical substance or force or spirit to distinguish any life from non-life. For all we know, all life phenomena obey to all the natural laws (physical and chemical) that adapted to the non-life world. There is no any extra natural law for the life world only. Life is no more unified than a collection of overlapping properties from overlapping disciplines, such as, biophysics, biochemistry, molecular biology, genetics, evolution, ecology, cytology, microbiology, physiology, anatomy and heredity, etc. However, the biophysics is poor result.

Farmer and Belin listed eight characteristics of the life: process, self-reproduction, information storage of self-representation, metabolism, functional interactions with the environment, interdependence of parts, stability under perturbations, and the ability to evolve. According to Farmer and Belin, life is a pattern of spacetime, rather than the specific identities of the atoms (Farmer, 1992).

Taylor described the properties of life: "Each property by itself, even when considered with others, is unable to clearly delineate the living from the non-living, but together they do help to characterize what makes living things unique." (Taylor, 1992).

Monod listed three characteristics of life: teleonomic or purposeful behavior, autonomous morphogenesis and reproductive invariance (Monod, 1971). Crick focused on the points related to: self-reproduction, genetics, evolution and metabolism (Crick, 1981). Kuppers pointed life as: metabolism, self-reproduction and mutability (Kuppers, 1985). Maynard Smith gave life two properties: metabolism and parts with functions (Maynard, 1986), and Ray cited two aspects: self-reproduction and the capacity for open-ended evolution (Ray, 1992).

Mayr thought that the process of living could be defined by a list of the kinds of characteristics by which living organisms differ from inanimate matter: (1) All levels of living systems have an enormously complex and adaptive organization. (2) Living organisms are composed of a chemically unique set of macromolecules. (3) The important phenomena in living systems are predominantly qualitative, not quantitative. (4) All levels of living systems consist of highly variable groups of unique individuals. (5) All organisms possess historically evolved genetic programs which enable them to engage in teleonomic processes and activities. (6) Classes of living organisms are defined by historical connections of common descent. (7) Organisms are the product of natural selection. (8) Biological processes are especially unpredictable (Mayr, 1982). (9) Life is continuum. (10) All life organisms are programmed to death naturally, which is called apoptosis (Ma, 2005b).

Schrödinger persisted that the second law of thermodynamics plays key role in the process of metabolization. The following sentences give his opinions: What is the characteristic feature of life? When is a piece of matter said to be alive? When it goes on doing something, moving, exchanging material with its environment, and so forth, and that for a much longer period than we would expect an inanimate piece of matter to keep going under similar circumstances. How does the living organism avoid decay? The obvious answer is: By eating, drinking, breathing and assimilating. Linguistically, the scientific term of life is metabolism. The essential thing in metabolism is that the organism succeeds in freeing itself from all the entropy (Schrödinger, 1969).

4 Life in the Timeless World

As it was described in another paper "The nature of time and space": From the ontology (or naturalism) angle, time and space are absolute (existed) and the universe is a timeless world, which means that all the past, the present and the future exist eternally. Everything in the universe will never change. Time and motion are nothing more than illusions. In the universe, every moment of every individual's life—birth, death, and anything in be-

tween—exists forever. Everyone is eternal. That means each and every one of us is immortal. The universe has neither past nor future. All the things in the past, present, and future exist forever. The concepts of past, present and future are depended on the human brain (Ma, 2003). Life is something (substance) existing in the timeless world. So that, all the life processes are the simple existence of something in the universe, like a movie in a tape, exist already and forever. This is the essential nature of life, in the ontology point. Under the timeless principle, there is only existence in the universe, not something complexity and other thing simplicity. The life is not more complex than non-life from the ontological concept. However, in the timeless world, there are natural connections among the all the existence. All the scientific studies, philosophical ratiocinations and religious believe are the trial to reveal the natural rules.

5 Life as Negative Entropy

The second law of thermodynamics was formulated in the middle of the last century by Clausius and Thomson, which could be formulated in four different ways: (1) Heat cannot flow from a colder body to a hotter one without energy input; (2) Entropy must increase in a closed system; (3) No cyclic process can convert heat entirely to work; (4) In any cyclic process the heat Q transferred to the system from its surroundings at the temperature T must obey an inequality: $\oint dQ/T < 0$ (Ma, 2003). Above the four points, the principle concept of the second law of thermodynamics is to say that in the closed system all the natural processes increase entropy (decrease order). So, the second law of thermodynamics can be called the entropy law or law of entropy. However, life violates second law of thermodynamics. In natural world, life process is negative entropy one. In the life process, the entropy decreases, which means that the order increases. More importantly, there is no evidence to say that the entropy decrease of life costs by the entropy increase of environment. The conclusion is that the life process does not obey the second law of thermodynamics. For all we know that all life phenomena obey to all the natural laws that adapted to the non-life world. How can we say that life violates the second law of thermodynamics? Is there any conflict? The answer is that there is no conflict here. As it was described in the article "The nature of time and space", "the second law of thermodynamics is a statistical result, ……, the basic statistical principles and the second law of thermodynamics are useful tools in human practice, but they are not the true natural existence" (Ma, 2003). The fact is that the life process does not obey the second law of thermodynamics, but it obeys all the natural laws. The second law of thermodynamics is not a natural law, but a technical tool.

6 Life as Autopoiesis (Auto-organizing)

Autopoiesis is the process whereby an organization produces itself. An autopoietic organization is an autonomous and self-maintaining unity which contains component-producing processes. The components, through their interaction, generate recursively the same network of processes which produced them. An autopoietic system is operationally closed and structurally state determined with no apparent inputs and outputs. A cell and an organism is an autopoietic system. Autonomy is the condition of subordinating all changes to the maintenance of the organization. Self-asserting capacity of living systems maintain their identity through the active compensation of deformations. Allopoiesis is the process whereby an organization produces something other than the organization itself. An assembly line is an example of an allopoietic system (Varela, 2005). Life is an emergent property of autopoietic, dissipative systems. Life is an autopoiesis (auto-organizing) complex, which can organize itself without energy input, even without information input. Active life process costs energy and uses information. However, the cost of energy is not the requirement of energy by the second law of thermodynamics. It cannot stay long period without energy and information input. After a while without exchange energy and information with outside world, the active life will die.

7 Evolution and Creation

Evolution theory is one of the most important theories in science. Evolution of life shows a remarkable growth in complexity. Simple prokaryotic one-celled life leads to more complex eukaryotic single-celled life, which then leads to multicellular life, then to large-bodied vertebrate creatures with sophisticated sensory processing capacities, and ultimately to highly intelligent creatures that use language and develop sophisticated technology as human. Creation theory says that life is not evolution but created by God, and all the species do not change forever. The interest thing is that many scientists are strongly believe creation in their non-work time, which means that the scientists believe Bible when they are in their churches in their religious time (normally in the weekend) or when they spend time in their Bible studies. However, these

scientists never do anything following creation theory in their work time, which means that they never do any experimental or publish any thing in the academic journals or teach students to support creation opinions. In the work time they need to do something that positive for their life as their income comes from the work, and non-work time they can do anything what they want.

Gene transfer is to transfer a gene from one DNA molecule to another DNA molecule, which can change the genetic background of an organism in anyway we want (Ma, 2005a). The evolution happens naturally, and also can happen artificially by gene transfer technique. Cloning creates a genetically identical copy of an organism, which can be done in all the kinds of living things, including human being. Transgenic animal and clone for the study of gene regulation and expression has become commonplace in the modern biological science now (Pinkert, 1999). The sheep Dolly was the world's most famous clone animal, but it was not the first one. Many animals—including frogs, mice, sheep and cows had been cloned before Dolly. Plants have been often cloned since ancient people. Human identical twins are also clones. Dolly was the first mammal to be cloned from an adult cell, rather than an embryo. This was a major scientific achievement of Dolly, but also raised scientific and ethical concerns. Since Dolly was born in 1996 many other animals have been cloned from adult cells, such as mice, pigs, goats and cattle. Cloning by interspecies nuclear transfer offers the possibility of keeping the genetic stock of those species on hand without maintaining populations in captivity (Lanza, 2002) and change the species, but also possibly creates the risk of biological calamity (Ma, 2004).

8 Adaptation

Adaptive evolutionary explanations are familiar to all of us from elementary school biology. A classic application of adaptationism is to explain the giraffe's long neck as an adaptation for browsing among the tops of trees, on the grounds that natural selection favored longer-necked giraffes over their shorter-necked cousins. There are alternatives to adaptive explanations, such as explanations appealing to allometry, genetic drift, developmental constraints, genetic linkage, epistasis, and pleiotropy. The presupposition that a trait is an adaptation and so deserves an adaptive explanation is usually treated as unfalsifiable. The adaptationist perspective on evolution emphasizes natural selection's role in creating the complex adaptive structures found in living systems.

The important feature for all life is the evolutionary process of adaptation. For the evolution, it is sometimes the blind operation of natural selection, sometimes the general process of evolution, and sometimes the adaptation produced by the evolution. Normally the life should have the ability to adapt appropriately to unpredictable changes in the environment. It is the force of adaptation and selection that makes the evolution happens. The adaptation is supple.

9 Emergence

Both living systems and artificial life models are commonly said to exhibit emergent phenomena. Emergent phenomena share two characterizations: they are constituted by and generated from underlying phenomena, and they are autonomous from those underlying phenomena. There are three main points for emergent properties. The first key point of emergence is simply the idea of a property that applies to wholes or totalities but does not apply to the component parts considered in isolation. The second key point of emergence is to insist that emergent properties are supervenient properties with causal powers that are irreducible to the causal powers of micro-level constituents. The third key point of emergence is poised midway between the other two.

10 Living Hierarchy

Living phenomena fall into a complex hierarchy of levels, what can be called the *vital hierarchy*. Even broad brush strokes can distinguish at least eight levels in the vital hierarchy: (1) ecosystems, (2) communities, (3) populations, (4) organisms, (5) organ systems (immune system, cardiovascular system), (6) organs (heart, kidney, spleen), (7) tissues, and (8) cells. Under the life hierarchy, there are molecules, atoms and quanta that are substance but not life constituents. Items at one level in the hierarchy constitute items at higher levels. Individual organisms are born, live for a while, and then die. The vital hierarchy raises two basic kinds of questions about the nature of life. First, we may ask whether there is some inherent tendency for living systems to form hierarchies. Why are hierarchies so prevalent in the phenomena of life? The second question concerns the relationships among the kinds of life exhibited throughout the vital hierarchy. Are there different forms of life at different levels, and if so then how are these related? How are they similar and different? Which are prior and which posterior? What is

the primary form of life?

The theory supple adaptation reveals a two-tier structure with connected but different forms of life. The first tier is the primary form of life—the supplely adapting systems. At the second tier, entities that are suitably generated and sustained by such a supplely adapting system branch off as different but connected secondary forms of life. These secondary forms of life include organisms, organs, and cells.

11 Continuum or Dichotomy

Can things be more or less alive? Serious reflection about life quickly raises the question whether life is a boolean property (zero or one)—whether it is a continuum property. We can say that a rat is alive and a rock is not alive. But it is difficult to say some condition of living body is alive or not, such as a virus which is unable to replicate without a host and spores or a frozen cell which remain dormant and unchanging indefinitely but then come back to life when conditions become suitable. Furthermore, we all agree that the original life forms somehow emerged from a pre-biotic chemical soup, and this suggests that there is very little, if any, principled distinction between life and non-life. In fact, life is continuum and it can be more or less alive. There is no absolute line between life and non-life. If life is considered as supple adaptation the most important life/non-life distinction involves a continuum because the activity of supple adaptability comes in degrees.

12 Apoptosis

For all the things existed, including the life cells in the earth and universe itself, there is a time to live and a time to die. There are two ways in which cells die: (1) Cells are killed by injury or disease. (2) Cells suicide. Programmed cell death is also called apoptosis, which is cell suicide. Apoptosis is a mechanism by which cells undergo death to control cell proliferation or in response to DNA damage. Some types of cancers, such as B-cell chronic lymphocytic leukemia, follicular lymphoma (Tsujimoto, 1985) and tumors infected by human T-cell leukemia/lymphoma virus-1 (Hengartner, 2000) are characterized by defects in apoptosis leading to immortal clones of cells. Other malignancies have defects in the apoptotic regulatory pathways such as p53 (Kaufmann, 2001).

Apoptosis can be triggered by the following internal signals: (1) In a healthy cell, the outer membranes of its mitochondria express the protein Bcl-2 on their surface. (2) Bcl-2 is bound to a molecule of the protein Apaf-1. (3) Internal damage to the cell (e.g., from reactive oxygen species) causes: Bcl-2 to release Apaf-1; a related protein, Bax, to penetrate mitochondrial membranes, causing; cytochrome c to leak out. (4) The released cytochrome c and Apaf-1 bind to molecules of caspase-9. (5) The resulting complex of cytochrome c, Apaf-1, caspase-9 and ATP is called the apoptosome. (6) These aggregate in the cytosol. (7) Caspase-9 is one of a family of over a dozen caspases. They are all proteases. They get their name because they cleave proteins—mostly each other—at aspartic acid (Asp) residues. (8) Caspase-9 cleaves and activates other caspases. (9) The sequential activation of one caspase by another creates an expanding cascade of proteolytic activity, which leads to digestion of structural proteins in the cytoplasm, degradation of chromosomal DNA, and phagocytosis of the cell.

Apoptosis can be triggered by external signals also: (1) Fas and the TNF receptor are integral membrane proteins with their receptor domains exposed at the surface of the cell. (2) Binding of the complementary death activator (FasL and TNF respectively) transmits a signal to the cytoplasm that leads to activation of caspase 8. (3) When cytotoxic T cells recognize their target, they produce more FasL at their surface. This binds with Fas on surface of the target cell leading to its death by apoptosis.

Apoptosis is a universal event in the universe, that happens in all the life bodies and azoic things in the universe, including the universe itself. To understand apoptosis clearly will be important to the understand of the basic nature laws (Ma, 2005b). Apoptosis is the nature of life, and apoptosis is also the nature of nature!

13 Artificial Life

Could robot do all the things what human do? Could artificial electronic life play all the functions what the organic life play? Up to now, nobody can answer these questions.

In 1966, John von Neumann made the first artificial life model with his famous creation of a self-reproducing, computation-universal entity using cellular automata. John von Neumann was pursuing many problems that are important in the artificial life today, such as understanding the spontaneous generation and evolution of complex adaptive structures. Originally, cybernetics applied two tools to the living system studies: the use of information theory and a deep study of the self-regulatory processes. Information theory typifies the abstractness and material-independence of artificial

life, and self-regulation is one of the hallmarks of living systems studied in artificial life.

Biology studies have provided rich knowledge about actual living systems. Physics and mathematics have had a strong influence on artificial life, especially in the study of complex systems. Statistical mechanics and dynamical systems theory have improved artificial life's methodology.

The real artificial life should be organic life, same as the natural life. Right now, people can synthesize simple organic molecules such as sugar and amino acids from the inorganic carbon, hydrogen and oxygen. Just after the technique developing, people will have the ability to make the real cells, tissues, organs and animals even a real human. This will be the real artificial life—everything is same as the natural life.

14 Matter and Form of Life

The advent of the field of artificial life has focused attention on a set of questions about the role of matter and form in life. On the one hand, certain distinctive carbon-based macromolecules play a crucial role in the vital processes of all known living entities; on the other hand, life seems more like a kind of a process than a kind of substance. Furthermore, much of the practice of artificial life research seems to presuppose that life can be realized in a suitably programmed computer. This raises a number of related questions: Can a computer play all the functions of the organic life play? Is the natural life just substance properties what the substance has or life has independent proper that performs by the substance? Functionalism captures the truth about life. Furthermore, there is no evident reason why the functional structure specified the theory could not be realized in a suitably structured computational medium. If so, then a computerized "life" could in principle create a real, literally living entity. In fact, a computer can play many functions of the organic life play, but could not play all the functions of the organic life play, because the matter is essential different. The natural life is dependent on the substance of the life bodies.

According to the classic science, there are two independent existences in the world: matter and space. Matter occupies space and moves about in it and it is the primary reality. Space is a backdrop or container. Without furnished by material bodies, it does not enjoy reality in itself. This common sense concept goes back to the Greek materialists and it was the mainstay also of Newton's physics. It has been radically revised in Einstein's relativistic universe (where spacetime became an integrated four-dimensional manifold), and also in Bohr's and Heisenberg's quantum world. Now it may be considered that matter and space are unified.

Advances in the new sciences suggest a further modification of this assumption about the nature of reality. In light of what scientists are beginning to glimpse regarding the nature of the quantum vacuum, the energy sea that underlies all of spacetime, it is no longer warranted to view matter as primary and space as secondary. In the modern concept there is no absolute matter, but only a matter generating energy field.

15 Life and Mind

It is an essential philosophical question whether there is any intrinsic connection between life and mind. Viruses, plants, bacteria, worms, animals and human have various kinds of sensitivity to the environment, various ways in which this environmental sensitivity affects their behavior, and various forms of inter-organism communication. Various kinds of what one could call mental capacities are present throughout the biosphere. Furthermore, the relative sophistication of these mental capacities seems to correspond to and explain the relative sophistication of those forms of life. It is reasonable to ask whether life and mind have some natural connection. The process of evolution establishes a genealogical connection between life and mind, but life and mind might be much more deeply unified. Since all forms of life must cope in one way or another with a complex, dynamic, and unpredictable world, perhaps this adaptive flexibility inseparably connects life and mind. In fact, the mind comes from brain that composes by the organic molecules and the organic molecules compose by inorganic matter. But, there is no evidence to say that the inorganic matter in the living organism is different from the inorganic matter out the living organism.

16 Life and Quantum

Up to now, no scientific evidence to show that life body and non-life body obey the different natural laws. By the classic physics and chemistry, there is no essential difference discovered in life and non-life. There is no lifeline defined by modern science, this means that we neither qualitate nor quantitate life by any current scientific method. However, all the researches are made by the methods of biology, biochemistry and molecular biology, etc., which means that all current biological and neurobiological descriptions of the life and brain are based on Newton's physics, even if it is well

known that Newton's physics has its limitations. Biophysics has started for several decades and it did not get many achievements. Up to now, nobody tried to reveal the nature of life under the quantum level. It is reasonable to think about that the life and non-life are essential different in the biophysics, i. e. the quantum level. The life phenomenon, especially consciousness, is unlikely to arise from classical properties of matter. Quantum theory allows for a new concept of matter altogether, which may well leave cracks for life and consciousness, for something that is not purely material or purely extra-material. Interactions with the quantum vacuum may not be limited to micro-particles: they may also involve macroscale entities, such as living systems. The recognition of openness is returning to the natural sciences. Traffic between our consciousness and the rest of the world may be constant and flowing in both directions. Everything that goes on in our mind could leave its wave traces in the quantum vacuum, and everything could be received by those who know how to tune in to the subtle patterns that propagate there.

All the life organisms compose by organic molecules plus their inner environment such as inorganic water and ions (and specific fields) inside and outside the cells. The whole life world finally composes by an organic world, and even though all organic molecules compose by inorganic substance. But, nobody knows if the water in alive cells and around cells is same or different from the water far away from the cells (under the living meaning). It is possible that the inorganic environment of living cell is different from non-living environment in the quantum level. This is the principle task for biophysics doing to reveal the nature of life.

17 Origin of Life

When the earth formed about 4.6 billion years ago, it was a lifeless place. A billion years later it was teeming with organisms such as blue-green algae. How did life begin? The discovery of self-replicating RNA was a critical milestone on the road to life. Before the mid-17th century, most people believed that God had created humankind and other organisms by mud. For the next two centuries, those ideas were subjected to increasingly severe criticism.

In 1903, Svante Arrhenius proposed that life on the Earth was seeded by spores originating from another planet. In 1905, the astronomer Simon Newcomb proposed that because the Earth was a representative planet orbiting a representative star Sun, life could be abundant throughout the universe (Zubay, 2000). But up to now, there is no discovery of the life existing in another planet.

All living things consist of similar organic compounds. Proteins in all organisms are consisted by one set of 20 amino acids. These proteins include enzymes that are essential to live, develop and reproduce, and the protein that essential to the organism structure. Organisms carry their genetic information in nucleic acids RNA and DNA, and use them as the same genetic code. This code specifies the amino acid sequences of all the proteins and peptides in each organism. The nucleotides consist of a sugar (deoxyribose in DNA and ribose in RNA), a phosphate group and one of four different bases. In DNA, the bases are adenine (A), guanine (G), cytosine (C) and thymine (T). In RNA, uracil (U) substitutes for T. The bases constitute the alphabet, and triplets of bases form the words as the genetic codes. As an example, the triplet CUU in RNA instructs a cell to add the amino acid leucine to a growing strand of protein when the protein is synthesized. Organisms store genetic information in nucleic acids that specified the composition of all sythesized proteins. It relies on proteins to play the biological metabolism processes.

There is a paradox. Nowadays nucleic acids are synthesized only with the catalyzing of proteins, and proteins are synthesized only with the coding of nucleic acids. It is impossible that proteins and nucleic acids arose spontaneously in the same place at the same time. It is also impossible to have one without the other. And so, at first glance, one might have to conclude that life could never have originated by chemical means. In the fact, RNA came first and established what is now called the RNA world—a world in which RNA catalyzed all the reactions necessary for a precursor of life's last common ancestor to survive and replicate. RNA has developed the ability to code amino acids to synthesize proteins. The modern RNA viruses are still use RNA as their genetic codes. The ribonucleotides in RNA are more readily synthesized than are the deoxyribonucleotides in DNA. Moreover, DNA could evolve from RNA and then take over RNA's role as the heredity. In fact, RNA came before proteins. In 1983 Thomas Cech at University of Colorado and Sidney Altman at Yale University discovered the first known ribozymes, enzymes made of RNA. The first ribozymes identified could do little more than cut and join preexisting RNA. Nevertheless.

As the experiments to reveal the original origin of life in the Earth, in the early 1950s Stanley Miller, working in the laboratory of Harold C.

Urey at the University of Chicago, did the first experiment to clarify the chemical reactions that occurred on the primitive earth. In the flask at the bottom, he heated water and forced water vapor to circulate through the apparatus. The flask at the top contained an atmosphere consisting of methane (CH_4), ammonia (NH_3), hydrogen (H_2) and the circulating water vapor. Next he exposed the gases to a continuous electrical discharge, causing the gases to interact. Water soluble products of those reactions then passed through a condenser and dissolved in the mock ocean. The experiment yielded amino acids and enabled Miller to explain how they had formed. For instance, glycine appeared after reactions in the atmosphere produced simple compounds formaldehyde and hydrogen cyanide that participated in the set of reactions that took place. For the above experiments, one heavy critics is that the so called amino acid products coming from bacteria contamination. Bacteria exist everywhere in the Earth and it is very possible to get the bacterial contamination in the experiments.

Stem cell is the origin of an orgnism's life. Stem cells have the remarkable potential to develop into many different cell types in life bodies, that are exciting to scientists because of their potential to develop into many different cells, tissues and organs. Stem cell is totipotent and it is a single cell that can give rise to progeny that differentiate into any of the specialized cells of embryonic or adult tissue. The ultimate stem cells (fertilized egg) divides to branches of cells that form various differentiated tissues or organs. During these early decisions, each daughter cell retains totipotency. Through divisions and differentiations the embryonic stem cells lose totipotency and gain differentiated function. During normal tissue renewal in adult organs, tissue stem cells give rise to progeny that differentiate into mature functioning cells of that tissue. Stem cells losing totipotentiality are progenitor cells. Except for germinal cells, which retain totipotency, most stem cells in adult tissues have reduced potential to produce cells of different types (Ma, 2005c).

18 Discussions

There are plenty of puzzles about the concept of life. The concrete objects ready to hand are usually easily classified as living or non-living. Fish and ants are alive while candles, crystals and clouds are not. Yet many things are genuinely puzzling to classify as living or not. Viruses are one borderline case, biochemical soups of evolving RNA strings in molecular genetics laboratories are another. Extraterrestrial life forms, if any exist, might well not depend on DNA-encoded information or, indeed, any familiar carbon chemistry processes. How would we recognize extraterrestrial life if we found it? We have no reason to suppose it will have any of the accidental characteristics found in familiar forms of life. What, then, are the essential properties possessed by all possible forms of life? The search for extraterrestrial life needs some answer to this question, for we can search for life only if we have a prior conception of what life is.

The phenomena of life raise a variety of subtle and controversial questions. Early life forms somehow originated from pre-biotic chemical soup. Does this imply that there is an ineliminable continuum of things being more or less alive, as many suppose? Another subtle question concerns the different levels of living phenomena, such as cells, organs, organisms, ecosystems and asks in what senses the concept of life applies at these various levels. Does the essence of life concern matter or form? On the one hand, certain distinctive carbon-based macromolecules play a crucial role in the vital processes of all known living entities; on the other hand, life seems to be more in the nature of a process than a kind of substance. The relationship between life and mind raises another question. When we consider plants, bacteria, insects, and mammals, for example, we apparently find different kinds of mental activity, and it seems that different degrees of behavioral sophistication correspond to different levels of intelligence. Might the various forms of life and mind be somehow connected? To answer questions like these above and make sense of the puzzling phenomena of life, we need a sound and compelling grasp of the nature of life. Can any property embrace and unify not only life's existing diversity but also all its possible forms? What is the philosophically and scientifically most plausible way to account for the characteristic life-like features of this striking diversity of phenomena? How can we resolve the controversies about life? The concept of life as supple adaptation, explained below, is my attempt to address these issues.

Notice that our ordinary, everyday concept of life does not settle what the true nature of life is. Thus, we are not concerned here with careful delineation of the paradigms and stereotypes that we commonly associate with life. We want to know what life is, not what people think life is. Glass does not fall under the everyday concept of a liquid, even though chemists tell us that glass really is a liquid. Likewise, we should not object if the true nature of life happens to have some initially counterintuitive consequences.

Four questions are important to answer: (1) How are different forms of life at different levels of the vital hierarchy related? (2) Is there a continuum between life and non-life? (3) Does life essentially concern a living entity's material composition or its form? (4) Are life and mind intrinsically connected?

For now, many people, including biologists and other scientists are still believing that God created the life, even they never publish any academic articles to describe that. The ridiculous things are that many biologists always write articles and teach students evolution in their work time but believe creation theory (deny evolution) in their weekend church time. Depending on the academic articles, they make their career and life, but depending on the Bible, they come back non-experiment believe. The fighting between science and religion is still a heavy topic in the modern time.

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MINIREVIEW

A Puzzle of the Effect of Magnetic Field on Biological Cells

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Abstract: The mechanism for interactions of magnetic field, particularly in extremely low frequency with biological cells is still puzzle to the scientific researchers. Our investigations have guided to the speculation of roles of the magnetic field for the cell-cell communication in physiological responses. To explain the complexity, the experimental evidences and observations and their associated theories have been included in this review. The result of a disruption of the homeostatic regulation of the cells responding to a specific strength and frequency of magnetic field can be as the signal that triggers signal transduction to modulate the cell-cell communication. This review leads into the stochastic systems in cell, for instance, ion channels on cell membrane, providing a basis for signal amplification to disrupt the cell homeostatic regulation. [Life Science Journal. 2005;2(1):16-21] (ISSN: 1097-8135).

Keywords: biological effect; cell; magnetic field

1 Introduction

Biological cells have been shown to respond low frequencies electromagnetic fields as well as in chemical and biochemical reactions (Dobson, 1996). Puzzle of the energy of the fields is too low to the noise in cellular level to produce observable effects (Adair, 1991; 1992; 1994). Therefore, the physical mechanism of primary interaction between the magnetic fields and the biological target sites, such as electrical charge in motion, molecules structure with magnetic moments and the application of Faraday law, generating local electrical current by a varying magnetic field has mainly concluded only amplification mechanisms can solve the puzzle. As we know, the interaction should be very weak at field strength less than 10 Gauss and the frequency below 100 Hz. If considering the stochastic resonance model, the amplification of weak electromagnetic interaction signals can be modulated by external fields (Jung, 1993). By considering ferromagnetic transduction model, the minimum applied magnetic fields would produce a torque on a biogenic magnetite particle coupling via the cytoskeleton to the ion gates. The deformation of the cell membrane and the closing of the ion gates would occur quickly enough to compensate for the forced opening of the gate as long as the frequency of the forcing field was below 100 Hz, regardless of the strength of the applied field. Dawson et al. (Dawson, 1996) used scalar potential finite difference code for low frequency electromagnetic computations and to model induction in anatomically realistic human in terms of average and maximum electric field intensities. The calculation revealed the induced dosimetry amount for various major organs upon exposing to extremely low-frequency electromagnetic fields. The whole body induced maximum current density is about 21 micro amp per meter square from a 50 Hz to a 60 Hz source frequency and from a 1 amp per meter to a 0.1 Gauss source strength. Comparatively, the cell culture dosimetry for low frequency magnetic fields study by Hart (Hart, 1996) has shown the induced current density averagely about 0.9 micro amp per meter square from a 60 Hz source of frequency and a 1 Gauss source of field strength. Accordingly, the induced effect between the human body and the cells *in culture* clarified the interaction mechanisms involved could be different. The mechanisms whereby ELF electromagnetic field stimulates changes in biological functions of cells *in culture* may not guarantee that would cause the same biological effect *in vivo*. Many researchers therefore switched their interest to the biological effect of very high frequency band of wireless communication protocol just because of the support funding being hard to continuous for ELF biological effect study.

2 The ELF Biological Effect

The concern of the exposure to extremely low frequency (ELF) electromagnetic fields may present a health hazard to workers and the public. The controversial and contradictory finding in the scientific research, especially from epidemiological studies is puzzle (EPRI, 1994). Research of ELF elec-

tromagnetic fields interaction caused biological effect in biological system includes experimental investigations on both *in vivo* and *in vitro*. In present, the researchers have been interested in both to the electric fields and magnetic fields as well as identifying a possible mechanism for ELF acting as a cancer initiator, promoter or co-promoter. On the other hand, epidemiological studies have shown very weak connection between ELF exposure and leukemia, brain cancers, breast cancer and lung cancer. Almost all related research studies were in flaws, for instance, numbers of cases were too low to look at cancer subtypes, lack of specific exposure, lack of reliability of data, lack of statistical power and lack of control for repeaters. There is no reliable supporting data for an association between ELF exposure and cancer risk in the public. There is no conclusive evidence so far from the epidemiological evidence that electric or magnetic fields cause a risk of cancer. The researcher only holds a possibility of the risk of cancer of ELF electromagnetic fields occupational exposures. Oppositely, experimental investigations with cellular systems have shown that electromagnetic fields can interact with biological systems. Several researchers have confirmed cellular effects involving the movement of calcium ions through cellular membranes under ELF interaction (Fewtrell, 1994). The significance of this effect as it relates to possible adverse health outcomes is still not understood. Direct effects on significant cellular molecules, such as DNA, have not been observed. No direct mutagenic or carcinogenic effects on animals have been observed. Current research has shifted to focusing on the role of ELF (particularly magnetic fields) as a tumor promoter or co-promoter. However, it is still no effects were observed on mice exposed without the chemical promoter yet. The overall view obtained from the research literature indicates that while some biological effects of exposure to ELF electric and magnetic fields occur, there are no resulting adverse health effects from these exposures. In the United States, a large number of research papers and overview reports have been produced along with numerous conferences over the past 15 years. Unfortunately, the findings remain controversial and contradictory. There is insufficient data to determine if a cause and effect relationship exists. The National Council on Radiation Protection and Measurements (NCRP) in Bethesda, Maryland, set up a committee chaired by Dr. W. Ross Adey to review the possible health effects of ELF. The National Academy of Sciences committee, chaired by Dr. Charles Stevens, Salk Institute, California released a report in 1996 concluding no clear, convincing evidence exists to show that residential exposures to electric and magnetic fields (EMFs) are a threat to human health. The United Kingdom's National Radiological Protection Board (NRPB) established an Advisory Group on Non-Ionizing Radiation in 1990 to review the scientific evidence and determine the extent to which this evidence suggests possible health risks. The International Non-Ionizing Radiation Committee (INIRC) of the International Radiation Protection Association (IRPA) in cooperation with the Environmental Health Division of the World Health Organization (WHO) developed recommendations for 50/60 Hz electric and magnetic field exposure limits. At the 8th Congress of the IRPA in May 1992, the IRPA established a new independent scientific organization, the International Commission on Non-Ionizing Radiation Protection (ICNIRP) as a continuation of the former IRPA/INIRC. In April 1998, ICNIRP published guidelines for limiting electromagnetic field exposures for frequencies up to 300 GHz, including 50/60 Hz.

3 Energy Transduction in Cell

The mitochondria in cell contain the series of catalysts known as the respiratory chain collect transport reducing equivalents to react with oxygen for forming water. The reducing equivalents,-H or electrons, are made from oxidation of carbohydrate, fatty acids and amino acids. The cell system couples respiration to generate the high energy intermediate ATP, termed oxidative phosphorylation involved NADH, Succinate, Ubiquinol, Ferrocytochrome and ATP synthase five protein lipid enzyme complexes. Three different mechanisms including chemical coupling hypothesis, the conformational coupling hypothesis and the chemiosmotic hypothesis have been proposed to explain the energy transfer between electron transport and ATP synthesis (Menendez, 1996). Accordingly, electron transport along the respiratory chain can be the source of electromagnetic field to exert forces on the proton. Through these forces, electromagnetic coupling hypothesis is proposed to describing the protons are moved from the mitochondrial matrix to the exterior. In the mean time, the protons moved by the field toward the inner membrane pass through its protein components plays the role of proton channel. Just as if in a motor, ELF triggers the electrical energy, transfer of electrons along the respiratory chain, to produce proton translocation, which is mechanical energy, be used in cell system.

4 Stochastic Resonance

Several resonance theories, for instance, ion cryotron resonance (Liboff, 1991), parametric resonance were proposed to describe the biological effect caused by ELF fields. For the problem of synchronization of the relaxation time and the noise, it is hard to find the evidence of resonance in cell system. Nevertheless, because of ionic channels of cell membranes are elemental molecular switches for channel states, open or close, in spite of the small size of the channel, high resolution ion current detection allows us to watch electrical activity of a single ionic channel isolated within a micrometer patch of a cell membrane. The state-transition behavior between the open and close states of an ion channel characterizes many biological processes. The experimental results suggested that the potential oscillation could be driven by the oscillation in the intracellular concentrations of cyclic AMP and calcium in two-state model system (channel closing and opening). The signal-to-noise ratio increases when the state-transition rate of membrane channel influenced by the frequency response of the intracellular sensing system. An applied ELF (electric or magnetic field) field is an important factor to perturb the rate of state-transition of membrane channel in opening or closing. Jung (1993) has developed stochastic resonance driven process for multichannel systems and shown the optimal choice of parameters can lead to signal amplification. From the stochastic resonance theory, time average of the total current through a set of N channels can be calculated. To access the magnitude of amplification of the responding signal caused by external ELF field in cell, the membrane with N voltage gated channels and biochemical oscillator (calcium oscillation) must be considered. Because of this modulation, the external ELF field signal is transformed into a periodic component in the ion current across the membrane. As we have focused in the study on the primary mechanisms that a biological cell can use to amplify weak external influences, a system of identical ion channels embedded in a membrane and synchronously modulated can significantly amplify the original signal. The amplification of the signal to noise ratio is proportional to the square root of the number of channels modulated and inversely proportional to the square root of the sum of the average channel relaxation times.

5 Role of Gap Junctions

In cell, six connexin 43 subunits oligomerze in the Golgi apparatus into a connexon, called hemi channel and be transported to plasma membrane of the cell. Before pairing process, hemi channels are closed to avoid leakage of cellular contents and entry of extra-cellular materials. During the pairing of connexons and aggregation into plaques at the plasma membrane, connexin 43 is phosphorylated at least twice and connexons are attracted to those located on the adjacent cells. Two connexons join in an end-to-end manner to form a complete channel. The channels aggregate into large gap junction plaques open to connect two cells for cell-to-cell communication and is called gap junctional intracellular communication (GJIC), which can be modulated by environmental factors, such as drugs, X-ray, electromagnetic fields etc. Since the function of the GJIC, cultured cells coupled *in vitro* except the stem cells and cancer cells (Trosko, 1991; 2001). Furthermore, in basic signal transduction, we want to know what the gap junction growth regulatory signal within cells is. The cAMP is a very important gap junction signal molecule. This signal molecule can pass between cells through gap junction channels and affects cell growth. The levels of the cAMP can oscillate within the cell and generates oscillation in growth control. The amplitude of the oscillations would be dampened by GJIC. However, cAMP is not the only signal molecule that may affect GJIC. Increasing drug penetration and dispersal in tumors would increase GJIC. Gap junction channels in cell membrane can create a stochastic two-state system, opening or closing of the channels, to amplify weak signal caused by ELF fields.

6 Physics Correlation between GJIC and ELF Magnetic Field

The diffusive current equation for connexin 43 channels can be written as

$$< I > = \sum_{k=1}^{m} kP(k) \tag{1}$$

where probability $P(k)$ indicates total m channels is taken into account for opening k channels from all cell-to-cell communications on the surface of the cell mono layer. Therefore,

$$P(k) = \frac{m!}{k!(m \div k)!} (P^{o})^{k} (P^{c})^{m-k} \tag{2}$$

$$\frac{dP^{o}}{dt} = r^{c}P^{c} - r^{o}P^{o} \tag{3}$$

$$\frac{dP^{c}}{dt} = r^{o}P^{o} - r^{c}P^{c} \tag{4}$$

where r^{c} is the rate of changing from c-state to o-state and r^{o} is the rate of changing from o-state to c-state of the connexin 43 channels activating totally on the cells mono layer surface. Generally, r^{o}

does not have to be same with r^c since the life times of the o-state and c-state may vary. To clarify the physical meaning, we further assume the current through an open channel as i. The diffusive current caused by GJIC channels can be rewritten as

$$<I>= miP_s^o \quad (5)$$

where P_s^o is the modulated probability for o-state by external ELF field signal. According to theory of Jung (1993), the power spectral component originated from the signal is given by

$$S_k=\frac{(mi)^2}{2}\sum_{q=1}^{\infty}|C_q|\delta(\omega-q\omega_k) \quad (6)$$

C_q is the Fourier expansion coefficients of P_s^o.

In comparison with equations (3) and (6), the signal-to-noise ratio (SNR) of the characteristic frequency of the cell system can be depicted (Galvanovskis, 1997).

$$\mathrm{SNR}=\left|\frac{\text{signal amplitude}}{\text{background amplitude}}\right|^2 =A^2\left|\sqrt{m\frac{\pi}{\Delta\omega}\frac{r^o r^c}{(r^o+r^c)}}\right|^2 \quad (7)$$

where m is the number of channels, A is the amplitude and $\Delta\omega$ is the bandwidth of the external ELF field signal.

7 Specific Inhibit and Promote of GJIC

Many researchers have developed new techniques to inhibit or promote the expression of a target gene in culture cells and animals. ELF magnetic field treatment is one of the main factors that scientists are interested. We proposed a new way to separate the background and signal and revealed the evidence of existing stochastic resonance system buried in biological cells by GJIC essay under ELF magnetic fields. Specific inhibit of GJIC within mouse osteoblast cells in culture under the exposure of ELF magnetic field (Teng, 2002) depicted several possibilities, blockage of connexin gene expression, connexin gene knockout and transfection of defective connexin genes. Additionally, specific enhancement of GJIC within mouse osteoblast cells under the exposure of different doses of ELF magnetic field (Hart, 1996) can be transfected of functional connexin genes.

8 SNR Spectrum

By using of the probe of Gauss-meter, cells-induced magnetic fluctuation can be shown as

$$\{B_i^c\}=\{B_1^c, B_2^c, \cdots, B_{2000}^c\} \quad (8)$$

Equation (8) contains the cellular response signal of the reaction to the external ELF magnetic field (Teng, 2003). The sampling time was 0.0005 second. The probe was located at the distance 10^{-4} m perpendicularly to the center of single layer of the cell surface in culture dish. The Gauss-meter was manufactured by F. W. Bell Company (series of 9550) in Florida of USA. Oscilloscope was manufactured by Agilent Company (54621A) and can be used to convert $\{B_i^c\}$ to voltage sequence $\{V_i^c\}$ as

$$\{V_i^c\}=\{V_1^c, V_2^c, \cdots, V_{2000}^c\} \quad (9)$$

Matlab and Fortran programming were used for power density spectrum analysis of these voltage sequences. $\{B_i^m\}$ for the first control was taken 2000 times per second at the distance 10^{-4} m perpendicularly to the culture dish with only medium in it. Geo-field control $\{B_i^n\}$ was taken with the same sample rate at the distance 10^{-4} m perpendicular to the empty culture dish recording local geomagnetic field fluctuation. The corresponding $\{V_i^m\}$ and $\{V_i^n\}$ can be obtained by the same way as $\{V_i^c\}$ previously. Furthermore, trial signals,

$$\Omega_i(n)=A_i\times\sin(\omega_i n), 1\mathrm{Hz}\leqslant\omega_i\leqslant 60\mathrm{Hz},$$

where signal amplitude $A_i = F\times V_{\max}$, F is the adjustable fraction factor and $V_{\max}$ is such, $V_{\max}=\max(\{V_i^c\})$, as to the maximum value of the sequence $\{V_i^c\}$. By taking into consideration of signal amplitudes at F values, for instance, $F=0.7, 0.4$, and 0.03 respectively, the corresponding amplitudes would be

$$A_{0.7}=0.7\times V_{\max},$$
$$A_{0.4}=0.4\times V_{\max},$$
$$A_{0.03}=0.03\times V_{\max}$$

for a given trial signal at ELF ω_i (1 Hz$\leqslant\omega_i\leqslant$60 Hz). We computed autocorrelation function of $\{V_i^c\pm\Omega_i(n)\}$ and its Fourier transforms to obtain their corresponding signal-to-ratio ratio. The SNR spectrum, then, for $\{V_i^c\pm\Omega_i(n)\}$ at frequency could be simply a second order equation as

$$a\times(S_{\omega_i}(F))^2+b\times S_{\omega_i}(F)+c=0 \quad (10)$$

Accordingly, substituting the SNR at different F (different amplitudes at frequency) into the equation, we can solve unknowns a, b and c. If c-value is much bigger than zero, then, the SNR of the intrinsic signal peaked at ω_i is detected (Teng, 2005). The value of c is about 0.07.

In the paper by (Galvanovskis, 1997), the SNR could be written as

$$S\omega_i(F)=(F\times V_{\max})^2\times m\times Q\times\frac{2\pi}{\omega_i(\tau_o+\tau_c)}$$

when the life time of c-state and o-state equal to 10^{-6} second. Under optimal condition, the quality factor $Q=\frac{\omega_s}{\Delta\omega}$ approximately equals to 100 at 60

Hz with bandwidth $\Delta\omega = 0.6$ Hz and $F = 0.6$. The numbers of GJIC channels are taken 1000 per cell (Galvanovskisz, 1997). The SNR value is fit to the value calculated from SNR spectrum suggested by Teng (Teng, 2005).

9 Discussion

We examined the role of the ELF magnetic field in the biological cells system. It is hard to imagine how the forces of evolution could have led to biological mechanisms that respond to ELF magnetic field in cells, because low frequency fields have not been present during virtually all of evolution. So far, there is still no direct evidence that ELF magnetic field can affect the physiological endpoints within cells in culture. However, enough evidences have shown the biological effect that the cell responded to the interaction of externally applied ELF magnetic field (Kaiser, 1996). Since GJIC affiliates with many physiological endpoints, changing of the characteristics of GJIC causes the biological effect within cells in culture. A confluent cell culture in a vertical magnetic field could receive several different exposures. If the cells are not tightly jointed to each other and to the walls, then the system is homogeneous. Different cell types could experience very different induced current and electric field distributions under the same magnetic field exposure in similar dishes. Gap junctions joint the cells and are open to allow current paths connect the cell interiors. They produce the current paths throughout the interiors of the cells in the cell culture. Most of the time, two dimensional model is oversimplify the current density distribution that is actually experienced by cells in a culture dish. Value for the induced current density, in effect, is the top surface perpendicular to the applied magnetic field. When the magnetic field is applied along the surface of the cells, perpendicular to the normal direction of the surface, the homogeneous system would be inapplicable around the central planes, but it could be used away from the central region.

10 Conclusion

If a cell culture is exposed to a vertical magnetic field for a relatively long period, the exposure received by the cells may change during the course of the experiment. Trusko et al. (Trosko, 2001; Upham, 1998) originated the perfusion of the dye produced by the function of the GJIC in cell culture to study the cell physiology. Teng et al. first proposed the signal-to-noise SNR spectrum calculation of the near magnetic field in 2002. They have revealed the possibility of signal transduction pathways perturbed by ELF magnetic fields at different characteristic frequencies and the stochastic resonance frequencies within the cells. The result of a modulation of the GJIC within the cells responding to a specific strength and frequency of magnetic field can be as the signal that triggers signal transduction in cell.

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Expression and Purification of a Human Tumor-Associated Protein Annexin V

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Abstract: Annexin V is a phospholipase A2 and protein kinase C inhibitory protein with calcium channel activity and an undefined role in cellular growth and differentiation. The product of *annexin* V gene is necessary for doing experiments to determine whether the tumor-associated *annexin* V protein can be used as a new molecular marker for diagnosis and prognosis of gastric carcinoma. This study describes the procedures which were used for cloning and expressing the human *annexin* V as a maltose binding protein (MBP) fusion polypeptide in bacteria. The expression plasmid for *annexin* V was constructed by ligation of the *annexin* V cDNA into the expression vector pMAL-c2x. The protein was expressed by *E. coli* strain TB1 cells and purified by amylose affinity column chromatography. The purity of the protein was assessed by SDS-PAGE and Western blot. The results showed that the molecular weight of the recombinant MBP-*annexin* V polypeptide was consistent with the calculated molecular weight, and that the purified protein appeared as an apparent single band of 77 kDa on the gel filtration column by SDS-PAGE. Our expression system allows the expression and purification of *annexin* V with MBP in high yield with no need of removal of the tag and gives pure protein in one purification step, and also makes it possible for the structural and functional studies of these proteins. [Life Science Journal. 2005;2(1):22 - 26] (ISSN: 1097 - 8135).

Keywords: *annexin* V; expression; purification; pMAL-c2x

1 Introduction

Annexins are Ca^{2+} and phospholipid-binding proteins forming an evolutionary conserved multigene family. Each member of this protein family contains a conserved protein core characterized by high alpha-helix content that includes the calcium and phospholipids binding sites, and a variable N-terminal domain that is specific in sequence and length for each *annexin*. Although the structure of the protein core is highly conserved, different *annexins* exhibit a wide biochemical and functional diversity, e. g., inflammatory response, membrane fusion and exocytosis, ion channel regulation, and inhibition of blood coagulation[1]. Despite an abundance of experimental evidence suggesting that *annexins* are associated with a plethora of biological processes, the exact physiological function of *annexins* remains to be investigated. Annexin V is a phospholipase A2 and protein kinase C inhibitory protein with calcium channel activity and an undefined role in cellular signal transduction, inflammation, growth and differentiation[2]. It has been previously isolated as placental anticoagulant protein[3]. Annexin V may be used to assess tumor response to chemotherapy[4]. We have previously described the overexpression of *annexin* V in gastric carcinoma tissues using proteome-based approach[5]. In this study we have made an *annexin* V expression construct, purified the protein and further determined the identity of the protein for the purpose of studying its role in tumor cells[6].

2 Materials and Methods

Construction of pMAL-Annexin V: Total RNA extracted from human placental by Rnasey Midi Kit (Qiagen, Valencia, CA, USA). The mRNA was then reverse transcribed to cDNA using AMV reverse transcriptase (Promega Co, USA) with 9-mers random primer. The cDNA encoding for human *annexin* V (Accession # J03745) was amplified by PCR using the upstream primer 5′-GC GAATTCATGGCACAGGTTCTCAGAGGCA-3′ designed with an upstream *EcoR* I restriction site (underlined), and the downstream primer 5′-GCGCTGCAGTTAGTCATCTTCTCCACA-3′ designed with a down-stream *Pst* I restriction site (underlined). Both PCR products contain an 'A' at the 3′ end, the 965-bp DNA fragments from the PCRs were directly cloned into pMD T vector

(TAKARA Bio Inc.) following standard procedures[7]. pMAL-c2x plasmid (New England Biolabs) contains an Ampr gene, tac promoter, the *lac Iq* gene, a polylinker, and the *mal* E gene, which encodes for maltose binding protein (MBP). pMD-*annexin* V was digested with the restriction enzymes *EcoR* I and *Pst* I (Promega Co., America), and pMAL was digested with *EcoR* I and *Pst* I with manufacturer's buffers at 37°C for 2 hours. The fragments from these restriction digests were gel-purified using the Qiaex II Gel Extraction Kit (Qiagen, Valencia, CA, USA). The cut pMAL vector was then ligated with the *annexin* V gene using T_4 DNA ligase (Promega Co., USA). All ligations were transformed into MAX efficiency *E. coli* strain TB1 cells. Bacterial cells were plated onto LB-Amp plates and incubated overnight at 37°C. A colony containing each of the constructs were inoculated into LB-Amp culture medium overnight and then midi-prepped using a Plasmid Midiprep Kit (Vitgene, China).

Verification of constructs: The recombinant plasmid was detected by restriction endonucleases and specific PCR. The midi-prepped constructs were sequenced on an ABI 3700 Sequencer to verify correct splicing of the gene into the plasmid. Sequence analysis was done with the program Sequencher, software available from Gene Codes Corporation (Ann Arbor, MI).

Expression of fusion proteins: TB1 competent bacterial cells were transformed according to manufacturer's instructions with the sequenced MBP-Annexin V[8]. Cells were plated onto LB-Amp plates and grown overnight at 37°C. The overnight culture was added to 900 ul 50% glycerol to make glycerol stocks. Overnight cultures were diluted 1:100 with pre-warmed LB-Amp media. The protein expression was induced by addition of Isopropyl-Beta-D-thiogalactoside (IPTG) to a final concentration of 0.2 mM when OD_{600} reached 0.4 − 0.5. After a 6 h post-induction period, the cells were harvested by centrifugation at 6,000 g at 4°C for 20 min. One ml of both uninduced and induced cultures were spun down and resuspended in 100 ul 2 × SDS-PAGE sample buffer and analyzed by 12% SDS-PAGE gel.

Purification of MBP fusion proteins: The fusion proteins were purified based on a protocol[9,10] as described (New England Biolabs, USA). One liter of cells transformed with the fusion construct was induced to produce protein as described above. The cultures were spun down and resuspended in 50 ml column buffer (20 mM Tris-Cl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT). Resuspended cultures were frozen at −20°C until purification. Frozen cultures were defrosted slowly in ice-cold water. 0.5 M DTT, 250 mM PMSF, and 100 × protease inhibitor cocktail from Sigma were added to the defrosted cultures to final concentrations of 5 mM, 0.1 mM respectively. The mixtures were sonicated in short bursts of 10 seconds for 20 minutes total[11,12]. Sonicates were spun down, and the supernatants were collected. 1.5 ml amylose resin beads (New England Biolabs, USA) were added to the supernatants and the protein was allowed to bind to the beads overnight on a rotary shaker at 4°C. The beads were washed with column buffer and protein was eluted with 100 mM maltose in column buffer overnight at 4°C. Samples for SDS-PAGE analysis were taken from induced culture, post-sonicate pellet, post-sonicate supernatant, supernatant after binding to amylase resin beads, and eluted protein was analyzed using a 12.5% SDS-PAGE.

Detection of recombinant proteins by western blot: Electrophoretically separated proteins were transferred to an Immobilon PVDF transfer membrane (pore size 0.45 μm, Millipore) by electrophoresis in transfer buffer (PBS, pH 7.4, 200 mM glycine, 20% methanol, 0.1% SDS) using a Transblot cell (Biorad, USA) at 275 mA for 30 min. After transfer the non-specific binding sites on the membrane were pre-blocked by incubating with blocking buffer overnight at 4°C. The primary rabbit anti-*annexin* V antibody (Abcam Ltd, USA) was diluted 1:300 in PBS and incubated with the pre-blocked membrane for 60 min at room temperature with constant shaking. Non-bound antibodies were removed by washing three times in wash buffer (0.05% Tween-20 in PBS) for a total of 60 min. The antibody antigen complex on the membrane was detected by incubating the membrane with a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (diluted 1:5,000) for 1 hour at room temperature. The membrane was then washed as before and rinsed with PBS[13-15]. Reactivity was visualized according to the manufacturer's instructions.

3 Results

Verification of constructs of MBP-Annexin V: As shown in Figure 1, the recombinant plasmid of pMAL-*annexin* V was digested into two fragments by *EcoR* I and *Pst* I which are 6.7 kb pMAL vector fragment and 0.96 kb *annexin* V gene fragment[15], and there was a single 7.0 kb fragment by the digestion of *EcoR* I alone. The 0.96 kb *annexin* V gene fragment could be ampli-

fied by specific PCR. It was demonstrated that recombinant plasmid of pMAL- *annexin* V was constructed successfully. Sequencing analysis showed that the construct, which was used for protein purification contains the full-length 965 bp *annexin* V gene. The cDNA opening reading frame of *annexin* V gene contained 965 bp which coded for a protein with 319 amino acids and a calculated molecular mass of 35,805 Da.

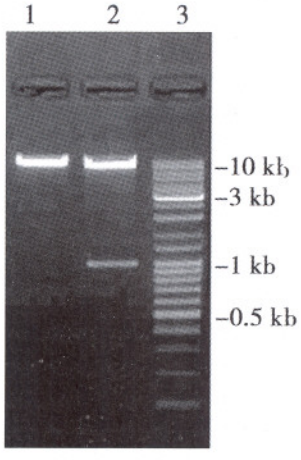

Figure 1. The recombinant plasmid was verified by restriction endonucleases. Lane 1, recombinant plasmid DNA was digested by *EcoR* I and *Pst* I; lane 2, recombinant plasmid DNA was digested by *EcoR*I alone; lane 3, DNA molecular markers (10 kb).

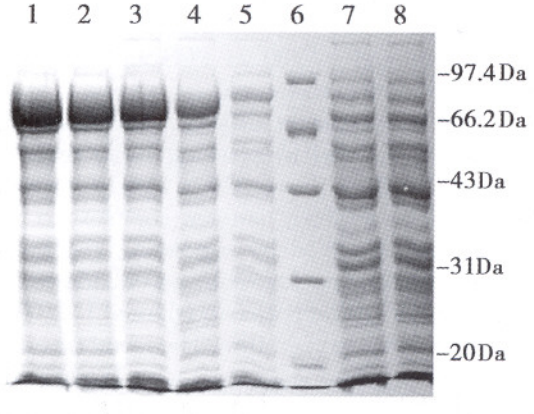

Figure 2. Expression of MBP-*Annexin* V fusion constructs. Lane 1, TB1 (pMA) 0.2 mM IPTG induced 5 hours; lane 2, TB1 (pMA) 0.2 mM IPTG induced 4 hours; lane 3, TB1 (pMA) 0.2 mM IPTG induced 3 hours; lane 4, TB1 (pMA) 0.2 mM IPTG induced 2 hours; lane 5, l TB1 (pMA) 0.2 mM IPTG induced 1 hours; lane 6, protein marker; lane 7, TB1 induced 4 hours; lane 8, TB1 (pMAL－c2x) un-induced 4 hours.

Expression of recombinant MBP-Annexin V fusion protein: Annexin V was cloned into the pMAL-c2x expression vector, as described in materials and methods. MBP-Annexin V constructs were readily expressed in TB1 cells. To obtain high Annexin V expression, Figure 2 shows the TB1 cellular content of Annexin V expressed in the different conditions. After SDS-PAGE analysis, the MBP-Annexin V protein was detected at 77 kDa (42 kDa MBP + 35 kDa Annexin V = 77 kDa) by Coomassie blue staining. As shown in Figure 3, the level of expressed Annexin V was apparently affected. Annexin V constituted approximately 39.9% of total cellular protein after IPTG induction for 4 hours.

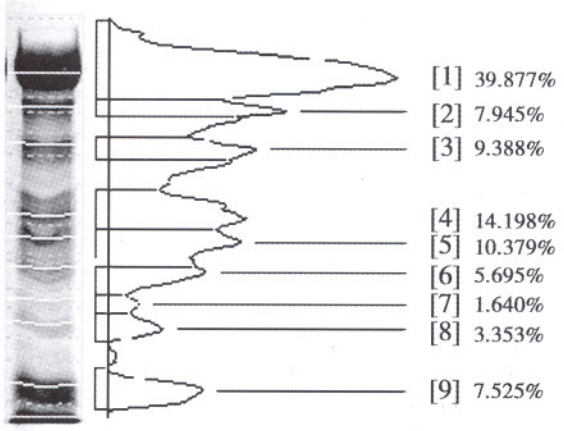

Figure 3. Contents analysis of the MBP- Annexin V fusion protein. Percentage of the each protein was showed in the figure. Annexin V constituted approximately 39.9% of total cellular protein after IPTG induction for 4 hours.

Purification of recombinant MBP-Annexin V fusion protein: The efficiency of the purification was dependent on appropriate amounts of equilibrated amylose resin. Due to the relatively binding capacity of the resin, we found that 1 ml crude extract (approximately 10 mg protein) needs 1 ml of equilibrated gel slurry. Prolonged incubation times of more than 15 min at 4℃ had to be used in order to achieve optimal recovery of bound protein. We observed that the first washing steps contained large amounts of MBP-Annexin V complexes. Most of the MBP-Annexin V protein produced by IPTG induction was found in the post-sonicate pellets, and not in the post-sonicate supernatants. Almost all of the protein contained in the supernatants was able to bind to the amylose resin and could be eluted from the beads with a high efficiency. After purification and SDS-PAGE analysis, the MBP-Annexin V protein was detected as a single band of 77 kDa, and it also immunoreacted with anti-Annexin V polycolonal antibody, The result showed that this protein was expressed and purified as a single chain MBP-Annexin V fusion protein. The final yield was 1.9 mg of purified protein per liter

culture cells and the purity of the preparation was estimated to be over 95% as judged by SDS-PAGE analysis.

4 Discussion

This study describes the process of bacterial expression and purification of a MBP-Annexin V fusion protein. The results indicate that fusion protein using the MBP purification system from New England Biolabs can be readily produced in bacteria and purified with high yield. The experiments which have been done in this study showed that the post-sonicate supernatant contains cellular soluble fractions from the rupture of cells. The appearance of more protein in this component of the sonicate implies that the number of cells that remained ruptured after sonication is greater than the number of cells that released their protein during sonication. The anti-Annexin V polyclonal antibody was used to assess the specificity of *annexin* V protein by Western blot analysis. The results demonstrated that the purified Annexin V protein had strong reactivity with the polyclonal anti-Annexin V antibody, suggesting that it could be used in the subsequent experiments to characterize the gene *annexin V* in terms of its function.

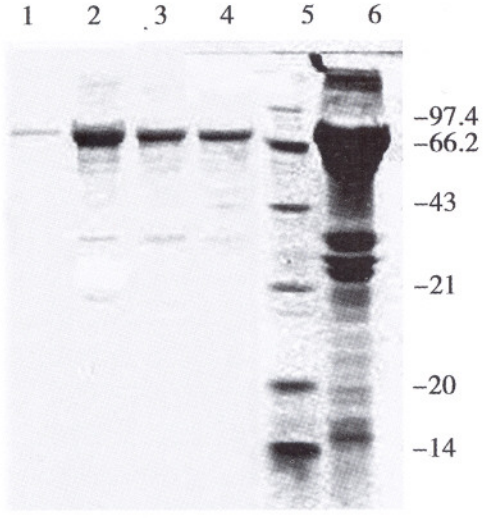

Figure 4. Purification of recombinant MBP-Annexin V fusion protein. Lanes 1 – 4, Purification of MBP-Annexin V protein from tubes 1 – 4; lane 5, Molecular marker; lane 6, TB1 (pMAL-annexinV) post-sonicate supernatants.

Some other experiments are currently underway to further investigate the function of Annexin V. Its probable role in regulation of the expression of the tumor-specific protein may make it as an intriguing subject of research[16]. This study only describes the procedure of expression and purification of recombinant Annexin V, which will be useful for further investigations of Annexin V. The purified Annexin V protein could be used as important experimental material for the studies that will be performed to gain insight into the functioning of Annexin V.

In summary, we reported a robust method to express, isolate and purify recombinant MBP-Annexin V fusion protein in bacterial cells. Due to its high soluble content, the Ca^{2+}-dependent phospholipid binding proteins have proven readily to express and isolate in sufficient quantities for structural and functional studies. The protocol which was used in this study allows the protein production of at least 2 mg per liter of culture, and also makes it possible for the structural studies of these proteins.

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Clinical Investigation in Improved Quality of Life with Medroxyprogestrone Acetate in Patients with Advanced Lung Cancer

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Abstract: Objective. This study was to investigate the effect of medroxyprogesterone acetate (MPA) in 50 cases with advanced lung cancer. **Methods.** 98 cases with advanced lung cancer were randomly divided into two groups, 50 cases of the therapeutic group undergoing routine chemotherapy plus taking MPA, and 48 cases of the controlled group undertaking routine chemotherapy alone. **Results.** In the therapeutic group, 78.0% cases showed improvement of appetite, 60.5% cases gained weight, and the score of Karnofsky rose 59.1% ($P<0.01$). **Conclusion.** MPA has beneficial effects to improve quality of life in patients with advanced lung cancer while its side effect is not obvious. [Life Science Journal. 2005;2(1):27-29] (ISSN: 1097-8135).

Keywords: MPA; chemotherapy; lung neoplasm; quality of life

1 Introduction

Dystrophia and increasing weight loss are common complications of advanced cancer, which are aggregated by Nausea, vomiting and Anorexia induced by chemotherapy, usually they constitute one of the main causes that prevent patients from continuing chemotherapy. To reduce the side effects of chemotherapy, improving quality of life in patients of advanced cancer during chemotherapy is one important aspect in present clinical investigation of cancer. From late 1980s, except intestinal and extraintestinal high nutrition and other supportive therapy can improve weight of patients with cancer, the synthesized drug of progesterone—Medroxyprogesterone Acetate (MPA) can remedy Anorexia and weight loss, so indirectly improved the effects of anti-tumor therapy. Now reports as follows from the results of clinically randomized investigation of our department in recent years.

2 Materials and Methods

2.1 Clinical data

98 patients with advanced lung cancer, from October 1998 to May 2002, were all diagnosed by histodiagnosis. There were 53 males and 45 females, the median age was 60.5 years old (From 17 to 78 years old), in which there were 32 patients with small cell lung cancer and 66 with non small cell lung cancer; 21 patientsin stage Ⅲ and 77 in stage Ⅳ. The average Karnofsky scores were 62.5 in the therapeutic group and 64.2 in the controlled group. The standards by which patients can be chosen were: ① not accepting hormonetherapy; ② without history of hypertension diabetes and thrombosis; ③ not accepting enteral or parenteral high nutrition (including transfusion of blood and some albuminoid biologic preparation); ④ with normal function of liver, kidney and blood routine.

2.2 Methods

Both chemotherapy schemes used cisplatin plus etoposide for two cycles at least. Patients in the therapeutic group began to have MPA of 500 mg twice a day, for 14 days continually when routine chemotherapy started. Patients in the controlled group were treated by routine chemotherapy plus usual supportive therapy. Improvement of appetite, changes of weight and changes of Karnofsky scores after treatment were investigated separately.

2.3 Statistic analysis

Statistic analysis adopted chi-square test (x^2-test).

3 Results

3.1 The amount of food that patients consumed and weight

The standard to evaluate the appetite of pa-

tients is: it can be divided into three groups on the basis of changes of the amount of food that patients consumed everyday after treatment. **Improvement**: the amount improved no less than 0.1 kg. **No change**: the amount change less than 0.1 kg. **Reduction**: the amount reduces no less than 0.1 kg. The result shows that appetite of patients began to improve after using MPA for 2 to 3 days. In which, the patients in the therapeutic group were 78.0% (39/50), and the patients in the controlled group were 10.4 (5/48). There was significance of difference by comparing the two groups (Table 1).

Table 1. Changes of appetite and weight in both groups

Group	Cases	Changes of appetite				Changes of weight			
		Improvement (cases)	Reduction (cases)	No change (cases)	Rate of effectiveness (%)	Improvement (cases)	Reduction (cases)	No change (cases)	Rate of effective (%)
The therapeutic group	50	39	2	9	78.0	32	3	15	64.0
The controlled group	48	5	31	12	10.4	6	3	39	12.5

3.2 Changes of Karnofsky score

Changes of Karnofsky scores can be classified into: improvement, the Karnofsky scores after therapy increased no less than 10; no change, the scores changed less than 10; reduction, the scores reduced on less than 10. The scores of Karnofsky in the therapeutic group increased in 29 patients, which is 58.0% (29/50). However the scores of Karnofsky increased in 11 patients in the controlled group, which is 22.92% (11/48). There was significance of difference by comparing both groups ($P<0.01$) (Table 2).

3.3 Bad effect

Among 50 patients that took MPA, four patients (8 percent) got slightly depressed edema and recovered after taking uretica and three patients' (6 percent) blood glucose increased. Among 23 female patients, 2 patients have vagina herrmorrhage after stopping taking MPA. Vascular obstructive disease, liver and renal function damage couldn't be found.

Table 2. Changes of scores of Karnofsky in both groups

Group	Cases	Scores reducing	Scores no change	Increasing 10	Increasing 20	Rate of effectiveness(%)
The therapeutic group	50	4	17	18	11	58.0
The controlled group	48	12	25	9	2	22.92

4 Discussion

Anorexia, weight loss, strength loss, hypoalbuminemia and so on are main factors that affect quality of life of advanced cancer patients. As a major therapy to conquer advanced cancer, chemotherapy is hindered because that myelosuppression and gastrointestinal tract toxicity caused by chemotherapy can reduce furtherly patient's quality of life. MPA is a kind of synthetic progestogen that can promote albumin assimilation. It is indicated in clinical study in recent 10 years that large dose of MPA can not only improve appetite, raise weight and promote albumin assimilation, but also palliate cancer pain, reduce toxicity and side effects that chemotherapeutic agents generate on marrow and gastrointestinal tracts and consequently develop quality of life during chemotherapy and tolerance to it completely. It is indicated that from 30 percent to 100 percent advanced cancer patients get negative nitrogen balance while MPA can improve appetite, raise absorption to protein, heat and sodium, keep positive nitrogen balance. In the therapeutic group, 39 (78.0 percent) patients showed improvement of appetite, 32 (60.5 percent) patients gained weight and 29 (58.0 percent) patients' score of Kamosfsky rose. Living quality of

therapeutic group improved more than control group significantly ($P<0.01$).

To sum up, cachectic syndrome like anorexia related with cancer and toxicity caused by chemotherapy are important factors that affects living quality of advanced cancer patients. Compared with singly using enternal feeding, parenteral hyperalimentation or other heteropathies that can improve advanced cancer patients' nutritional state and toxicity caused by chemotherapy, MPA can not only stimulate appetite, raise weight, promote albumin assimilation, raise strength and spiritual state but also be characterized by convenient use, no pain and long term use. So that, MPA can not only treat enzyme sensitive tumor but also improve quality of life of cancer patients during chemotherapy completely if it was accurately and reasonably taken by intermediate and advanced cancer patients. So it is an effective medicine worthy spreading to improve quality of life advanced lung cancer patients.

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Expression of the Antigenic Gene TspE1 of *Trichinella spiralis* in Skin and Muscle of BALB/c Mice

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Abstract: Trichinellosis is a serious zoonosis with a worldwide distribution and pork is still the most common source of human trichinellosis. Vaccines are needed to control this disease in swine. The recombinant eukaryotic expression plasmid, pcDNA3-TspE1, containing a gene encoding a 31 kDa antigen from *T. spiralis* was constructed. BALB/c mice were immunized with plasmid DNA vaccine by intramuscular injection and via gene-gun delivery. The transcriptional activity of the pcDNA3-TspE1 in skin and muscle at the site of inoculation was detected by RT-PCR using gene specific primers. Protein expression from the TspE1 gene in skin and muscles at the site of inoculation was detected by immunohistochemistry and indirect fluorescencent antibody test (IFAT), respectively. The results indicated that the recombinant plasmid pcDNA3-TspE1 was successfully transcribed and expressed in skin and muscle at the site of inoculation of mice. Thus, the plasmid encoding 31 kDa antigen may be of value for further development of a DNA vaccine against swine trichinellosis. [Life Science Journal. 2005;2(1):30-36] (ISSN: 1097-8135).

Keywords: *Trichinella spiralis*; DNA vaccine; gene-gun delivery; expression; mice

1 Introduction

Trichinellosis, one of the most serious helminthic zoonosis, is still considered to be endemic in many parts of the world. Human acquire the disease by ingesting raw or insufficiently cooked meat containing *Trichinella* larvae. Outbreaks of trichinellosis have occurred in many areas around the world over the past 20 years, even though the veterinary public health efforts have focused on the control and eradication of the disease for more than a century. The global prevalence of trichinellosis is difficult to evaluate, but it is estimated that as many as 11 million people may be infected (Dupouy-Camet, 2000). More than 10000 cases of human trichinellosis were reported by the International Commission on Trichinellosis from 1995 to June 1997 and about 10000 porcine infections were reported by the Office International des Epizooties in 1998 (Dupouy-Camet, 2000). Thus, trichinellosis has been regarded as emerging or re-emerging disease in both developed and developing regions (Murrell and Pozio, 2000). The disease is particularly problematic in some parts of China. Since the first patient with trichinellosis was recorded in Tibet in 1965 (Huang, 1965), there have been 548 outbreaks reported from 12 provinces, autonomous regions or municipalities (P/A/M) of China during 1966-1999 (Wang and Cui, 2001a). Pork is the main source of *Trichinella* infection for humans in China (Wang et al., 1998). The transmission of *T. spiralis* via household garbage is a main feature of the epidemiology of swine trichinellosis in China (Wang and Cui, 2001b). Vaccination of swine against *T. spiralis* could provide an alternative to prevent the risk of human infection. The development of vaccines capable of preventing swine from becoming infected would thus make a substantial contribution towards the ultimate goal of disease elimination.

In order to control trichinellosis, vaccines have been developed based upon irradiated- or ultraviolet-attenuated infective first stage larvae (Agyei-Frempong & Catty, 1983; Nakayama et al., 1998), autoclaved larvae (Eissa et al., 2003), antigens from different life-cycle stages (Darwish et al., 1996; Aucouturier et al., 2001), and a synthetic peptide (Robinson et al., 1995; McGuire et al., 2002). In each case, high levels of specific antibodies against *Trichinella* were induced in experimental animals, but failed to protect these animals fully against infection. In addition, the attenuated vaccine is impractical for field use.

DNA vaccination has been used to deliver a variety of parasite antigens to both small and large animal species, and the protective efficacy of antibodies generated in response to DNA vaccines has been shown in several challenge models (Donnelly et al., 1997; Rothel et al., 1997; Zhang et al., 2001;

Dumonteil et al., 2003). DNA vaccination has the attraction of enabling antigen expression to remain in a eukaryotic cell, increasing the probability that expressed antigen will be correctly glycosylated and generate conformation-specific antibodies. *Trichinella* antigens used as recombinant-protein vaccines have been identified, and demonstrated capable of eliciting host protective humoral immune responses (Arasu, et al., 1994; Sun, et al., 1994). In our previous study, the structural gene (TspE1) encoding 31 kDa antigen of *T. spiralis* was cloned and expressed in the prokaryotic expressing vector, pGEMEX-1. High specific antibody against the recombinant 31 kDa protein was identified by Western blot, and *Trichinella*-specific antibody was induced in mice immunized with the fusion protein (Cui et al., 2002). Production and analysis of a DNA vaccine encoding the 31 kDa antigen of *Trichinella spiralis* in skin and muscle of BALB/c mice is described in this paper.

2 Materials and Methods

2.1 Mice

Male BALB/c mice aged 4 – 6 weeks were obtained from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) and were raised in plastic micro-isolator cages before and after immunization. Anti-*Trichinella* antibodies were not detected by ELISA using secretory-excretory (ES) antigens of *T. spiralis* muscle larvae in all mice before immunization.

2.2 Parasite

T. spiralis used in this study was obtained from a swine source in the Henan Province of China and was maintained by serial passage in Sprague-Dawley (SD) rats every 6 – 8 months. Each rat was orally infected with 500 *T. spiralis* larvae.

2.3 Generation of anti-31 kDa fusion protein polyclonal sera

Anti-31 kDa fusion protein polyclonal mouse sera, used as positive control sera in IFAT, were obtained from BALB/c female mice injected intraperitoneally on two occasions 2 weeks apart with 60 μg (20 μg each injection) of 31 kDa fusion protein in complete Freund's adjuvant. Sera were collected 28 days after the second immunization and stored at −80℃ until use.

2.4 Plasmid construction

The recombinant eukaryotic expression plasmid pcDNA3-TspE1 was constructed as previously described (Cui et al., 2004). In brief, the larvae of *T. spiralis* in skeletal muscles were collected by acid-pepsin digestion. The target gene encoding the 31 kDa protein was prepared by RT-PCR from *T. spiralis* muscle larval RNA and cloned into the pUC18 vector. The positive clones were examined for the presence of a correct size insert by double digestion with *BamH* I and *Hind* III and single digestion with *Eco* RI, and also by PCR using gene specific primers. The target gene was sub-cloned into the eukaryotic expression vector pcDNA3 (Invitrogen, CA, USA). The pcDNA3 vector, which contains the human cytomegalovirus (CMV) promoter and an ampicillin-resistance gene, was used for the DNA vaccination studies. After transforming *Escherichia coli* strain JM109, the recombinant clones were selected on LB plates containing 100 μg/ml ampicillin overnight at 37℃ and plasmid DNA was extracted by the alkaline lysis method (Maniatis et al., 1982). The colonies containing inserts of the appropriate size in the right orientation were identified by electrophoresis of PCR products using gene specific primers. An 876 bp fragment was obtained after the recombinant plasmid pcDNA3-TspE1 was digested by *BamH* I and *Hind* III and as expected. Nucleotide sequencing was carried out to confirm the authenticity of the insert.

The recombinant plasmids were maintained and propagated in *E. coli* JM109 cells. The endotoxin-free plasmid DNA was purified from bacterial cells using a plasmid purification kit (Qiagen China Representative Office, Shanghai, China). The pcDNA3 plasmid without insert was also purified for use as a control. The purified plasmids were stored at −20℃ until use.

2.5 Production of DNA coated gold beads for immunization

Using protocols developed for the Hilos Gene Gun System (Bio-Rad, CA, USA), pcDNA3-TspE1 DNA and the control, pcDNA3 DNA, were precipitated onto gold beads (1.6 μm average diameter) and used to coat the inner surface of plastic tubing. The tubing was cut into a half inch length and stored dry at 48℃ until required. The quantity of gold and DNA comprising each immunizing "shot" was adjusted to produce the 1 mg DNA/0.5 mg gold "shots" for use in the immunization.

2.6 Immunization of mice

Male BALB/c mice, aged 4 – 6 weeks (10 per experimental group), were anesthetized and immunized. For intramuscular vaccination, 50 μg of pure plasmid DNA (in 50 μl of PBS) were injected into the left and right quadriceps of each leg, using a 1 ml tuberculin syringe fitted with a 28 G needle; The control animals received only PBS or pcDNA3. The mice were sacrificed 2 weeks after the primary intramuscular inoculation of DNA vaccine or pcD-

NA3 alone. For vaccination by gene-gun delivery, mice were anesthetized, their abdomens shaved, wiped with damp gauze and "shot" with the Gene Gun using 300 psi of helium gas. DNA vaccine doses of 3 μg (three shots) per mouse were used for epidermal inoculation. Controls mice were immunized similarly with pcDNA3 alone. The mice were sacrificed 4 h, 8 h, 24 h, 3 d, 5 d and 7 d after the initial inoculation of DNA vaccine or pcDNA3 alone by gene-gun delivery.

2.7 Detection of transcriptional activity of the pcDNA3-TspE1 in skin and muscles

To determine the duration and site of antigen presentation to the host, RNA was extracted from the skin and muscles of immunized mice and subjected to RT-PCR to detect evidence of transcription of the parasite DNA in transfected skin and muscle cells. Skin and muscles at the site of inoculation were excised at various intervals after DNA immunization, frozen on dry ice and stored at −80℃. The tissue was pulverized with mortar and pestle into powder and total messenger RNA was extracted using a commercial kit (Invitrogen, CA, USA). The extracted mRNA was treated with DNase I (RNase-free, Promega) to prevent PCR priming from plasmid a genomic DNA for 30 min at 37℃ and subjected to AMV reverse transcription reaction (Promega, WA, USA) for 60 min at 42℃. The reverse transcribed material was PCR amplified with primers specific to the encoding region of 31 kDa protein (using the thermal cycle protocol: 2 min 94℃; 35 cycles of 94℃ for 30 sec, 55℃ for 30 sec and 72℃ for 40 sec, and terminating with a 5 min extension step of 72℃). The resultant RT-PCR products were examined in 1.5% agarose electrophoresis gels.

2.8 Immunohistochemical staining

The expression of TspE1 gene in skin at the site of inoculation was assayed by immunohistochemical staining. The abdominal skin of vaccinated mice was excised and embedded in paraffin and 5 μm thickness of sections were prepared. After deparaffinization, the sections were processed to unmask the antigens by conventional microwave oven heating in 10 mg/ml citric acid buffer (pH 6.0) and subsequent detergent treatment using polyoxyethylene sorbitan monolaurate in PBS for 30 min. In order to observe the expression of TspE1 antigen in epidermis, immunohistochemical staining of 5 μm paraffin sections was performed with the Strept-Avidin- Biotin-Peroxidase complex (SABC) method (DGBio Co, Bejing, China). Briefly, 5 μm paraffin sections were treated with 3% hydrogen peroxide at room temperature for 10 min to remove endogenous peroxidase and incubated with 10% normal goat serum at room temperature for 5 min to block non-specific binding. The sections were then incubated with sera from mice infected with *T. spiralis* (diluted 1:50 in PBS) or normal mouse serum at 4℃ overnight. Section were washed in 0.01 M PBS three times and exposed to biotinylated goat anti-mouse IgG, followed by treatment with the SABC and stained with diaminobenzidine (DAB) with 0.15% hydrogen peroxide. Counterstaining was performed with haematoxylin.

2.9 Indirect fluorescencent antibody test (IFAT)

The expression of the TspE1 antigens in muscles at the site of inoculation in mice was detected by IFAT. The mice were sacrificed 2 weeks after the initial intramuscular inoculation of DNA vaccine and the muscles at the site of inoculation were separated and stored at −80℃. Then, 4 μm thick of frozen sections were cut, collected on gelatinized slides, and fixed with cold acetone for 10 min to prevent detachment of the sections during the following procedures. IFAT with frozen sections of muscles was carried out as previously described (Cui et al., 1999). Briefly, the frozen sections were incubated with 1:10 dilution of sera from mice immunized with recombinant fusion protein or infected with *T. spiralis* at 37℃ for 30 min. After washing with PBS, the sections were incubated with 1:16 dilution of goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) at 37℃ for 30 min. Sera from normal mice were used as negative control. Slides were examined under fluorescent microscope (Olympus, Tokyo, Japan). When a yellow green fluorescence staining appeared on the section, the reaction was defined as IFAT positive.

3 Results

3.1 Detection of mRNA for TspE1 in skin and muscle of DNA immunized mice

RT-PCR products were obtained only from the skin samples of the pcDNA3-TspE1 immunized mice at 8 h post-immunization (Figure 1), but not obtained at 4 h, 24 h and 72 h post-immunization. As expected, there was no TspE1 transcriptional activity in the skin samples from the control mice immunized with pcDNA3. These results demonstrate that detectable transcriptional activity of DNA transfected skin cells is very transient and confined to the site of immunization. mRNA was extracted from muscle of pcDNA3-TspE1 immunized mice 2 weeks following injection and was analyzed by RT-PCR using 31 kDa antigen-specific primers. The transcriptional activity of TspE1 gene

was clearly visible in muscle tissue from mice injected with pcDNA3-TspE1 but not in those mice injected with control plasmid (Figure 2).

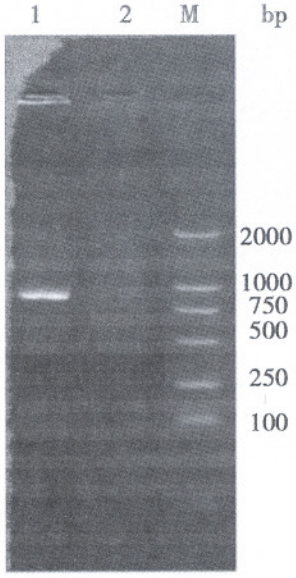

Figure 1. RT-PCR analysis of mRNA in skin of BALB/c mice immunized with pcDNA3-TspE1 and pcDNA3 plasmid. Mice were immunized with 3 μg of indicated plasmid DNA by gene-gun delivery. Skin at the site of inoculation was excised 8 h after the initial inoculation of DNA vaccine or pcDNA3 alone. The mRNA was isolated from the muscles and used for RT-PCR.
Lane 1: pcDNA3-TspE1; Lane 2: pcDNA3; Lane M: high molecular weight biomarker.

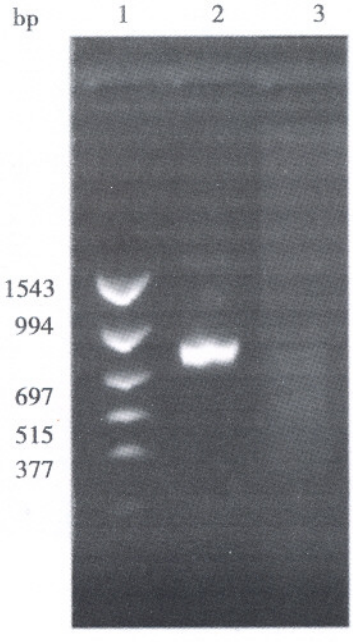

Figure 2. RT-PCR assay on RNA extracted from muscle two weeks after immunization with pcDNA3-TspE1 and pcDNA3 plasmid (1.5% agarose gel electrophoresis). Lane 1: DNA marker; Lane 2: RNA extracted from quadriceps muscle after immunization with pcDNA3-TspE1; Lane 3: RNA extracted from quadriceps muscle after immunization with pcDNA3.

3.2 Detection of TspE1 protein in skin at the site of inoculation

The results of immunohistochemical staining showed that specific brown round particles were seen among cells and in the cytoplasm of epidermial cells in all of the mice immunized with pcDNA3-TspE1 at 8 h post-immunization, but not in the mice immunized with pcDNA3 (Figure 3). Sections of *T. spiralis* muscle larvae as positive control were reacted with sera from the infected mice. No deep brown particles were observed when sections from the immunized and the infected mice were reacted with PBS or normal mouse sera (not showed in the figure).

3.3 Expression protein in muscle at inoculation sites of mice

The results of IFAT showed that the frozen section of inoculation site of mouse muscle 2 weeks after the initial intramuscular inoculation of pcDNA3-TspE1 reacted with sera from mice immunized with recombinant fusion protein or infected with *T. spiralis*. The fluorescence did not appear on the frozen section of inoculation site of mouse skin with only empty plasmid pcDNA3 (Figure 4).

4 Discussion

Both conventional protein vaccines and DNA vaccines (normally constituted of a naked DNA plasmid) elicit antibody responses, however, DNA vaccines have the additional advantage of stimulating cytotoxic T cells because the host is producing the antigenic protein intracellularly, thereby facilitating presentation of the antigen in the context of MHC class I molecules. Development of cellular immunity is important in fighting intracellular pathogens, giving DNA vaccines a clear advantage over protein vaccines. Another advantage of a DNA vaccine is its ease of administration and production. DNA is easily produced in large quantities with great purity, minimizing the risk of vaccine contamination with potential pathogens (McDonnell and Askari, 1996). Moreover, DNA can be readily introduced into tissues by DNA-coated microprojectiles through particle-mediated epidermal delivery (PMED), which facilitates easy epidermal administration and avoids the use of needles (Williams et al, 1991). Additionally, DNA vaccines may result in expressed antigens that resemble native antigens more closely than do antigens in conventional vaccines because manufacturing techniques can alter epitopes and reduce antigenicity. DNA vaccines may also be safer than some live attenuated vaccines, particularly in immunocompromised hosts.

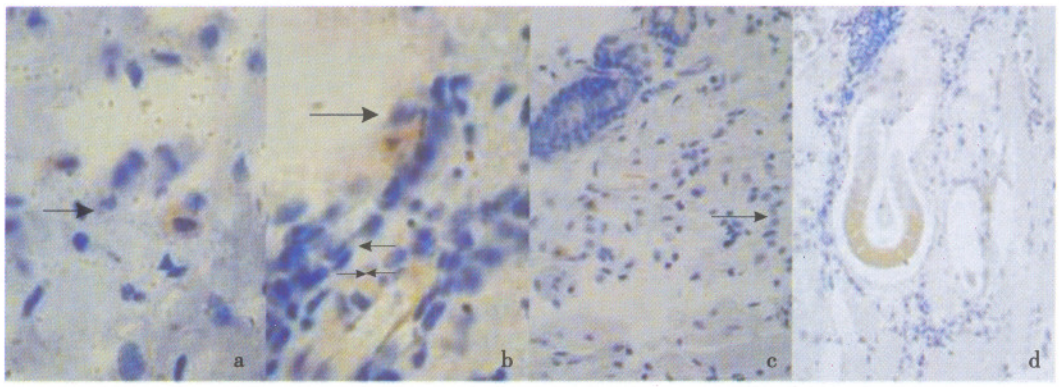

Figure 3. Immunohistochemical staining in skin of BALB/c mice inoculated with pcDNA3-TspE1 by gene-gun delivery.
a－b. Immunohistochemical staining of skin tissue of mouse at 8 h after gene-gun injection (1000×), arrows showing brown particles in cytoplasm (a) and extracells (b).
c. Negative control of pcDNA3 expressed in skin tissue of mouse at 8 h after gene-gun injection (400×).
d. Positive control of immunohistochemical staining of skeletal muscle of mouse infected with *T. spiralis* (400×), arrow showing the larva stained as brown.

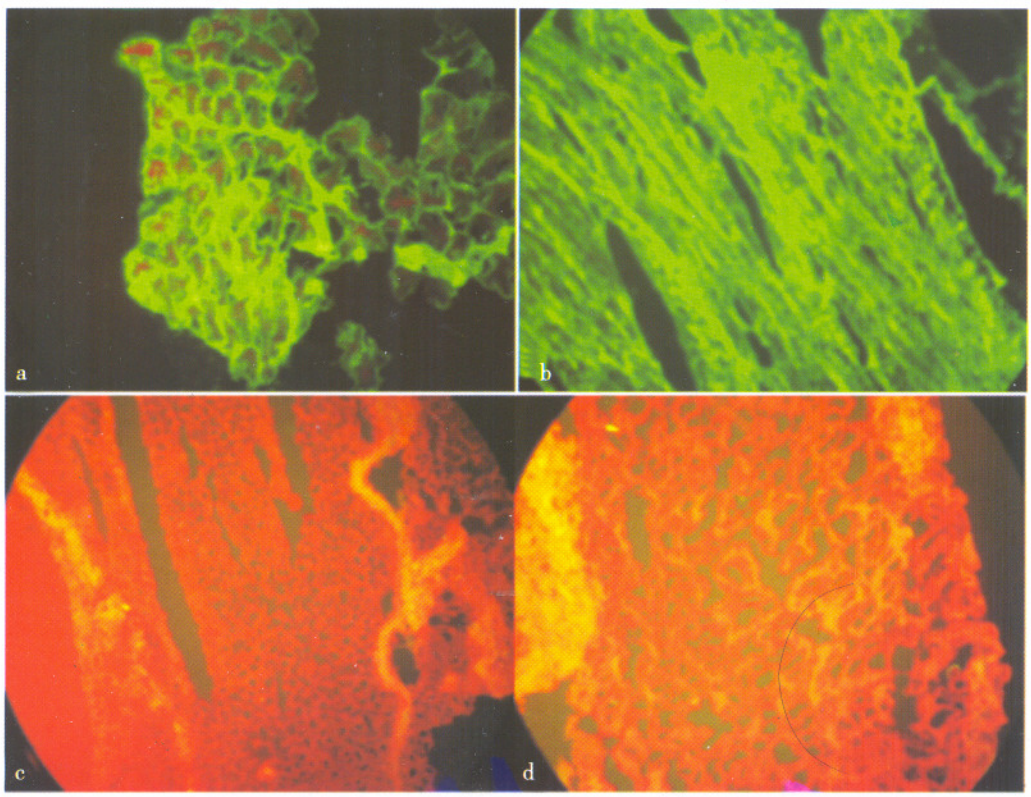

Figure 4. IFAT showing the antigenic protein expression of TspE1 gene in muscles 2 weeks after the initial intramuscular inoculation of recombinant plasmid pcDNA3-TspE1.
a. Section of muscle carrying pcDNA3-TspE1 reacted with sera from mice immunized with the recombinant fusion protein (200×).
b. Section of muscle carrying pcDNA3-TspE1 reacted with sera from mice infected with *T. spiralis* (200×).
c. Section of muscle carrying empty plasmid pcDNA3 reacted with sera from mice immunized with the recombinant fusion protein (100×)
d. Section of muscle carrying empty plasmid pcDNA3 reacted with sera from mice infected *T. spiralis* (200×).

Furthermore, several antigen genes could be included on one plasmid, reducing the total number of vaccinations that must be administered. Initial studies suggest that DNA vaccines can be administered safely and without overt toxicity (Tacket et al., 1999; Roy et al., 2001).

DNA immunization represents a novel method for generating the in situ expression of vaccine antigens and is now a widely reported means of generating immune responses. The administration of plasmid DNA via intramuscular, intradermal or gene gun delivery routes has been shown to stimulate T helper cells, cytotoxic T cells (CTL) and antibodies specific to the plasmid encoded antigen (Donnelly et al., 1997). The protective role for antigen-specific antibodies induced by genetic immunization has been manifestly demonstrated in a variety of challenge models against parasitic infection (Zhang et al, 2001; Dumonteil et al., 2003). However, up to date, DNA vaccines against Trichinella infection have not been reported in the literature.

We selected DNA vaccine as a strategy to prevent swine trichinellosis. The recombinant eukaryotic expression plasmid pcDNA3-TspE1 contained the gene encoding a 31 kDa antigen of *T. spiralis* was constructed. BALB/c mice were immunized with plasmid DNA vaccine by intramuscular injection and via gene-gun delivery. The transcriptional activity of the pcDNA3-TspE1 in skin and muscles at the site of inoculation was detected by RT-PCR using gene specific primers. The expression of TspE1 gene in skin and muscles at the site of inoculation was confirmed by immunohistochemistry and iFAT, respectively. These results showed that the recombinant plasmid pcDNA3-TspE1 was successfully transcribed and expressed in skin and muscles at the site of inoculation of mice. Thus, the plasmid encoding 31 kDa antigen may be of value for further development of DNA vaccine against challenge infection with *T. spiralis*.

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The Endocrine Disorder by Smoking Inhalation

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Abstract: The smoking inhalation reduced the secretion levels of blood serum, testosterone (T), luteinizing hormone (LH), and follicle stimulating hormone (FSH) of the rats. Prolactin level was reduced under the long-term regular smoking inhalation for female rats. The histology analysis of the testis of the male rats was performed to exam the degree of lesion by smoking. Conclusively results revealed that smoking inhalation causes the endocrine disorder. It took long time to recover from endocrine disorder if stopped smoking. [Life Science Journal. 2005;2(1): 37-39] (ISSN: 1097-8135).

Keywords: endocrine disorder; smoking; smoking inhalation

1 Introduction

Smoking is one of the important factors to affect the human health. The smoking may cause the organ system acting abnormally. Chemicals in smoking inhalation causing the endocrine disorder of the human body have been discussed for two decades. The main purpose of this report is to confirm the levels of endocrine disorder by smoking.

2 Material and Method

36-male and 36-female Wistar rats were divided into three types of group as smoking inhalation, control and natural recovery group respectively. Each group has 12 rats and the weight of them was between 180 g to 220 g. All the animals were provided by Henan Experimental Animal Center (Zhengzhou, China). Hongxi cigarette, made in Henan Ruzhou Cigarette Manufacture Company, and the home-made inhalation box, sized as 1740 mm × 1100 mm × 1500 mm with two 2 mm × 3 mm air holes on the two vertex of topside diagonal line were used to provide smoking inhalation. The nicotine amount of Hongxi cigarette is 1.1 mg. The amount of tar is 17 mg per 84-mm-long cigarette as well. The experimental test kits of testosterone (T), luteinizing hormone (LH), follicle stimulating hormone (FSH) and prolactin were provided by Tianjin Depu Biomedical Technique Company. The SM-696 γ-measure machine was made in Shanghai Nuclear Research Institute, Rihuan Equipment Manufactory Company. Six rats were kept in one cage. Two cages designed to fit into the smoking inhalation box. Four pieces of cigarette were tightened as a bundle. A piece of bundle cigarettes were hanged and burned for smoking in the cage. Changing the bundle-cigarette every 15 minutes. It was scheduled to burn up 32 pieces of cigarette in 50 minutes, twice a day for first 38 days. And then once a day for another 38 days for 76 days, we sacrificed the animals by coeliac-injection of ketamine for checking the levels of T, LH, FSH and prolactin for female and the visceral coefficients of the testis and epididymis for male. The data were analyzed by SPSS 10.0 program with u test (Ridit analysis), student t test and expressed the results as $x + s$ (standard error). Visceral coefficient is defined as the ratio of the weight of organ to the body. The histology study was followed up by the standard procedures.

3 Results

Table 1 and Table 2 have shown the level of T, LH, FSH and prolactin in control, natural recovery and smoking inhalation group. The natural recover group did not show any significant difference in comparison with the control ($P > 0.05$). Others have shown the significant difference. The data of visceral coefficients of testis and epididymis for male rats ($x \pm s$) presented in Table 3. Figure 1 depicted the normal histology structure of testis for rats control group. Spermatogonium cells, Stertoli cells, primary spermatocytes and secondary spermatocyte all could be seen in seminiferous tubles. Sperms were in tubli recti and Laydig cells were also seen in rete testis. Minor lesions were found in Figure 2. Histoplasmosis and less sperms were depicted for 50% rats in smoking inhalation group. In Figure 3, seminiferous tubles presented to be

thinner than normal, much less primary spermatocytes and secondary spermatocytes were found. Sperm and sperm cells disappeared. However, Spermatogonium cells still could be seen for 25% rats in smoking inhalation group. Necrosis of spermatocyte was seen clearly. In Figure 4, all cells necrosis was seen clearly in serious lesions for 25% rats in smoking inhalation group. For natural recovery group, in comparison with smoking inhalation group, we were not able to find significant difference. All the lesions were very similar ($P>0.05$).

Table 1. In comparison to the level of T and LH for female rats ($x \pm s$)

Type of the group	n	T (n mol/L) level	LH (IU/L) level
Control	12	4.86 ± 2.29	1.74 ± 0.66
Smoking inhalation	12	$2.1 \pm 1.12^{**}$	$1.06 \pm 0.41^{*}$
Natural recovering	12	$2.3 \pm 0.97^{**}$	$1.18 \pm 0.36^{*}$

Note: in comparison with control statistically, $*P<0.05$, $**P<0.01$

Table 2. In comparison to the level of FSH and Prolactin for female rats ($x \pm s$)

Type of the group	n	FSH (IU/L)	Prolactin (μ g/L)
Control	12	1.45 ± 0.34	2.76 ± 1.08
Smoking inhalation	12	$1.02 \pm 0.31^{*}$	3.13 ± 1.16
Natural recovering	12	$1.06 \pm 0.33^{*}$	3.08 ± 1.21

Note: in comparison with control statistically, $*P<0.05$

Table 3. The visceral coefficients of testis and epididymis for male rats ($x \pm s$)

Type of the group	n	Testis (g/kg)	Epididymis (g/kg)
Control	12	9.78 ± 1.29	4.45 ± 0.33
Smoking inhalation	12	$8.59 \pm 0.98^{*}$	$4.05 \pm 0.26^{**}$
Natural recovering	12	$8.66 \pm 0.79^{*}$	$4.10 \pm 0.27^{*}$

Note: in comparison with control statistically, $*P<0.05$, $**P<0.01$

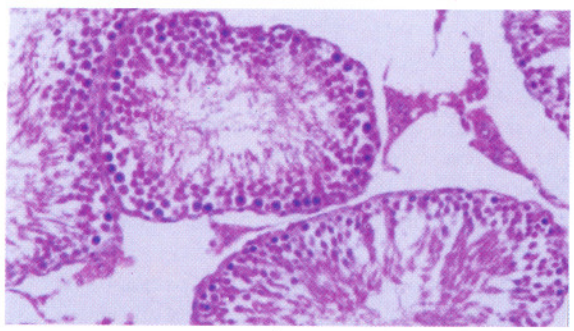

Figure 1. The normal testis histology microscopic picture (HE×400)

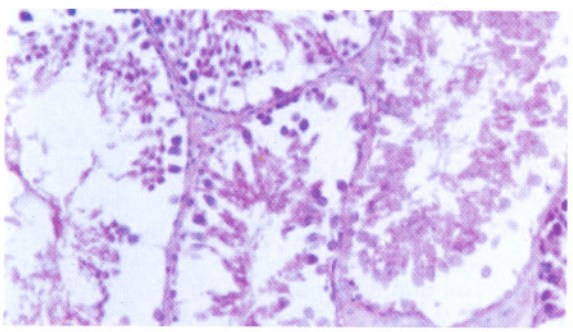

Figure 2. Minor lesions of the testis of the male rat caused by smoking inhalation (HE ×400)

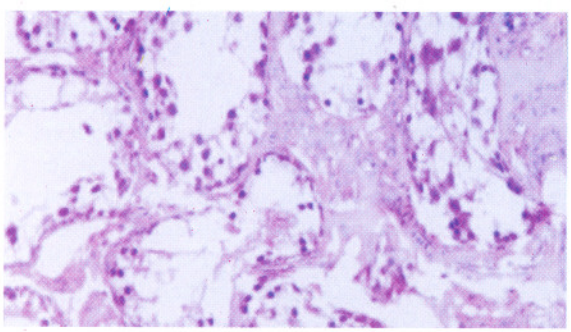

Figure 3. The second degree lesion of the testis of the male rats (HE×200)

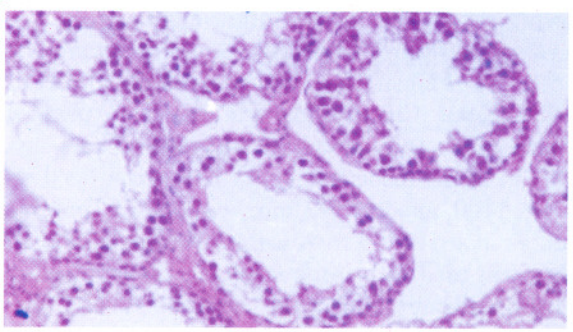

Figure 4. The serious lesion of testis of the male rat under smoking inhalation (HE ×200)

4 Discussion

The effect of the smoking inhalation to human reproductive endocrine, special for the male becomes more and more concern to the most of smokers. The experimental data revealed that the quality and quantity of the sperms being influenced by smoking[2,3]. This report also revealed that the smoking could cause the different degrees of lesion on testis of the male rats, which is consistent to the research results all around the world[4]. The cigarette smoking includes about 400 different chemicals that may affect the human organ[5,6]. The mechanism of the lesion occurred in testis

tissue may be originated by the long term increasing of anti-oxidizes[7] and the level of plasma endothelin-1[8], which may cause the vasoconstriction to insufficiently provide the blood to the organ. This may be the reason that smoking can cause the cell shrinkage of the testis. By Goerre, plasma endothelin-1 is the chemical to cause most vasoconstriction. Plasma endothelin-1 causes vasoconstriction in organs including testis of the male rat. Therefore, the supply of the blood may be insufficient for the function of testis.

5 Conclusion

This report presents the result of decreasing body level T, which is consistence with the research result that Wei did in the year of 2000[9,10]. The synthesis of testosterone is in the endoplasmic reticulum (ER) of interstitial cell and mitochondria. In the mean time, the synthesis of testosterone is also involved hypothalamic-pituitary unit. Zhou[11] suggested HCG may inhibit the secretion of testosterone and was confirmed by our experiments[12]. The smoking may also affect the characteristics of Leydig cells, which indirectly affect the secretion of testosterone and caused the decrease of serum concentration. The analysis of the data from natural recovery group revealed the existence of the mechanism for recovery if smoking is stopped in the long run. However, the detail mechanism is still not clear and the recovery effect was slow in comparison with the damage of the organs. The further research will be focused on the mechanism of the recovering process of the stopped smoking.

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Study of Physiologically Required Selectivity Coefficients of Potentiometric Sensors in Clinical Assays

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Abstract: Required selectivity coefficients of ion-selective electrodes (ISEs) in physiological analysis are studied based on the conventional Nicolski-Eisenman equation and a newly proposed consistent approach with the worst allowable error (1%). The physiologically required selectivity coefficients from both methods are compared in this work. The revised consistent approach gives out a more reasonable description of selectivity even in case of the unequally charged primary and interfering ions. An Mg^{2+}-selective electrode using ionophore ETH 5504 is tested to measure the selectivity of free Mg^{2+} against the interfering ions in physiological levels. Obviously the required selectivity coefficients derived from the consistent approach shows the more accurate description of selectivity than the conventional one. For evaluating the applicability of ISEs in physiological solution, the consistent required selectivity coefficients provide the reasonable criteria especially regarding the primary and interfering ions of different charges. [Life Science Journal. 2005;2(1):40-45] (ISSN: 1097-8135).

Keywords: selectivity coefficients; ion selective electrodes; magnesium; physiological assay; ETH 5504

1 Introduction

Potentiometric ion-selective electrodes (ISEs) based on neutral inonphore are widely used in clinical assays and also integrated in diagnostic analyzers[1-3]. The selectivity pattern of any ion-selective electrode is clearly the most important character that often reflects whether a sensor may be reliably employed in analysing the target electrolyte over other coexisting interfering ions. One important application of ISE is detecting electrolyte ion activities in whole blood or serum in which a small concentration range of electrolyte extremely mandates the measuring accuracy. The ISE should achieve a sufficient selectivity pattern of the primary ion over coexisting interfering ions (secondary ions) to meet the physiological requirement of worst allowable error of 1%[4]. Conventionally, the required selectivity coefficients for physiological analysis are derived from the well-known semi-empirical Nicolsky-Eisenman (N-E) equation[5,6]:

$$E = E_i^0 + \frac{RT}{z_i F}\ln(a_i + K_{ij}^{pot} a_j^{z_i/z_j}) \qquad (1)$$

where E is the measured electromotive force (EMF) of the electrode in the sample, all constant potential contributions are included in E_i^0; K_{ij}^{pot} is the selectivity coefficient; z_i and z_j are the charges of primary ion I and interfering ion J; a_i and a_j are the sample activities of I and J; and R, T and F have their usual meanings. It is well known that the selectivity coefficient is, in theory, a constant parameter for a particular electrode, irrespective of the charges of primary and interfering ions since K_{ij}^{pot} is a function of E^0 values respectively for two ions of interest.

Although the N-E equation is, with some exceptions[7,8], accurate for ions of the same charge, the selectivity coefficient of ISEs derived from this equation may lead to an inconsistent description for unequally charged primary ion and interfering ion. For instance, taking primary ion as interfering ion and vice versa does not give the same predicted electrode potential[9]. In fact, when the monovalent and divalent ions considered as primary and interfering ions, this discrepancy can amount to 8 mV or more than 30% activity variation. Such discrepancy of selectivity is a serious limitation of N-E equation that may lead to wrong practical predictions. A lot of discussions on this problem have been conducted and many new modification approaches of N-E equation have also been proposed[8,10,11]. In 1998, a consistent formalism was proposed by Zhang et al[9] with modifying the conventional N-E equation with introduction of consistent selectivity coefficient K_{ij}^{cons}

$$E = E_i^0 + \frac{RT}{F}\ln(a_i^{1/z_i} + K_{ij}^{cons} a_j^{1/z_j}) \qquad (2)$$

where K_{ij}^{cons} is defined as consistent selectivity coefficient and

$$K_{ij}^{cons} = (K_{ij}^{pots})^{1/z_i} \qquad (3)$$

With this modification, the selectivity coefficient K_{ij}^{cons} is completely independent of the charge number of the ions involved.

In measuring ion activity in intercellular fluid samples, ion-selective electrode should specifically respond to the activity of target ion with a sufficient discrimination over the co-existing interfering ions. The minimum selectivity requirement of ISEs determines the applica-

bility of certain ISEs and the accuracy of assays. The physiologically required selectivity coefficients are calculated assuming an allowable error of 1% (0.01) in the worst case of a maximum quantity of interfering ion and a minimum quantity of primary ion within physiological range.

Traditionally, the required selectivity coefficients are calculated based on the conventional Nicolsky-Eisenman equation, assuming an allowable error of 1% (0.01) in the worst case of a maximum quantity of interfering ion and a minimum quantity of primary ion within the physiological range by the following equation[12]:

$$(K_{ij}^{pot})_{required} \leqslant 0.01 \cdot \frac{a_{i,min}}{a_{j,max}^{z_i/z_j}} \quad (4)$$

or

$$\log(K_{ij}^{pot})_{required} \leqslant \log(0.01 \cdot \frac{a_{i,min}}{a_{j,max}^{z_i/z_j}}) \quad (5)$$

When electrode is calibrated by physiological background solutions, the variations of interfering ion activity around its medium value need to be considered. The difference between the activity of the calibrator $a_{j,cal}$ and the unknown solution a_j, $(a_{j,cal}^{z_i/z_j} - a_j^{z_i/z_j})$, contributes to the actual interference. Then, Eq. (5) can be then changed to:

$$\log(K_{ij}^{pot})_{required} \leqslant \log\left(0.01 \cdot \frac{a_{i,min}}{a_{j,cal}^{z_i/z_j} - a_j^{z_i/z_j}}\right) \quad (6)$$

Obviously, the required selectivity coefficients in Eq. (5) and (6) are inaccurate in case of unequal charge numbers of the concerned ions ($z_i \neq z_j$), which is originated from the discrepancy of the conventional N-E equation.

In order to get the consistent selectivity criteria for physiological assay with ISEs, the consistent required selectivity coefficients are derived from the newly modified N-E equation for a given allowed relative error, 1% (0.01), in the measurement of physiological solution[2]:

$$\log(K_{ij}^{cons})_{required} \leqslant \log\left(0.01 \cdot \frac{a_{i,min}^{1/z_i}}{a_{j,max}^{1/z_j}}\right) \quad (7)$$

With the physiological background calibration, the consistent required selectivity coefficient is described as:

$$\log(K_{ij}^{cons})_{required} \leqslant \log\left(0.01 \cdot \frac{a_{i,min}^{1/z_i}}{a_{j,max}^{1/z_j} - a_j^{1/z_j}}\right) \quad (8)$$

It is shown that Eq. (7) and (8) are obviously different from Eq. (5) and Eq. (6). For primary and interfering ions of different charges ($z_i \neq z_j$), the consistent required selectivity coefficients $\log(K_{ij}^{pot})_{required}$ gives out the reliable criteria to determine the applicability of ISEs, which gets rid of the discrepancy from the conventional N-E equation. In this work, an Mg^{2+}-selective electrode with iononphore ETH 5504[13] was used to check the selectivity coefficient in physiological measurements.

2 Experiments

2.1 Reagents

All chemicals were of analytical reagent grade or higher purity. The following chemicals were purchased from Fluka Chemie AG, Buchs, Switzerland: $MgCl_2$, $CaCl_2$, KCl, NaCl, KT*p*ClPB (potassium tetrakis (4-chlorophenyl)borate), PVC (poly(vinyl chloride), high molecular weight) and tetrahydrofuran (THF, distilled prior to use). The Mg^{2+}-selective ionophore ETH 5504 (1,3,5-tris[10-(methyl-7,9-dioxo-6,10-diazadecyl) benzene]) and the plasticizer ETH 8045 (12-(4-ethylphenyl)dodecyl-2-nitrophenyl-ether) were synthesized as described in[2,14]. Doubly distilled water was used throughout.

2.2 Membrane preparation and EMF measurement

The PVC-based Mg^{2+}-ISE membrane was prepared as described elsewhere[13,15] using 1 wt% Mg^{2+}-selective ionophore; 155 mol% (relative to the ionophore) lipophilic anionic sites KT*p*ClPB; 65 wt% plasticizer, ETH 8045; and 33 wt % PVC. The working electrode was assembled by cutting disks of 7 mm diameter out of the membrane (thickness is about 150 μm). The disk was mounted into a Phillips electrode body (ISE-561, Glasbläserei Möller, Zürich). A solution of 0.1M $MgCl_2$ was used as the internal filling solution for the Mg^{2+}-selective electrode.

After conditioning the electrode in 0.1M $MgCl_2$ for 24 hours, EMF measurements were conducted with following cell: Hg, Hg_2Cl_2 | KCl (satd.) MMKCl (3.0 M) MM sample solution ‖ membrane ‖ internal solution ($MgCl_2$, 0.1 M) |AgCl, Ag.

The calomel reference electrode corresponded to the free-flowing double-junction type[16]. The cell potential was measured using an Apple IIGS (Apple Computer, Cupertino, CA, USA) equipped with a 7150 Digital Multimeter (Solartron Instrumentation, UK). The single-ion molal molar activities in physiological electrolyte situation were calculated using the Pitzer program[17]. The selectivity coefficients were measured based on the IUPAC recommended procedure of separate solution method. All EMF values were corrected for changes in the liquid-junction potential, E_j, using the Henderson formalism[18]. All potentiometric measurements were performed at ambient temperature (~25°C).

2.3 The main cations in physiological solution

In physiological fluids, the main cations are Na^+,

K^+, Ca^{2+} and Mg^{2+} which play important roles in biological functions. In human blood serum, the intercellular concentrations of these cations vary in certain ranges that are listed in Table 1. In calculating the required selectivity coefficients of ISEs for physiological assays, an allowable measuring error of 1.0% is considered with minimum level of a physiological molar activity. The required selectivity coefficients for detecting main inorganic cations in physiological solution are calculated assuming an allowable error of 1.0% at the minimum level of a physiological molar activity of the detecting ion. The sensors are measured with- and without the calibration of physiological ion background (Dc, assuming variation of the background ion molality level around the mean physiological points, Table 1).

The calculation without background calibration (Eq. (5) and Eq. (7)) is based on the maximum level of interfering ion $a_{j,\max}$ and the minimum level of primary ion $a_{i,\min}$. For obtaining the required selectivity coefficients with the background calibration (Eq. (6) and Eq. (8)), the minimum level of the primary ion $a_{i,\min}$ and the medium level of the interfering ion $a_{j,\mathrm{cal}}$ is considered for the background calibration.

3 Results and Discussion

3.1 Comparison of methods for calculating the required selectivity coefficients

The required selectivity coefficients of ISE for different primary ions are calculated based on the conventional N-E equation and the modified consistent N-E equation. The obtained required selectivity coefficients with and without the physiological background calibration (Eq. (6) and Eq. (8)) are compared in Table 2.

Table 1. The main cation levels in physiological solution

Cation		min. level	Medium level	max. level
Mg^{2+}	Concentration c (M)	3.0×10^{-4}	4.5×10^{-4}	6.0×10^{-4}
	molar activity a (M)	1.1×10^{-4}	1.6×10^{-4}	2.1×10^{-4}
Ca^{2+}	Concentration c (M)	1.0×10^{-3}	1.15×10^{-3}	1.3×10^{-3}
	molar activity a (M)	3.4×10^{-4}	3.9×10^{-4}	4.4×10^{-4}
K^+	Concentration c (M)	3.3×10^{-3}	4.05×10^{-3}	4.8×10^{-3}
	molar activity a (M)	2.4×10^{-3}	2.9×10^{-3}	3.5×10^{-3}
Na^+	Concentration c (M)	0.134	0.138	0.143
	molar activity a (M)	0.099	0.103	0.106

Table 2. Comparison of the required selectivity coefficients of ISEs for measuring the main inorganic cations in physiological solutions, assuming an allowable error of 1.0% at the lower level of the physiological range of the detecting ion.

	Conventional SSM	$(\log K_i^{\mathrm{pot}})_{\mathrm{required}}$		Consistent SSM	$(\log K_i^{\mathrm{pot}})_{\mathrm{required}}$	
Primary ion	Secondary ion	no calibration	with calibration	Secondary ion	no calibration	with calibration
Mg^{2+}	Ca^{2+}	−2.4	−1.5	Ca^{2+}	−2.2	−1.0
	K^+	−0.9	−0.3	K^+	−1.4	−0.6
	Na^+	−3.8	−2.6	Na^+	−2.9	−1.4
Ca^{2+}	Mg^{2+}	−1.8	−1.1	Mg^{2+}	−1.9	−0.9
	K^+	−0.6	−0.0	K^+	−1.3	−0.5
	Na^+	−3.5	−2.3	Na^+	−2.8	−1.2
K^+	Mg^{2+}	−2.8	−1.8	Mg^{2+}	−2.8	−1.8
	Ca^{2+}	−2.9	−1.7	Ca^{2+}	−2.9	−1.7
	Na^+	−3.6	−2.1	Na^+	−3.6	−2.1
Na^+	Mg^{2+}	−1.2	−0.2	Mg^{2+}	−1.2	−0.2
	Ca^{2+}	−1.3	−0.1	Ca^{2+}	−1.3	−0.1
	K^+	−0.5	0.3	K^+	−0.5	0.3

Because the modified consistent N-E equation removes the influence from the ion charge-dependence, the consistent required selectivity coefficient $(K_{ij}^{cons})_{required}$ reasonably illustrates the preference of the sensor to the primary ion over interfering ions, especially for measuring the unequally charged primary and interfering ions. It can be seen from Table 2 and Table 3 that when monovalent ion (K^+ or Na^+) is considered as the primary ion, two methods give out the equal required selectivity coefficients $(K_{ij}^{cons})_{required} = (K_{ij}^{pot})_{required}$. However, the consistent coefficient values of $\log (K_{ij}^{cons})_{required}$ for divalent primary ions (Mg^{2+} or Ca^{2+}) are significantly different from the conventional ones of $\log (K_{ij}^{pot})_{required}$. The logarithm of the required selectivity coefficient for Mg^{2+} over Ca^{2+} changes from -2.4 to -2.2 and the one for Mg^{2+} over K^+ from -0.9 to -1.4. As well, the $\log (K_{ij}^{pot})_{required}$ for Mg^{2+} over Na^+ decreases from -3.8 to -2.9. Such differences lead to the necessity of applying the consistent formulism of the required selectivity coefficient for divalent primary ions.

3.2 Mg^{2+}-selective electrode and the required selectivity coefficients in physiological analysis

In recent years, studies of developing selective Mg^{2+} potentiometric sensors have been intensively conducted in many groups and the adequate discrimination between Mg^{2+} and Ca^{2+} is still the main target[19-21]. Suzuki et al[22] synthesised a selective Mg^{2+} ionophore K22B5 which logarithm of selectivity coefficient of Mg^{2+} over Ca^{2+} was reported as -2.5 measured with the IUPAC recommended separate solution method (SSM)[23]. The inadequate selectivity against monovalent cations (Na^+ and K^+) of this kind of Mg^{2+} ionophores made the corresponding Mg^{2+} membrane electrodes hard to be applied in physiological assays. Spichiger et al developed an Mg^{2+} selective electrode based on ionophore ETH 5504 which has high selectivity over Ca^{2+}, K^+ and Na^+ while its high lipophilicity prevents ETH 5504 leaching from plasticized PVC membrane. So far, many Mg^{2+} selective microelectrodes based on various Mg^{2+} ionophores have also been reported in measuring the physiological systems[24-28].

In serum samples, the intercellular Mg^{2+} molar activity range (0.46～0.66 M) is much lower that Ca^{2+} level range (1.01～1.26 M) and the discrimination of Mg^{2+} over Ca^{2+} is very critic for accurate measurement of Mg^{2+} in physiological assays. The selectivity coefficients of Mg^{2+}-selective electrode based on ionophore ETH 5504 was determined according to the IUPAC recommended separate solution method (SSM)[23] as well as the newly modified consistent SSM[9].

In order to ensure the selectivity coefficients of Mg^{2+}-ISEs to reflect the "worst case" in physiological situation, the minimum level of Mg^{2+} and the maximum levels of interfering ions were used in calculating the selectivity coefficients. EMF values of the minimum physiological level Mg^{2+} were compared with that of maximum physiological levels of other interfering ions, Ca^{2+}, Na^+ and K^+ separately (Figure 1). Two sets selectivity coefficients for Mg^{2+} – ISE, $\log K_{Mg,J}^{pot}$ and $\log K_{Mg,J}^{cons}$, were calculated based on both the conventional N-E method and the modified consistent N-E method. As shown in Figure 2, the selectivity coefficients (SSM) of Mg^{2+}-selective electrode based on both methods were obtained by using electrolyte solutions of standard concentration (0.1 M) and of physiological medium levels of primary and interfering ions (Table 1).

It can be seen from the results in Figure 2 that the selectivity pattern of Mg^{2+}-selective electrode with ETH 5504 were measured based on both approaches of the conventional and the modified consistent approaches. The selectivity of Mg^{2+} against Ca^{2+} reaches -1.0 ～ -1.8 with conventional SSM approach and -1.0 ～ -1.2 with the consistent method. Both values satisfied the physiologically required selectivity coefficients calculated from Eq. (6) (conventional approach, $\log(K_{ij}^{pot})_{required} = -1.5$) and Eq. (8) (consistent approach, $\log(K_{ij}^{cons})_{required} = -1.0$) with physiological background calibration. However, for the direct measurements without background calibration, the selectivity still has space to improve. Regarding the interfering monovalent cations of K^+ and Na^+, this Mg^{2+}-ISE showed the adequate selectivity over K^+ even for non-calibration measurement of Mg^{2+} either with conventional method ($\log(K_{ij}^{pot})_{required} = -0.9$) and consistent method ($\log(K_{ij}^{cons})_{required} = -1.4$). However, the discrimination against Na^+ of this Mg^{2+}-ISE also requires the physiological background calibration so as to satisfy the physiological requirement (with background calibration, $\log (K_{ij}^{pot})_{required} = -2.6$ and $\log(K_{ij}^{cons})_{required} = -1.4$). According to this comparison, it is obvious that the physiological background calibration is really needed for using the Mg^{2+}-ISE with ionophore ETH 5504 to measure free intercellular Mg^{2+} level. This requirement can predict the necessary enhancing space of selectivity pattern for developing future Mg^{2+}-ISEs in direct measurements in physiological assays.

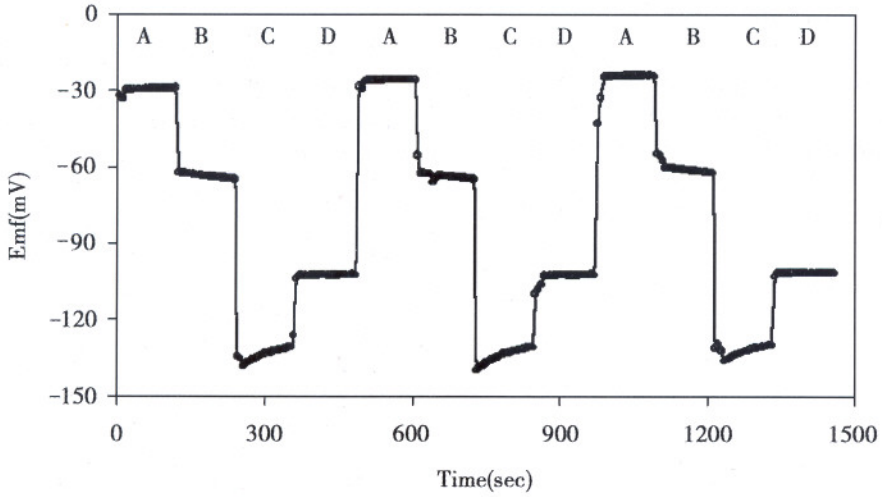

Figure 1. Selectivity measurement in the "worst case" for Mg^{2+}-ISE in physiological electrolyte situation
A:0.3 mM $MgCl_2$; B:1.3 mM $CaCl_2$; C:4.8 mM KCl; D:140 mM NaCl.

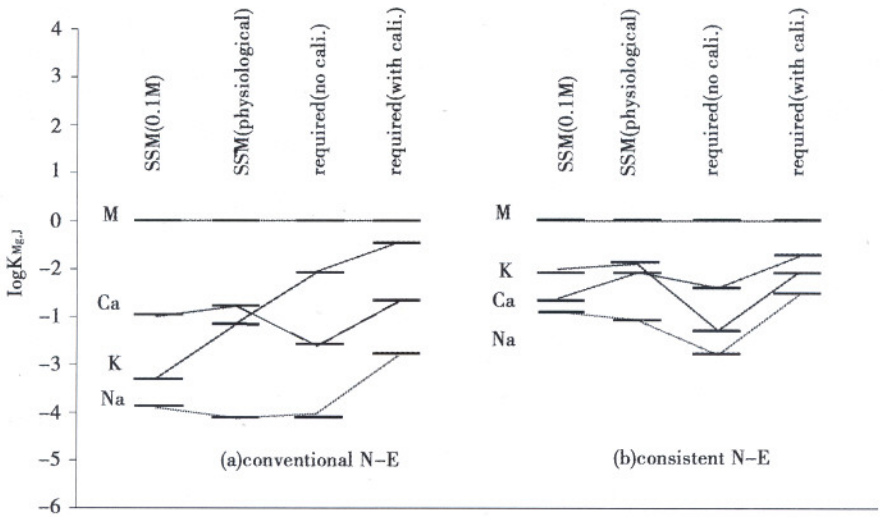

Figure 2. The comparison of the Mg^{2+}-selectivity coefficients with required selectivity coefficients.
(a) Conventional N-E method; (b) Consistent N-E method.

4 Conclusion

In conclusion, the physiologically required selectivity coefficients of ISEs for physiological analysis were studied by comparing the conventional N-E method and the modified consistent N-E method. The modified consistent method eliminates the discrepancies originated from the conventional N-E equation for unequally charged primary and interfering ions. Indeed, the consistent required selectivity coefficients for the divalent primary ion (Mg^{2+} or Ca^{2+}) gives out a more accurate calculation that the conventional one and provides a reasonable criteria for evaluating the applicability of ISEs in physiological assays. However for the monovalent primary ion (K^+ or Na^+), the required selectivity coefficients calculated with two methods are identical. For comparing the performances of ISE in the physiological solution with the required selectivity pattern, the electrode was checked in the "worst case" of minimum level of primary ion and maximum level of interfering ion in physiological solution. Indeed, the modified consistent required selectivity coefficients are more accurate than the ones derived from the conventional N-E equation, which provides the more reliable criteria for evaluating the applicability of ion-selective electrodes in physiological assays.

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Comparison of Patients' Psychological Status between Controlled Seizures and Uncontrolled Seizures by Symptom Checklist 90

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Abstract: Purpose. Psychological treatment is one of the most important treatment options available to patients with epilepsy. It is vital to determine whether long-term psychological treatment is necessary. The purpose of this study is to compare the psychological status of patients' in which seizures are controlled with that of patients who experience uncontrolled seizures by Symptom Checklist 90 (SCL 90). **Method.** Patients with epilepsy were divided into two groups: Controlled seizure group ($n=64$, seizure free 1－5 years), and uncontrolled seizure group ($n=61$). The two groups were matched in gender, age, duration of disorder, education and personality (Eysenck personality questionnaire, EPQ). SCL 90 was used to test psychological status and a social support questionnaire used to assess social support status. **Result.** Compared with the average in the Chinese population, the factor scores of somatization (mean 1.65±0.57 SD), obsessive-compulsive (mean 2.08±0.79 SD), depression (mean 1.90±0.85 SD), anxiety (mean 1.80±0.74 SD), hostility (mean 1.84±0.97 SD), photic anxiety (mean 1.58±0.59 SD), and psychotic tendencies (mean 1.60±0.59 SD), were significantly higher in the uncontrolled seizures group ($P<0.05$); the factor scores of obsessive-compulsive (mean 2.01±0.65 SD), hostility (mean 1.68±0.61SD), photic anxiety (mean 1.41±0.42 SD), and psychotic tendencies (mean 1.53±0.55SD) were significantly higher in the controlled seizure group ($P<0.05$). However, only the factor score of somatization was significantly higher in the uncontrolled seizure group than that of the controlled seizures group (mean 1.33±0.25 SD, $P<0.05$). At the same time, there was no significant difference in social support between the two groups ($P>0.05$). The score of each group was below 35 (mean 25.67±4.97 SD for controlled seizures and mean 24.38±4.53 SD for uncontrolled seizures). This result indicates that both groups were short of good social support. **Conclusion.** Patients with epilepsy need long-term psychological treatment and good social support. [Life Science Journal. 2005;2(1):46－48] (ISSN: 1097－8135).

Keywords: epilepsy; seizure; psychology; Symptom Checklist 90; social support

1 Introduction

Epilepsy is a chronic neurological disease. As in other chronic conditions, more attention should be paid to the quality of life (QOL) of patients with epilepsy. Psychological functioning is one of the most important factors in the health-related quality of life model[1]. Lots of studies show that there are psychological disorders in the patients with epilepsy. But there are few articles on the study of the psychology of patients with controlled epilepsy. Symptom Checklist 90 (SCL 90) is a major measure of mental health symptoms[2].

The present study was designed to compare the psychological status of patients' in which seizures were controlled with that of patients who experienced uncontrolled seizures by the Symptom Checklist 90 (SCL 90) and social support questionnaire.

2 Patients and Methods

2.1 The criteria of patients and groups

Patients were considered suitable for the study if they met the following criteria: Age >12 years, education >6 years, and had a normal intelligence level. If patients had history of psychiatric illness or were on psychiatric medication, they would be excluded.

Patients with epilepsy were chosen in two groups. Group one (controlled seizure group) consisted of 64 patients known to be seizure free for 1 – 5 years. Group two comprised 61 subjects who continued to experience seizures. The two groups were matched in gender, age, duration of disorder, education and personality.

2.2 The explanation of questionnaire

Eysenck personality questionnaire (EPQ) (Table 1): SCL 90 was used to test psychological status and social support status was assessed with a social support questionnaire. The patients recruited from community. The SCL 90 and EPQ are translated versions. They are validated and self-report inventory.

Table 1. The conditions matched in two groups

	Controlled seizure group	Uncontrolled seizure group
Gender	Female: $n=24$; male: $n=40$	Female: $n=24$; male: $n=37$
Age	Mean: 24.6 years	Mean: 25.8 years
Duration of disorder	Mean: 6.4 years	Mean: 10.1 years
Education	>6 years: $n=64$	>6 years: $n=61$
Personality (mean ± SD)	P: 5.44 ± 3.08	P: 6.39 ± 3.91
	E: 10.65 ± 4.48	E: 11.25 ± 5.31
	N: 10.06 ± 4.93	N: 11.23 ± 5.61
	L: 13.12 ± 3.62	L: 12.65 ± 4.31

$P>0.05$: Personality (P, E, N, L) of two groups compared with each other. P is the abbreviation of psychoticism; E is the abbreviation of Extroversion; N is the abbreviation of neuroticism; L is the abbreviation of lie.

2.3 Statistical methods

The factor scores of EPQ and SCL 90 in two groups compared with the averages within the Chinese population respectively, and compared each other used paired t-test.

3 Results

Compared with the averages within the Chinese population[2], the factor scores of somatization, obsessive-compulsive, depression, anxiety, hostility, photic anxiety, and psychoticism were significantly higher in the uncontrolled seizure group ($P<0.05$); the factor scores of obsessive-compulsive, hostility, photic anxiety, and psychoticism were significantly higher in the controlled seizure group ($P<0.05$). However, only the factor score of somatization was significantly higher in the uncontrolled seizure group than that of the controlled seizure group (Table 2). At the same time, there was no significant difference in social support between two groups ($P>0.05$). The scores from both groups were below 35 (Table 3). This suggests that both groups were short of good social support.

Table 2. Factor scores of the SCL 90 in the two groups

Factor	Uncontrolled seizures group		Controlled seizures group	
	Mean ± SD	P	Mean ± SD	P
Somatization	1.65 ± 0.57	<0.05	1.33 ± 0.25	*P<0.05
Obsessive-compulsive	2.08 ± 0.79	<0.05	2.01 ± 0.65	<0.05
Depression	1.90 ± 0.85	<0.05		
Anxiety	1.80 ± 0.74	<0.05		
Hostility	1.84 ± 0.97	<0.05	1.68 ± 0.61	<0.05
Photic anxiety	1.58 ± 0.59	<0.05	1.41 ± 0.42	<0.05
Psychoticism	1.60 ± 0.59	<0.05	1.53 ± 0.55	<0.05

$P<0.05$: Compared with norm of Chinese; * $P<0.05$: somatization in uncontrolled seizure group compared with that in controlled seizure group.

Table 3. Social support scores of the two groups

	Uncontrolled seizures group (mean ± SD)	Controlled seizure group (mean ± SD)	P
Social support	25.67 ± 4.97	24.38 ± 4.53	>0.05

$P>0.05$: Social support scores of two groups compared each other.

4 Discussion

There are specific measurement parameters for the efficacy of treatment of epilepsy. Current seizure frequency is one of the most important predictors. Psychological factors, as well as the Quality-of-life (QOL) of the patient, are also important to achieve an accurate prognosis[3]. Research has confirmed that patients with epilepsy inevitably have psychological disorders such as depression and anxiety, as such symptoms accompany the diagnosis of epilepsy. It is important to understand the relationship between the frequency of epilepsy and the psychological symptoms presented. For example, in cases where seizures are controlled, do psychological symptoms disappear or decrease drastically? It is necessary to determine whether a long-term psychological treatment is required? We compared the psychology status of patients in whom seizures were uncontrolled with that of patients in whom seizures were controlled (seizure-free 1 – 5 years), by Symptom Checklist 90 (SCL 90). We found the factor score of somatization to be significantly higher in the uncontrolled seizure group than that of controlled seizure group. It is suggested that the patients still present many psychological symptoms in the controlled seizure group and need long-term psychological treatment. Somatization may be the factor that can easily change in patients with 1 – 5 seizure-free years.

Psychological status is related to the frequency of epilepsy in patients. The more frequently that seizures occur, the poorer the psychological status of epileptic patients is expected to be[4,5]. However, the patient's perception of seizure severity has a stronger relation to psychosocial adjustment than actual seizure frequency[6-11]. Our results indicate that epileptic patients continue to present psychological symptoms even though their epilepsy has been seizure-free for 1 – 5 years. There are some reasons for these results: fear of repeat seizures; lack of confidence in the future; lack of social support; and additional factors.

Depression and anxiety are the major factors causing psychological distress in epilepsy[12-14]. These factors were shown to be significantly higher in the cohort that experienced uncontrolled seizures compared with the average in the Chinese population. However, the scores for the group in whom seizures are controlled were not significantly different from the population average. This indicates that depression and anxiety are to some extent related to the frequency of epilepsy. Gramer has studied the influence of depression on seizure severity[15]. He found that patients with depression reported higher levels of perceived severity and distress from seizures than those patients without depression experiencing similar types of seizures.

However, Attarian, studying the relationship between depression and intractability of seizures, found that patients with epilepsy have a higher prevalence of depression than the general population, the intractability of the seizure disorder does not seem to be an independent risk factor for the occurrence of depression[16]. There is no relationship perceived between the severity of depression and monthly seizure rate. Therefore attention should be paid to both seizure severity and depression.

Social support plays an important role in health and good mood[17,18]. Hence we investigated the level of social support experienced by the two groups. We found that in both the uncontrolled seizure group and the controlled seizure group the level of social support was low. Thus it is suggested that patients with epilepsy need long-term psychological treatment and good social support.

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Changes of Left Ventricular Dysfunction and Cardiomyocyte Apoptosis in Losartan-treated Heart Failure Rats

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Abstract: Objective. This study is to determine whether angiotensin II (Ang II) I type receptor (AT1R) retarder inhibits cardiomyocyte apoptosis and attenuates left ventricular (LV) dysfunction in the chronic heart failure rats. **Methods.** 40 rats of health Sprague-Dawley (SD) were divided into 4 groups randomly. Group Ⅰ was sham-operated group ($n=8$). Group Ⅱ was heart failure group ($n=12$). Group Ⅲ was losartan treated group 1 ($n=10$). Group Ⅳ was losartan treated group 2($n=10$). Besides group Ⅰ, other 3 group models of heart failure were established by part constriction of abdominal aorta of rats. After 6 weeks, group Ⅲ and group Ⅳ were treated by gavage of losartan 10 $mg \cdot kg^{-1} \cdot d^{-1}$ and 30 $mg \cdot kg^{-1} \cdot d^{-1}$ respectively. Group Ⅰ and group II were gavaged by normal saline (NS). After 14 weeks, the parameters of hemodynamic and LV remodeling were detected. Ang Ⅱ of plasma and cardiomyocyte were measured by radioimmuneoassay. Cardiomyocyte apoptosis were stained in situ by using TUNEL. The expression of Bcl-2, Bax and procaspase-3 protein of cardiomyocyte was determined by Western blot. **Results.** The Left ventricular end-diastolic pressure(LVEDP) of group Ⅱ was 7.2 mmHg higher than that of group Ⅰ ($P<0.01$)in 14 weeks. Left ventricular weight(LVW)/ Body weight (BW) of group Ⅱ was 0.42 mg/g higher than that of group Ⅰ ($P<0.01$). Ang Ⅱ of plasma and cardiomyocyte of group Ⅱ were 135.31 pg/ml and 94.4 pg/g higher than that of group Ⅰ respectively ($P<0.01$). The exponent of cardiomyocyte apoptosis of group Ⅱ was 12.11‰ higher than that of group Ⅰ ($P<0.01$). When group Ⅱ compared with group Ⅰ, the expression of cardiomyocyte Bax of group Ⅱ was increased significantly while expression of Bcl-2 and procaspase-3 decreased significantly($P<0.01$). When group Ⅲ and group Ⅳ compared with group Ⅱ, the indexes above mention have statistical significance($P<0.01$ or $P<0.05$), and the index changes are related to losartan by treated dose. **Conclusion**. The results indicate that heart failure is associated with LV dysfunction and cardiomyocyte apoptosis involving activation of procaspase-3, and increased Bax/Bcl-2 ratio in the rat heart. AT1R retarder attenuates LV dysfunction in the chronic heart failure rats through decreasing cardiomyocyte apoptosis and changing expression of apoptosis-related proteins. [Life Science Journal. 2005;2(1):49-54] (ISSN: 1097-8135).

Keywords: apoptosis; heart failure; losartan; rat

1 Introduction

An important character heart failure is neurohormonal activation. Neurohormonal activation is closely related with cardiac apoptosis. Enhancement of circulation and local Ang II are important factors[1]. The receptor of Ang II also is increased[2]. Although the available evidences suggest that apoptosis can be induced in cardiomyocyte by a variety of stimulation including pressure overload, most of models are used to acute heart failure models. The relationship between Ang II and apoptosis are ambiguous. Many studies use cultured cells. However, results of clinical studies and animal experiments are disagreement. A number of genes have been identified that regulate the apoptotic process. The Bcl-2 proto-oncogene family is critical for the regulation of apoptosis[3]. Bcl-2 family members come in 2 functional categories, that are inhibiting apoptosis (i.e., Bcl-2) and induce apoptosis (i.e., Bax). The relative abundance of proapoptotic and antiapoptotic proteins determines the susceptibility to cell death. Relationship between these proteins and Ang II is ambiguous. Losartan is AT1R retarder which mostly treats hypertension in clinic. Mechanism of it in pressure overload by constriction of abdominal aorta of rats partly is indistinct in inducing cardiomyocyte apoptosis.

In the present study, we delineated the losartan role and changed in Bax, Bcl-2 and procaspase-3, in pressure overload by part constriction of rats abdominal aorta, critical steps involved in death signaling pathways.

2 Materials and Methods

Animal and design: The healthy male SD rats (BW 100 – 110 g) were provided by Henan Province Experiment Animal Center. 40 rats of health SD were divided into 4 groups randomly. Group Ⅰ was sham-operated group ($n = 8$). Group Ⅱ was heart failure group ($n = 12$). Group Ⅲ was losartan treated group 1 ($n = 10$). Group Ⅳ was losartan treated group 2 ($n = 10$). Besides group Ⅰ, other 3 group models of heart failure were established by part constriction of abdominal aorta of rats. Rats were anaesthetized by 2% butaylone 40 mg/kg abdominal injection. After abdominal operation, upside of renal artery, an injector pinhead (internal diameter 0.80 mm) was placed around the abdominal aorta. After ligating pinhead and abdominal aorta, pinhead was drawn out. Rats of sham-operated group only separate abdominal aorta but didn't constrict abdominal aorta. After 6 weeks, group Ⅲ and group Ⅳ were treated by gavage of losartan 10 $mg \cdot kg^{-1} \cdot d^{-1}$ and 30 $mg \cdot kg^{-1} \cdot d^{-1}$ respectively. Group Ⅰ and group Ⅱ were gavaged by NS.

Determination of cardiac function: After 14 weeks, all animals were killed. The animals were anaesthetized by 2% butaylone abdominal injection of 40 mg/kg before killed, and arterial blood pressure was measured directly via the left carotid artery. LV hemodynamics was measured by inserting a PE 50-catheter through the free LV wall into the left ventricle. Pressures were registered with a transducer and an amplifier. LV contractility was obtained from the ventricular pressure curves. Hemodynamic measurements were performed, the hearts were removed, and ventricles were dissected free, weighed and kept in liquid nitrogen until further analysis.

Determination of LV remodeling: BW and heart weight (HW) and LVW were measured after 14 weeks. Heart quality index is HW/BW. LV quality index is LVW/BW.

Determination of Ang II contents of plasma and myocardium: Ang II was measured by radioimmunoassay. Plasma samples were extracted in phenyl-encapped cartridges, washed with 1 ml distilled water, eluted with 0.5 ml methanol, lyophilized, and reconstituted. For extraction of tissue Ang II, samples were pulverized frozen, boiled for 5 minutes in 10 volume of acetic acid (1 mol/L)/HCl (20 mmol/L), and homogenized at high speed (PT 1200, Polytron). The homogenate was then ultracentrifuged at 27 kg at 4°C, and the supernatant was stored at −20°C until radioimmunoassay.

Detection of apoptosis: In five animals from each group, myocardial apoptosis was assessed using a commercially available method, which relies on terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenintagged dUTP-biotin end-labeling of 3′-OH DNA ends generated by DNA fragmentation in situ. In brief, 6 μm tissue sections were deparaffinized in xylene, and rehydrated through graded ethanol and distilled water. The sections were then incubated for 1 min at 22°C in equilibration buffer. The equilibration buffer was then replaced by TdT solution and incubated for 1 h at 37°C in a humidified chamber. The slides were then washed in phosphate-buffered saline (PBS, pH 7.4) and incubated with anti-digoxigenin antibody conjugated to horseradish peroxidase, washed with PBS and further incubated with diaminobenzidine and hydrogen peroxide. For negative controls, TdT enzyme was replaced with distilled water in the labeling reaction. Myocardial nuclei were considered apoptotic only if they displayed both TUNEL stain positive and appropriate nuclear morphology (condensation and/or fragmentation). Five sections from each specimen were examined. At high magnification (×400), five random fields per section from noninfarcted, remodeled regions were examined to calculate the number of apoptotic nuclei per 1000 total nucented. Specimens were read in a blinded fashion.

Western blot analysis: For Western blots, frozen LV myocardium was pulverized in liquid nitrogen and homogenized in a lysis buffer containing 100 mM NaCl, 50 mM Tris (pH 7.4), 0.5 mM Triton X−100, 1 mM dithiotreitol, 50 mM NaF, 0.5 mM $NaVO_3$, and an EDTA-free protease inhibitor cocktail (Roche). Protein homogenates were centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatants were used for Western blotting. After determining protein concentrations by the Bradford Method (Bio-Rad), 30 μg of protein/sample were denatured by boiling for 5 min in a loading buffer containing 0.25 M Tris (pH 6.8), 20% glycerol, 4% SDS, and 0.05% bromophenol blue, and subjected to 10% SDS-PAGE. Following electrophoresis, the separated proteins were transblotted on to PVDF membranes (Immobilon P, Millipore). The membranes were blocked with 10% normal goat serum (preimmune serum, DAKO) for 1 h at 22°C. The membrane was then

incubated with primary antibody and then with secondary antibody for 2 h each. Signals were revealed with chemiluminescence using the ECL-detection system (Amersham). Quantification of the signals was performed using NIH image. Rabbit polyclonal IgG antibodies against human Bcl-2 (cross reactive with rat) were used at a concentration of 5 μg/ml.

Statistical analysis: All results are presented as means ± SD. Differences among the 4 groups of rats were tested by a one-way ANOVA. Comparison between groups was performed with the multiple comparison Student-Newman-Keuls test. P values < 0.05 were considered statistically significant. The detection of specific protein binding was preformed with enhanced chemiluminescence Western blot detection system.

3 Results

Hemodynamic and weight: Because rats suffer from acute heart failure, dead rats were 4 in group Ⅱ in the experiment course. Because rats suffer from chronic heart failure, there were 2 dead rats in group Ⅲ and group Ⅳ respectively. There was no dead rat in groupⅠ. After 14 weeks, LVEDP and $\pm dp/dt_{max}$ were significantly lower in groupⅡ, compared with group Ⅰ, confirming the presence of LV dysfunction. LVEDP and $\pm dp/dt_{max}$ have statistical significantly groupⅢ, compared with groupⅡ. Along with increased doses, above-mentioned indexes were more significantly (Table 1).

BW of group Ⅱ rats was lower than that of group Ⅰ rats. Heart quality indexes and LV quality indexes in group Ⅱ rats were markedly higher than that in group Ⅰ rats. Heart quality index and LV quality indexes in group Ⅲ rats were lower than that in group Ⅱ rats. Along with increased doses, above-mentioned indexes were more significantly (Table 2).

Ang II contents of plasma and myocardium: Ang II of plasma and myocardium in the group Ⅱ rats was higher than that in the group Ⅰ rats ($P < 0.01$). Above-mentioned index was obviously decreased in the group Ⅲ rats than that in the group Ⅱ rats ($P < 0.01$). Along with increased doses, above-mentioned index was significantly different (Table 3).

Table 1. Effects of losartan on hemodynamic indexes

Group	LVSP (mmHg)	LVEDP (mmHg)	$+dp/dt_{max}$ (mmHg/s)	$-dp/dt_{max}$ (mmHg/s)
Ⅰ	123.6 ± 15.2	5.7 ± 1.8	4853.7 ± 411.7	3817.3 ± 262.8
Ⅱ	105.5 ± 12.4[c]	12.9 ± 1.8[f]	3862.1 ± 436.6[f]	3283.8 ± 247.8[f]
Ⅲ	119.7 ± 12.3	9.5 ± 2.1	4402.7 ± 352.6	3544.1 ± 193.7
Ⅳ	120.9 ± 13.5[d]	7.64 ± 1.5[d]	4829.3 ± 309.7[d]	3788.8 ± 220.9[d]

Mean ± SD, [c]$P < 0.05$ vs group Ⅰ and group Ⅲ and groupⅣ; [f]$P < 0.01$ vs group Ⅰ and group Ⅲ and group Ⅳ; [d] $P < 0.05$ vs group Ⅲ Ⅰ: sham-operated group ($n = 8$); Ⅱ: heart failure group($n = 8$); Ⅲ: losartan treatment group 1($n = 8$); Ⅳ: losartan treatment group 2($n = 8$). LVEDP: left ventricular end-diastolic pressure; LVSP: left ventricular systolic pressure; dp/dt_{max}: the maximal rate of rise of left ventricular pressure; $-dp/dt_{max}$: the maximal rate of drop of left ventricular pressure

Table 2. Effects of losartan on BW, and ratio of heart weight and BW, and ratio of LV weight and BW

Group	BW(g)	HW/BW(mg/g)	LVW/BW(mg/g)
Ⅰ	386.6 ± 38.5	3.17 ± 0.29	2.34 ± 0.25
Ⅱ	346.1 ± 26.1[c]	3.86 ± 0.36[f]	2.76 ± 0.36[f]
Ⅲ	359.5 ± 39.4[d]	3.49 ± 0.25[d]	2.46 ± 0.32[d]
Ⅳ	372.8 ± 34.8	3.19 ± 0.38	2.35 ± 0.24

Mean ± SD, [c]$P < 0.05$ vs group Ⅰ and group Ⅲ and groupⅣ; [d]$P < 0.05$ vs group Ⅱ and groupⅣ; [f]$P < 0.01$ vs group Ⅰ and group Ⅲ and group Ⅳ.
Ⅰ: sham-operated group ($n = 8$); Ⅱ: heart failure group ($n = 8$); Ⅲ: losartan treatment group 1($n = 8$); Ⅳ: losartan treatment group 2($n = 8$). BW: body weight; HW: heart weight; LVW: left ventricular weight.

Table 3. Effects of losartan on contents of Ang II and index of apoptosis

Group	Plasma AngII (pg/ml)	Myocardium AngII (pg/g)	Index of apoptosis (‰)
Ⅰ	105.17 ± 24.79	214.2 ± 75	4.02 ± 0.78
Ⅱ	240.48 ± 31.24[c]	308.6 ± 59[c]	16.13 ± 1.17[c]
Ⅲ	175.47 ± 38.50	263.6 ± 65	13.57 ± 1.14
Ⅳ	128.14 ± 42.71[f]	229.7 ± 60[f]	11.32 ± 1.34[f]

Mean ± SD, [c]$P < 0.01$ vs group Ⅰ and group Ⅳ; [f]$P < 0.05$ vs group Ⅲ.
Ⅰ: sham-operated group ($n = 8$); Ⅱ: heart failure group ($n = 8$); Ⅲ: losartan treatment group 1($n = 8$); Ⅳ: losartan treatment group 2 ($n = 8$).

Apoptosis of cardiomyocyte: The apoptotic index decreased ($P < 0.01$) after treatment with losartan. Along with increased losartan doses, the apoptotic index was significant (Table 3). The apoptotic index decreased ($P<0.05$) in group Ⅳ, compared with group Ⅲ.

Expression of Bcl-2, Bax and precaspase-3: Bax expression diminished after treatment with losartan ($P<0.01$) (Figure 1). On the contrary, Bcl-2 expression increased after treatment with losartan ($P < 0.01$) (Figure 1). As a consequence, the ratio Bax/Bcl-2 decreased after treatment with losartan. Procaspase-3 was decreased in heart failure group rats, compared with sham-operated group and losartan treatment group rats ($P<0.01$) (Figure 1).

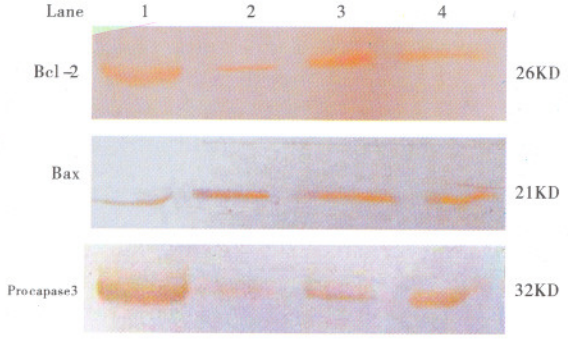

Figure 1. Effects of losartan on expression of apoptosis relative protein. Losartan up-regulate Bcl-2 expression and down-regulated Bax expression significantly. The size of molecular weight marker is shown on the right, and the names of proteins are shown on the left respectively. Lane 1: group Ⅰ; lane 2: group Ⅱ; lane 3: group Ⅲ; land 4: group Ⅳ. Bcl-2 26kD, Bax 21kD, procapase-3 32kD.

4 Discussion

This experiment's results show that losartan decrease cardiomyocyte apoptosis, as evidence by decrease in Bax to Bcl-2 ratio, caspase-3 activation profile, accumulation of TUNEL-positive nuclei. Losartan attenuates LV dysfunction in the heart failure rats through decreasing cardiomyocyte apoptosis and changing expression of apoptosis-related proteins. LV hypertrophy is an adaptive cardiac response to the imposition of pressure overload to the heart[4]. The initial benefits of cardiac hypertrophy, such as normalization of wall stress and preservation of systolic force generation, may be offset during the late stages of chronic hemodynamic overload due to progressive cell loss, which may lead to deterioration of cardiac function[5]. The results show that left ventricle has taken place hypertrophy and cardiomyocyte of left ventricle has apoptosis at the same time. Left ventricle hypertrophy and cardiomyocyte apoptosis remarkable decrease after losartan treatment. Along with increase of doses, above-mentioned indexes are significantly. Theses results show that cardiomyocyte apoptosis and left ventricle remodeling have a strong correlation. These results support important role of apoptosis in heart failure. Contractive function of LV obvious decline because left ventricle have a mass of cardiomyocyte apoptosis. These results illuminate that the relation of LV dysfunction and cardiomyocyte apoptosis is close.

Currently, the most popular model, rapid ventricular pacing, is simple to produce and represent a condition that is not frequently found in human heart failure. Also the physiological changes do not persist beyond removal of the pacing stimulus and ventricular dilatation occurs without an initial hypertrophic response[6]. Other methods include volume overload and coronary ligation. These studies do not calculate heart failure as a chronic process. Therefore, we have developed a reproducible model of chronic heart failure that results in marked systolic dysfunction in this study. This model is similar with clinical syndrome and is successful from hemodynamic assessment. This is perfect model to replicate heart failure.

The mechanism of Ang II evocable apoptosis is disputed. Several observations suggest that AT2 stimulation mediates apoptosis through extracellular signal-regulated kinase (ERK) inhibition[7], ceramide accumulation, activation of mitogen-activated protein kinase phosphatase 1 (MKP1) with subsequent inhibition of mitogen-activated protein kinase, and Bcl-2 dephosphorylation[8]. Apoptosis can be blocked by PD-123319 and PD-123177[9], which are specific AT2 blockers. In contrast, some authors[10] have shown that AT1 blockade with losartan can equally protect from apoptosis. AT1 stimulation can lead to an increase in Fas, together with a fall in constitutive NO synthase and Blc-2 levels. Similar observations have been made in the hearts of spontaneously hypertensive rats[11]. The ACE inhibitor captopril reduced apoptosis in spontaneously hypertensive rats with CHF[12]. Our results show that Ang II of plasma and myocardium are increased in heart failure rats. But these results are decreased after losartan treatment. At the same time, Bcl-2 and Bax have taken place huge changes after losartan treatment. Because losartan is selective AT1R blockade, we support AT1 stimulation can lead to an increase in Bax, together with a fall in Blc-2 levels. These results show that Ang II is an important factor in inducing cardiomyocyte apoptosis. Because losartan is a selective AT1 re-

ceptor inhibitor, we think that induced apoptosis of Ang II is through AT1 receptor in heart failure by part constriction of abdominal aorta of rats.

Cardiac apoptosis can proceed via either death-receptor or mitochondrial-dependent pathways[13], either of which activates specific caspases, ultimately resulting in cell death. The death-receptor pathway proceeds when extracellular death signal ligands, such as TNF-a, FasL, bind to their specific cell membrane receptors. Mitochondrial-dependent pathways, initiated by the release of cytochrome c from mitochondria, are closely regulated by the Bcl-2 family of proteins, which has both anti-apoptotic (e. g. Bcl-2 and Bcl-XL) and pro-apoptotic (e. g. Bax and Bcl-XS) members. The cellular balance between anti-apoptotic and pro-apoptotic Bcl-2 family proteins is an important determinant of cell survival or death[14,15]. In this study, our results show that anti-apoptotic Bcl-2 and pro-apoptotic Bax have taken placed huge changes. Above-mentioned index presents opposite changes after losartan treatment. Several lines of evidence indicate that myocardial apoptosis contributes significantly to remodeling of failing myocardium. Caspases, which are activated in various stages of HF, are the key effectors molecules for apoptosis. Cellular caspases exist as inactive precursors and need proteolytic cleavage for activation. Caspase activation co-localizes to apoptotic areas and precedes DNA degradation and the development of apoptotic morphology[16]. After a death signal, Bax can associate with the mitochondria to induce the release of cytochrome c and formation of the apoptosome complex in association with caspase-9 activation. Furthermore, caspase can also influence the contractile machinery of myocytes through cleavage of troponin[17]. This can result in contractile dysfunction. Indeed, overexpression of caspase-3 induces contractile dysfunction in mice. Furthermore, caspase-3 transgenic mice showed increased infarct size and a pronounced susceptibility to die[18]. Therefore, activated caspase-3 lead to contractile dysfunction in rats. Contractile dysfunction of heart obvious ameliorates after losartan. These results show that losartan improve heart function through decreasing cardiomyocyte apoptosis.

Pathophysiologic mediators of remodeling, such as catecholamines , Ang II[15], myocardial stretch[15,19], inflammatory cytokines[14], and oxidative stress[20] can all induce cardiomyocyte apoptosis. In animal models of HF[16,20,21] and human end-stage cardiomyopathy[22], remodeled myocardium displays apoptotic cardiomyocyte loss, variably associated with enhanced expression of p53[21], a transcriptional protein involved in cell cycle control, downregulation of Bcl-2, and upregulation of Bax[14,15]. However, apoptosis in HF is not limited to cardiomyocytes. Indeed, a recent study of canine-pacing tachycardia HF[20] demonstrated progressive apoptosis of myocytes, endothelial cells, and fibroblasts indicating that both parenchymal and interstitial cell loss occur during myocardial remodeling. Although earlier studies reported apoptotic (TUNEL-positive) nuclei in failing hearts as high as 35%, most have reported rates less than 1%[20-22]. Consistent with these prior studies, we consider that these are a strong correlation between LV remodeling and apoptotic rate. Our data indicate that increased apoptosis in failing myocardium occurs concomitantly with increased activity of mitochondrial pathways. The changes in apoptosis-related proteins suggest that caspase-3 activation and Bcl-2 down regulation acted upstream of Bax and caspase-9. After a death signal, Bax can associate with the mitochondria to induce the release of cytochrome c and formation of the apoptosome complex in association with caspase-9 activation[23].

In summary, this study also provides direct evidence that anti-apoptotic Bcl-2 is downregulated in failing myocardium, indicating a shift in the regulatory balance of the Bcl-2 family proteins favoring apoptosis, and suggesting attendant activation of mitochondrial-dependent pathways.

Our data indicate that Bcl-2 expression is markedly decreased in failure hearts. Losartan ameliorates contractile function of LV dysfunction in the heart failure rats. The mechanism may be decrease cardiomyocyte apoptosis through balance of the Bcl-2 family proteins.

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The Radiosensitive Therapy for Colorectal Cancer

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Abstract: The colorectal cancer is one of the most common cancers in the United States. Surgical resection is appropriate as the first component of treatment. However, only less than half of the patients are cured by primary resection. Neoadjuvant radiation can decrease tumor size before surgery, and can enable patients previously deemed unresectable, to undergo curative surgical resection. Unfortunately since many tumors have hypoxic areas or genetic mutations that enable them to be radioresistant, only 22 – 44% of rectal cancers will respond to neoadjuvant radiation. To overcome the radioresistance of tumors, multiple drugs have been tested as potential radiosensitizers. 5-Fluorouracil is one of the most widely used radiosensitizers, which enhances radiosensitization of colorectal cancer due to alteration of cell kinetics. Nitric Oxidize is a promising potential radiosensitizer. AdiNOS treatment of HCT-116 tumors significantly delayed tumor doubling time and growth when combined with single or multifractionated radiation. Other radiosensitizers, such as, Protein Kinase C-specific Inhibitor PKC412, Survivn, Caffeine, Bromodeoxyuridine and Iododeoxyuridine are also briefly discussed in this review. [Life Science Journal. 2005;2(1):55 – 60] (ISSN: 1097 – 8135).

Keywords: colorectal cancer; neoadjuvant radiation; radioresistance; radiosensitizers

1 Introduction

The colorectal cancer is the third leading cause of cancer in the United States with approximately 150,000 new cases annually. The primary modality for treatment for colorectal cancer is surgical resection. For every 100 patients initially evaluated, 45 are cured by primary resection[1]. Even though remarkable progress has been made in the treatment of the colorectal cancer during the past two decades, approximately 44% of patients with colon cancer will present with stage III or IV disease[2]. Neoadjuvant radiation can decrease tumor size before surgery, enabling a greater chance of obtaining a tumor-free surgical margin and can enable patients previously deemed unresectable, to undergo curative surgical resection[3]. Several studies have examined preoperative irradiation alone as a neoadjuvant regimen for the treatment of rectal cancer. The European Organization for Research and Treatment of Cancer studied randomized patients with T2 – T4 tumors to preoperative radiation or surgery alone. Although the radiation dosage of 3,450 cGy was lower than the standard dosage given today, there was a 50% reduction in the local recurrence rates in patients treated with radiation[4]. The first prospective, randomized controlled study documented that preoperative therapy reduces local recurrence and improves survival[5]. Despite these benefits, unfortunately only 22 – 44% of rectal cancers will respond to neoadjuvant therapy[6]. To overcome the radioresistance of tumors, multiple drugs such as, fluorodeoxyuridine, caffeine, and nitric oxidize have been tested as potential radiosensitizers[7-9]. In this article, we review some potential radiosensitive agents in colorectal cancer.

2 Molecular Mechanisms of Radiosensitization

Radiation is considered to have ionizing potential. The radiation used in radiation therapy (RT) produces several hundred thousand ionization events per cell per gray. The absorbed energy causes ejection of primary electrons that go on to ionize other molecules leading to a complex chain reaction. DNA is the most important target for RT. The evidence from experiments shown that irradiation of the nucleus, but not the cytoplasm, results in cell death. The extent of initial DNA damage and the persistence of this damage are presumably critical factors in determining the cellular response to radiation. In the process, free radicals, which are neutral atoms or molecules that have an unpaired electron, are generated. Because of their unpaired electrons, free radicals are very reactive and can reduce or oxidize biological molecules and break their chemical bonds. Since the most abundant molecule in cells is water, the most common free radicals

that are generated in a cell after exposure to ionizing irradiation are reactive oxygen species (ROS). Biological agents, such as growth factors, cytokines, monoclonal antibodies to cell surface receptors, can alter molecular pathways within a cell. Chemotherapeutic and physical agents, such as hyperthermia, hypoxia and radiation itself, can activate pathways that can also affect the outcome.

An important consequence of the involvement of free radicals in radiation damage is that oxygen plays a major role as a modifier of radiation responses. Oxygen influences the nature of the free radicals and the lesions that are formed. The peroxides and hydroperoxides, in particular, inhibit repair. Under hypoxic conditions the cells are typically 2.5 −3 times more resistant to irradiation than in the presence of oxygen[10]. A number of studies have documented the importance of hypoxia to the outcome of RT. At the molecular level, hypoxia induces expression of a number of genes, in particular genetic programs that are under the control of hypoxia inducible factor (HIF-1). Some of these (such as erythropoietin, vascular endothelial growth factor and tumor necrosis factor alpha (TNF-α)) are clearly aimed at increasing angiogenesis and increasing oxygen delivery. Hypoxia can therefore play an important role in driving angiogenesis and tumor expansion. Other hypoxia-induced genes (such as p53) are part of a stress response that encourages cells to undergo cell death by apoptosis. Acting in this way, hypoxia serves as a selective force to favor expansion of cells mutated in p53[11].

Expression or knockout of proto-oncogenes, tumor-suppressor genes, cytokines, cytokine receptors, cell adhesion molecules, redox-active genes and many other genes that are important in determining cell behavior, can influence the outcome of irradiation[11-14]. For example, transfer of genes for growth factors or growth factor receptors that cause cell proliferation can often achieve radiation resistance. A dominant negative approach antisense, or antibody directed against, for example EGFR, can result in cellular radiosensitization and the level of expression of EGFR by a tumor can determine radiocurability[15-17]. Radiosensitization often results from transfer of cytokine genes or receptors that slow cell cycle progression or encourage apoptosis.

Radiosensitivity is also related to histological classes. Leith studied the *in vitro* X-ray radiation survival characteristics of 181 cell lines from 12 different classes of exponentially growing human tumor cells (sarcomas, lung cancers, colorectal cancers, medulloblastomas, melanoma, breast cancers, prostate cancers, renal cell cancers, grades III and IV brain tumors, ovarian, head and neck cancers). Radiosensitivities could roughly be divided into two groups: the more radiosensitive group and the more radioresistant group. The intrinsic radiosensitivity of human tumor cells exists among different histological classes of neoplasm. If, however, tumors contained on average 20 percent hypoxic cells, the dose needed for equivalent cell killing increased by about a factor of 2.6−2.8. Also, there was no correlation between the rankings of relative radiosensitivities of the various classes of tumor cells at high doses (as in radiosurgery) to the sensitivity at low doses (as in conventional fractionated radiotherapy)[18].

3 Radiosensitive Agents in Colorectal Cancer

3.1 Fluorodeoxyuridine

5-Fluorouracil (5-FU) is one of the most widely used chemotherapeutic agents, and is known to be a radiosensitizer. The combination of fluoropyrimidines and radiation has resulted in increased control of colorectal cancer in the clinic. In 1980 the North Central Cancer Treatment Group and the Mayo Clinic compared adjuvant combination chemotherapy and irradiation to radiation alone. Patients undergoing combination chemotherapy and irradiation had an improvement in both disease-free and overall survival rates compared with those undergoing irradiation alone[19]. Crane compared the outcome from preoperative chemoradiation and from radiation therapy in the treatment of rectal cancer in two large, single-institutional experiences. Multivariate analysis of the patients in these groups showed that the use of concurrent 5-FU with preoperative radiation therapy for T3 and T4 rectal cancer independently increases tumor response and may contribute to increased sphincter preservation in patients with low rectal cancer[20]. More recently, 5-Flurouracil has been shown to be a radiosensitizer and acts in part to increase the susceptibility of tumor cells to the damaging effects of radiation[21]. Combined radiation with 5-FU-based chemotherapy is more efficacious than radiation alone in patients with squamous cell cancer of the anus. Similarly, combined chemoradiation has been shown by Minsky et al. to be more effective than radiation therapy as a neoadjuvant regimen in patients with rectal cancer[22]. Minsky and colleagues compared two groups of unresectable patients who were nonrandomly treated with combined chemoradiation or radiation alone. In patients undergoing chemoradiation ($n=20$), there was a higher com-

plete pathologic response compared with the 11 patients who underwent radiation alone (20% vs. 0%).

The radiosensitization by 5-fluorodeoxyuridine is in part due to alteration of cell kinetics and redistribution of cells throughout the cycle. In laboratory preliminary work showed that 2 h exposures of HT 29 human colon carcinoma cells to relatively low levels of 5-fluorodeoxyuridine resulted in extended thymidylate synthase inhibition after the drug was removed (up to 30 h after treatment with 0.5 microM 5-fluorodeoxyuridine). The low cytotoxicity associated with this treatment simplified efforts to test the effects of extended thymidylate synthase inhibition on radiosensitivity of HT 29 cells. Although thymidylate synthase was completely inhibited at the end of the 2 h exposure, an increase in the radiosensitivity of the cells was not evident until 16 h after the removal of drug. Flow cytometric analysis showed that cells accumulated in early S phase over time, and the increase in radiation sensitivity of the entire population followed the increase of the proportion of cells in early S phase, a relatively radiosensitive phase of the cell cycle. This treatment schedule was compared with 24 h continuous exposure, and was found that the same maximum increase in radiosensitivity was achieved by both treatment strategies. However, more cytotoxicity was associated with continuous exposure[23].

Adenoviral transduction of the Escherichia coli uracil phosphoribosyltransferase (UPRT) gene induced marked sensitivity in human colon cancer cells to 5-FU. Kayama investigated the efficacy of virally directed UPRT and 5-FU to enhance the radiosensitivity of HT 29 human colon cancer cells. In vivo chemoradio-gene therapy using the UPRT/5-FU/radiation system showed tumor regressive effects even against large HT 29-established subcutaneous tumors in nude mice[24].

3.2 Protein kinase C-specific inhibitor PKC412

The cellular response to ionizing radiation is governed by the DNA-damage recognition process but is also modulated by cytoplasmic signal transduction cascades that are part of the cellular stress response. Growth-promoting protein kinase C activity antagonizes irradiation-induced cell death, and, therefore, protein kinase C inhibitors might be potent radiosensitizers. The antiproliferative and radiosensitizing effect of the novel *N*-benzoylated staurosporine analogue PKC412 was tested *in vitro* against genetically defined p53-wild type (+/+) and p53-deficient (−/−) murine fibrosarcoma cells and *in vivo* against radioresistant p53 −/− murine fibrosarcoma and human colon adenocarcinoma tumor xenograft (SW480, p53-mutated). PKC412 sensitized both p53 +/+ and p53 −/− tumor cells *in vitro* and *in vivo* for treatment with ionizing radiation but with a different mechanism of radiosensitization depending on the p53 status. In p53 +/+, cells combined treatment with PKC412 and ionizing radiation drastically induced apoptotic cell death, whereas no apoptosis induction could be observed in p53-deficient cells *in vitro* and in histological tumor sections. Combined treatment resulted in an increased G_2 cell cycle distribution in p53 −/− cells at PKC412 concentrations that did not alter cell cycle distribution when applied alone. In vivo, a minimal treatment regimen during 4 consecutive days of PKC412 (4×100 mg/kg) in combination with ionizing radiation (4×3 Gy) exerted a substantial tumor growth delay for both p53-disfunctional tumor xenografts and showed that the clinically relevant protein kinase C inhibitor PKC412 is a promising new radiosensitizer with a potentially broad therapeutic window[7].

3.3 Survivin

Spontaneous apoptosis has been shown to predict tumor response to radiochemotherapy in rectal cancer *in vivo*. Recently, a novel member of the inhibitor of apoptosis protein family, designated survivin, was identified. The inverse correlation of survivin-expression with spontaneous and radiation-induced apoptosis suggests that survivin is an important inhibitor of apoptosis in colorectal cancer cell lines. Analysis of survivin mRNA or protein expression may therefore provide predictive information on radio- and chemoresistance of individual colorectal tumors[25]. Rodel investigated the impact of survivin expression on tumor cell apoptosis in three colorectal cell lines of different intrinsic radiosensitivities. *In vitro* analysis revealed higher spontaneous and higher radiation-induced apoptosis rates in the radiosensitive line (SW 48), as compared with the more resistant line (SW 480). SW 480 was characterized by a higher spontaneous expression and a pronounced induction of survivin 48 h after irradiation, whereas survivin expression was low when untreated and not increased after irradiation in the most radiosensitive line SW 48.

3.4 Caffeine

Boonkitticharoen investigated the effect of caffeine, the methylated xanthine, in sensitizing the lethal action of ionizing radiation *in vitro* in human cancer cells. Plateau phase cultures of colon adenocarcinoma, after absorbing doses of 2 Gy, survived at a rate of 56.30 per cent for colon cancer[26].

3.5 Bromodeoxyuridine and iododeoxyuridine

Bromodeoxyuridine (BrdU) and iodo-deoxyuridin (IdU) have similar the radiosensitizing effects on colorectal cancer. Miller conducted concurrently to characterize its effects on the shape of the radiation survival curves of cells of two human colon cancer cell lines, HT 29 and HCT 116. The efficiency of radiosensitization by BrdU, expressed as a function of percentage thymidine replacement, was lower when compared to IdU in both cell lines. The major radiosensitizing effect of BrdU was manifest as an increase in the initial slope (alpha), just as observed for IdU. However, with BrdU, in contrast to IdU, an increase in curvature (repairable damage) was also evident. Cells of the more radiosensitive line, HCT 116, showed less sensitization by either BrdU or IdU than cells of the more radioresistant line, HT 29. These results were consistent with the proposed mechanism of radiosensitization being an increase in the single-hit character of low-LET radiation. The radiosensitizing effects of both analogs were largest in the low-dose region of the survival curve[27].

3.6 Nitric oxidize

Nitric oxidize (NO), is another potential promising radiosensitizer. Multiple studies using NO donors have examined its effects in radiosensitizing tumor cells. Initial studies examining NO donors indicated that NO enhanced the radiosensitivity of hypoxic mammalian cells *in vitro*[28]. NO's radiosensitizing property was first demonstrated in 1957 by Howard Flanders in Nature[29]. Flanders interest in NO was based on the fact that like oxygen, NO had a reactive electron making it a free radical. He hypothesized and demonstrated that NO because of this property could effectively substitute for oxygen as an electrophile and sensitize bacteria to radiation under anaerobic conditions. Subsequently others have shown that NO could also radiosensitize normal human cells[28,30,31]. NO was found to be as effective as oxygen in radiosensitizing hypoxic mammalian cells[30]. One group demonstrated that cytokines could induce endogenous NO production and radiosensitize hypoxic breast cancer cells[32].

NO itself also has effect on colorectal cancer. In one large study of colorectal cancers, iNOS activity and protein expression correlated inversely with advanced stage of disease[33]. Pre-malignant colorectal adenomas may have the highest iNOS activity. iNOS overexpression in these polyps is associated with a specific point mutation in the p53 gene, suggesting that NO may function to initiate development of colorectal cancer rather than stimulating cancer progression[34]. Other studies in human colorectal cancer corroborate this inverse relationship between iNOS expression and tumor progression[35]. Lack of iNOS in knock-out mice promotes intestinal tumors further substantiating the role of iNOS in host defense against colorectal cancer[36]. Given these studies, the use of iNOS gene transfer would be a rationale means to improve the impairment in tumor defense mechanisms that utilize NO.

Although NO is a promising radiosensitizer, and NO itself induce apoptosis, the use of NO donors to augment the effects of radiation *in vivo* has significant limitations, since *in vivo* administration of these agents results in systemic hypotension and may increase tumor perfusion and oxygenation, potentially promoting tumor growth[37]. Overexpression of iNOS in tumors by localized direct intratumoral injection of the iNOS gene has the potential of minimizing the systemic side effects of NO while maintaining the salutary tumoricidal effects of high output paracrine NO release. We have previously examined the effects of direct intratumoral gene delivery of iNOS combined with both single and multifractionated irradiation on growth of HCT-116 colorectal tumors in nude mice. AdiNOS treatment of HCT-116 tumors significantly ($P \leqslant 0.005$) delayed tumor doubling time and growth when combined with single or multifractionated radiation in nude mice. We have previously demonstrated that adenoviral delivery of the iNOS gene enhances radiation-induced apoptosis in colorectal cancer cells[38]. We have also demonstrated that overexpression of the human inducible nitric oxide synthase gene by adenoviral gene delivery radiosensitizes both human colorectal cancer cells and tumors associated with increased apoptosis in nude mice[39]. The mechanism of NO radiosensitization may be that NO increases angiogenesis and then increases oxygen delivery.

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B-type Natriuretic Peptide in Predicting Short-term Mortality in Patients with Acute Coronary Syndromes: A Preliminary Report

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Abstract: Objective. This study was designed to investigate the usefulness of B-type natriuretic peptide (BNP) in predicting the short-term mortality in patients with acute coronary syndromes (ACS). **Methods.** A total of 106 patients with ACS, whose blood BNP concentration were measured with Triage BNP test, within 1 – 3 days after onset of ischemic symptoms, were divided into two groups: the survival and the non-survival group, according to the results of 4-week follow-up. **Results.** The blood BNP concentration was significantly higher in the non-survival than in the survival ($P<0.0001$) group; univariate analysis showed that BNP ($\geqslant$ 172 ng/L, median) and Killip class (Ⅱ-Ⅳ) were prognostic factors of the short-term cardiac death in patients with ACS ($P<0.0005$ and $P=0.001$); stepwise logistic regression analysis indicated that smoking and BNP ($\geqslant$ 596 ng/L, 75% percentile) were independent predictors of short-term cardiac death in patients with ACS (OR=5.5, $P=0.028$; OR=21.19, $P<0.0005$). **Conclusion.** BNP might predict the 4-week mortality in patients with ACS. [Life Science Journal. 2005;2(1):61 – 64] (ISSN: 1097 – 8135).

Keywords: B-type/brain natriuretic peptide; acute coronary syndrome; prognosis

1 Introduction

Acute coronary syndromes (ACS) encompass a continuum of cardiac ischemic events, ranging from unstable angina pectoris with no biochemical evidence of myocardial necrosis to ST-elevation acute myocardial infarction (AMI)[1]. Acute prediction of the mortality in patients with ACS is critically important to facilitate the application of preventative measures. B-type/brain natriuretic peptide (BNP), which is mainly secreted by ventricular myocytes, is increased in patients with systolic or diastolic heart failure[2,13]. Numerous clinical trials have demonstrated that BNP could be used to diagnose cardiac dysfunction[4,5]. It has also been shown that an increase in BNP values is associated with a higher mortality and morbidity rate in patients with ventricular failure or ACS[6-10]. In this study, we investigated the usefulness of blood BNP in predicting 4-week cardiac death in patients with ACS.

2 Materials and Methods

Patient selection

Patients were included if they presented within 72 hours after onset of ischemic discomfort and met one or more of the following criteria: electrocardiographic changes (ST-segment depression or elevation of at least 0.5 mm, T-wave inversion of at least 3 mm in at least three leads, or left bundle-branch block), elevated levels of cardiac markers, or a history of coronary disease.

One hundred and six patients admitted to the coronary care unit (CCU) at the First Affiliated Hospital of Zhengzhou University, were enrolled in the study between September 2003 and May 2004. Thirty-three patients had ST elevation myocardial infarction (STEMI), 7 had non-ST elevation myocardial infarction (NSTEMI) and 66 had unstable angina pectoris (UAP). Seventy-one patients were male and 35 were female. Mean age was 62 years (37 – 85). They were divided into 2 groups ac-

cording to the 4-week follow-up results: the survival ($n = 93$, 88%) and the non-survival ($n = 13$, 12%).

2.2 Measurement of blood BNP level

A point-of-care test of fluorescence immunoassay for the quantification of BNP was used (Biosite Diagnostics Inc, USA), 2 ml of intravenous blood was collected at the early morning after 1 – 3 day on admission and BNP was determined within 20 min. The range of measurement was 5 – 5000 ng/L.

2.3 Statistical analysis

Data of BNP were presented with categorical variables and with continuous variables. The difference of circulating BNP between the survival and the non-survival were compared by Wilcoxon signed rank test. Categorical variables were compared by χ^2-test. Both univariate and stepwise multivariate Logistic forward regression analysis were used to evaluate the prognostic value of the parameters. The criterion for inclusion in the regression equation was $P<0.05$. The criterion for exclusion from the regression equation was $P>0.1$. A value of $P<0.05$ was considered statistically significant. All data analysis was performed using the Statistical Package for Social Sciences (SPSS 11.0).

3 Results

The mean of BNP for all patients was 511.05 ± 799.57 ng/L (5 – 5000 ng/L), median was 172 ng/L, and quartile range was 37.5 – 596 ng/L. There were 12 patients whosecirculating BNP was above 172 ng/L (median) and 10 patients whose circulating BNP was above 596 ng/L (75 percentile) in the non-suverial ($n = 13$). Patients with circulating BNP above 172 ng/L and those with circulating BNP above 596 ng/L had a mortality of 23.1% (12/52) and 40.0% (10/25) at 4 weeks, respectively. Rank correlation analysis demonstrated that the circulating BNP levels and Killip class were positively correlated with cardiac death ($r = 0.429$, $P<0.0005$; $r = 0.316$, $P = 0.001$).

The circulating BNP level in the non-survival group was significantly higher than the survival group (median 1169 vs 126 ng/L, $U = 148$, $P<0.0001$, Figure 1). Univariate analysis (Table 1) showed that BNP (≥172 ng/L, median) and Killip class (Ⅱ – Ⅳ) were prognostic factors of short-term cardiac death in patients with ACS ($P<0.001$ and $P = 0.001$).

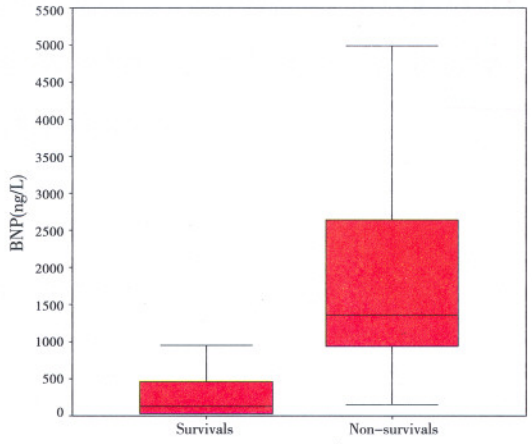

Figure 1. Comparison of circulating B-type natriuretic peptide between the survival and non-survival groups.

Table 1. Comparison between the survival and non-survival groups

Variables	Non-survival group($n = 13$)	Survival group($n = 93$)	P value
Sex(men/women)	9/4	62/31	0.99
Age(<60/≥60)	2/11	33/60	0.26
Smoking	6	19	0.09
Drinking	5	17	0.19
Family history	1	11	0.99
H/O DM	3	12	0.56
H/O Hypertension	7	45	0.71
H/O HF	5	19	0.27
H/O MI	3	13	0.66
Killip class (stage Ⅰ/Ⅱ – Ⅳ)	1/12	52/41	0.001
BNP (<172 ng/L/≥172 ng/L)	1/12	52/41	<0.0005

H/O: history of; DM: diabetes mellitus; HF: heart failure; MI: myocardial infarction.

Stepwise logistic forward regression analysis demonstrated that smoking and circulating BNP (above 596 ng/L, 75 percentile) were independent predictors of cardiac death at 4 weeks (OR = 5.5, 95% confidence interval 1.20 – 25.30, $P = 0.028$; OR = 21.19, 95% confidence interval 4.53 – 99.06, $P<0.0005$); covariates were age, sex, smoking, drinking, history of heart failure, history of myocardial infarction, hypertension, diabetes mellitus, Killip class and circulating BNP (above 596 ng/L). The risk of death in patients with smoking and circulating BNP level above 596 ng/L were 5.5 times and 21 times higher than non-smokers and those with circulating BNP level under 596 ng/L, respectively.

4 Discussions

BNP is a 32-amino-acid polypeptide secreted by the cardiac ventricles in response to increased stretch or wall tension[3,4]. Its diverse actions include natriuresis, vasodilation, inhibition of the rennin-angiotensin-aldosterone system, and inhibition of sympathetic nerve activity[8]. Previous studies have shown that BNP could be used to diagnose and to evaluate the prognosis of congestive heart failure[6]. This study showed that BNP is an independent predictor of short-term prognosis in patients with ACS. The risk of death in patients with circulating BNP level above 596 ng/L was 21 times higher than those with circulating BNP level under 596 ng/L.

A recent study found that the base-line level of BNP was correlated with the risk of death, heart failure, and myocardial infarction 10 months after the ischemic event[7]. In patients with non-ST elevation ACS, the mortality at 7 day and 6 month also increased significantly in patients with BNP above 80 ng/L[8]. Our preliminary study on 4-week mortality yielded similar results, indicating that measuring the circulating BNP levels within 3 days of ischemic event can give clinical physicians critical information in evaluating the severity of the disorder. For patients with very high circulating BNP levels (above 596 ng/L), regardless the severity of ischemic symptoms, physicians should pay great attention to these patients and treat them aggressively, in order to decrease mortality, and improve the short-term prognosis.

It is unclear why BNP is elevated in our patients with ACS who did not have noticeable heart failure. The release of BNP from myocardial cells is provoked by a variety of stimuli, including hyposia, ischemia, increased wall stress, and dilation of ventricles[10]. In rats, rapid induction of ventricular BNP gene expression and BNP production was found in the infarct region and the ischemic periinfarct region. BNP expression was also identified in the nonischemic surrounding myocardium 4 hours after ischemia or infarction[11]. Transient increase in BNP secretion can be found in patients undergoing percutaneous transluminal coronary angioplasty (PTCA)[12]. Above studies indicate that myocardial ischemia can induce synthesis and secretion of BNP, even without myocyte necrosis and overt left ventricular dysfunction.

The pathophysiologic mechanisms and clinical significance that inducing BNP synthesis and secretion by myocardial ischemia are follows[10]. First, elevation of BNP may result from acute ventricular dysfunction caused by myocyte necrosis, and the risk associated with elevated levels could therefore be related to the effect of ventricular impairment on mortality or to a new or progressive heart failure. Second, elevation of BNP levels may result from acute myocardial stretch caused by ischemia without myocyte necrosis, and the risk associated with elevated levels could therefore be related to the extent of ischemia and the consequent risks of future infarction and arrhythmia.

This study also demonstrated that smoking was a significant predictor of cardiac death in patients with ACS. Smoking and hyperlipidemia were the main clinical risk factors for coronary spasm among Chinese[13]. Brummett's study indicated that depressive symptom, smoking, and sedentary behavior were independent predictors of mortality in patients with coronary artery disease[14]. This study accords with above two studies.

The limitations of this study are that the samples were relatively small and the time of follow-up was very short. Larger and double-blinded trials may be required to validate the values of BNP in the risk stratification for patients with ACS.

5 Conclusions

Measuring blood BNP within 1 – 3 days of admission could provide important clinical information for predicting the prognosis of ACS. The risk of death in patients with circulating BNP level above 596 ng/L is 21 times higher than those with circulating BNP level under 596 ng/L. Therefore, aggressive treatment should be applied to patients with very high circulating BNP levels.

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Transposition of Pedicled Adrenal for the Treatment of Cushing's Disease

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Abstract: Objective. To explore a rational surgical treatment for the Cushing's disease. **Methods.** 68 patients with Cushing's disease were treated in our department with a new operation designed by authors between October 1990 and October 2004. All the patients presented typical Cushing's syndrome. The level of 24 hour urinary excretion of 17- hydroxycorticosteroids (17-OHCS) fluctuated from 40 – 125 μmol/24 h (62.5 ± 27.8). The diurnal rhythm of the plasma cortisol level was lost in all cases. In the operation, left adrenal gland was freed from surrounding tissue with the tissues between upper pole of adrenal and diaphragm was saved. Thus, a pedicle about 1.5 cm in width and 4 – 6 cm in length could be formed and the gland could be transposed into dorsal subcutaneous tissue through 11th intercostal space. Then right total adrenalectomy was performed. Postoperative histological diagnoses were bilateral adrenocortical hyperplasia. Adrenocortical substitution therapy was administered after operation and stopped completely 10 to 14 postoperative days in all patients. **Results.** All of patients recovered well after operation. 56 patients have been followed for six months to nine years. 52 patients were well. In these patients, plasma and urinary cortisol values had returned to normal levels and all clinical signs of Cushing's disease were absent. 42 patients had been followed up more than 3 years. The level of 24 hour urinary excretion of 17-OHCS decreased to 22.6 ± 9.2 μmol/24 h ($P < 0.001$) in 3 postoperative years. Hypoadrenocorticism developed in 4 patients and skin hyperpigmentation was observed. The patients required adrenocortical replacement therapy. No recurrent Cushing's syndrome was observed in all patients. **Conclusions.** It's simple to make a vascular pedicle on upper pole of the adrenal and transpose the pedicled adrenal through 11th intercostal space into the dorsal subcutis. Second operation is easy to perform if relapse of syndrome occurs. Nowadays, this procedure may be a rational technique for the treatment of Cushing's disease. [Life Science Journal. 2005;65 – 67] (ISSN: 1097 – 8135).

Keywords: adrenal; Cushing's disease; surgical treatment

1 Introduction

It's well recognized that Cushing's disease is caused by overproduction of cortisol. The majority of cases (85%) are due to bilateral adrenocortical hyperplasia stimulated by overproduction of pituitary adrenocorticotropic hormone (ACTH). Though many treatments for Cushing's disease are presented, there is no ideal one up to now[1]. In the clinical practice, we have found that adrenal vascular and the surrounding tissue are very abundant and the superior adrenal artery and vein with their surrounding tissues can be dissected and formed a vascular pedicle which is about 4 – 6 cm in length. Therefore, an operation with transposition of pedicled adrenal beneath dorsal subcutaneous was designed. Form October 1990 to October 2004, 68 cases of Cushing's disease were treated with this technique and satisfactory results have been achieved.

2 Operative Technique

Under extradural anesthesia, 11th intercostal incision is employed. The left adrenal is operated on firstly. The dorsal and upper parts of Gerota's fascia are incised and Gerota's fascia is dissected. The adrenal gland is exposed and explored to demonstrate the hyperplastic adrenal gland. The gland is freed laterally, inferiorly, anteriorly, posteriorly and medially. The tissues between upper pole of adrenal and diaphragm are saved as much as possible for protection of superior adrenal artery and vein that come from inferior phrenic vessels. The freed gland is pulled upwards and laterad. If the medial parts of tissues are felt tense, some of them could be divided. Then, a satisfactory pedicle is formed and sometimes several little vessels that pass into gland or its surrounding tissues can be seen. The pedicle is about 1.5 cm in width and 4 – 6 cm in length, so the gland can be transposed into dorsal subcutaneous tissue though 11th intercostal space easily. The bases attach to diaphragm as a fan. The way of the transposition ought to be as

short as possible to reduce the pedicle tense. If diaphragm is in the way of transposition, parts of crus of diaphragm should be divided. This procedure makes the transposition way shorter and straighter remarkably. The pedicle is fixed with sutures with nearby intercostal muscle. 10% of adrenal is removed for histological examination. The remained adrenal is fixed beneath the dorsal subcutis. The gland is marked with silver clips for X-ray location. Then right total adrenalectomy is performed. If the transposition on left gland is unsatisfactory or unsuccessful, a transposition on right adrenal could be performed as procedures above, but it's more difficult than on the left due to the proximity of the liver and vena cave. Adrenocortical substitution therapy was administered after operation and stopped completely 10 to 14 postoperative days in all patients.

3 Clinical Material

We reviewed the records of 68 patients with Cushing's disease treated in our department between October 1990 and October 2004. Cases of pituitary adenomas were excluded. Fifty-three of patients were female and fifteen were male, and their ages ranged from 11 – 50 years (mean 36.5). All the patients presented typical Cushing's syndrome. The level of 24 hour urinary excretion of 17-OHCS fluctuated from 40 – 125 (62.5 ± 27.8) μmol/24 h. The diurnal rhythm of the plasma cortisol level was lost in all cases. Skull roentgenography and tomography of the sella turcica were performed to exclude the presence of a pituitary tumor. However, no pituitary abnormality was demonstrated. Studies to localize adrenal hyperplasia with tomography were also performed. Postoperative histological diagnoses all were bilateral adrenal adrenal cortical hyperplasia. 56 patients have been followed for six months to nine years. 52 patients were well. In these patients, plasma and urinary cortisol values had returned to normal levels and all clinical signs of Cushing's disease were absent. 42 patients have been followed up more than 3 years. The level of 24 hour urinary excretion of 17-OHCS decreased to 22.6 ± 9.2 μmol/24 h ($P < 0.001$) in 3 postoperative years. Hypoadrenocorticism developed in 4 patients and skin hyperpigmentation was observed. The patients required adrenocortical replacement therapy. No recurrent Cushing's syndrome was observed in all patients.

4 Discussion

It's well known that the vessels of adrenal are so multiple and variable that it is difficult to dissect and utilize them. Our experience reveal that it is possible to moderately free superior adrenal artery and vein 4 – 6 cm long. According to literature[2], the superior adrenal artery comes from inferior phrenic artery that is the main blood supply for adrenal and the branches of it form a vascular circle around adrenals with other artery. The venous drainage of adrenal gland is almost exclusively via the inferior adrenal vein. The superior, medial and inferior adrenal arteries all paired by their relative veins. Generally superior, medial veins don't take main role in adrenal vein drainage, but when inferior adrenal vein is obstructed, they can exert drainage function[2,3]. Therefore the blood circulation in upper pole of adrenal may be maintained and the necrosis can be avoided providing superior artery and vein are preserved. Between upper pole of adrenal and diaphragm, there are a lot of connective tissues. Superior adrenal vessel passes through them. In order to form a satisfactory pedicle, these tissue should be preserved as much as possible. In this group of patients, the length of pedicle was about 4 cm in children and 5 – 6 cm in adults. In some pedicles there were several vessels to be seen and oozing of blood on severed gland edges was visible in some cases, but these was not obvious in other cases. In the following up the patients, we found that the adrenal function in these patients was similar. Therefore, we realized that it's easy to form a vascular pedicle on the upper pole of adrenal and it's not important for survival and function of transposed adrenal if oozing of blood on severed edges is visible or not, providing the pedicle are thick enough.

It's important for this procedure to form a satisfactory vascular pedicle. An appropriate operative approach is a key. Anterior transabdominal approach was employed on first patient. It was difficult to expose the upper pole of adrenal gland and make a 5 cm long pedicle. When we transposed this pedicled adrenal into dorsal subcutis, the pedicle felt tension as the way is longer. From then on, 11th intercostal incisions were employed routinely. Through 11th intercostal space and external lateral edge of sacrospinal muscle, the transposition way is straight and short for fixing gland into the dorsal subcutis with flaccid pedicles. Therefore, we recommend 11th intercostal incision for this procedure.

About the problems of the amounts of remained adrenal, traditionally 10 – 20% of adrenal are remained in subtotal adrenalectomy and may meet the daily needs of human bodies. In this group, more glands were remained[4]. 90% of each

adrenal gland was remained. The reason for this is that we remained 30% of gland in the operation in first several patients and hypoadrenocorticism developed in 2 patients. Thereafter, we try our best to remain more glands. Up to now, we don't find any cases of hyperadrenocorticism. We think the reasonable explanations are that: (1) Media and inferior adrenal vessels are divided completely and superior adrenal vessel is divided partially in the operation. The blood supply to the adrenal is not good, only parts of upper pole gland can survive postoperatively no matter how many glands are preserved. (2) The best place for the development of adrenal is its anatomy location. Dorsal subcutis is not suitable for its development.

In brief, we conclude that it's simple to make a vascular pedicle on upper pole of the adrenal and transpose the pedicled adrenal through 11th intercostal space into the dorsal subcutis successfully. Second operation is easy to perform if relapse of syndrome occurs. Nowadays, this procedure may be a rational technique for the treatment of Cushing's disease.

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Study of Remanent Activated Sludge Used as Biosorbent (I) Effect of pH on Zeta Potential of Dissociative Bacteria and Its Application in the Adsorption of Activated Sludge

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Abstract: The thesis researched the effect of pH value on the adsorbability of activated sludge and on zeta potential of dissociative bacteria in it. We found that with the change of pH, the changing trend of the two was consistent. Considering the cell wall as functionary target, the author discussed the chemistry reaction between acid or alkali and peptidoglycan or teichoic-acid of the cell wall. From this, the author explained the changing trend of bacterial zeta potential, and expounded the relation between it and the adsorbability of activated sludge. [Life Science Journal. 2005;2(1):68-71] (ISSN: 1097-8135).

Keywords: biosorbent; activated sludge; zeta potential; dissociative bacteria

1 Theory

1.1 The surface zeta potential of bacteria in activated sludge

There are many sorts of microorganism, but most are bacteria[1]. The chemical components of bacterial cell wall and polymer on the surface of the cell decide that the surfaces of bacteria are electronegative. So bacteria are the main reason why activated sludge has adsorbability.

Activated sludge possesses adsorbability, which is related with the zeta potential of bacteria in it[2]. And the value of zeta potential is tied up with the value of charge on bacterial surface. The value of zeta potential is determined by dint of electrophoresis phenomenon of electriferous particulate with the function of electric field. From the Helmohohz-smolachowski formula[3], it has direct ratio with electrophoresis velocity of electriferous particulate. So it can be calculated by determining the value of the velocity.

In this article, we studied the effect of pH value on the zeta potential of bacteria in activated sludge. And our purpose, for one thing, it was to research how to change the charge value of bacteria and improve the adsorbability. For another, it was important for the research of microbe ecology to investigate the optimum pH condition for microbe to develop. The reason was that pH value directly affected the microbe's variety, quantity and life activity, mode of metabolization and also type of production metabolized, surface speciality and so on[4]. Moreover, the significance of the problem studied consists in providing theoretic groundwork and thinking direction for recycle investigation of remnant activated sludge.

1.2 Structure and chemistry component of bacterial cell wall

Bacteria have special structure of cell wall which other organisms have not. Substance constituting the framework of bacteria is peptidoglycan composed of peptide chain and amino sugar chain. And add other components such as amylose, muramic acid, protein, mucopolysaccharide, and then bacterial cell wall with different kinds of function has been made up[5].

2 Materials and Methods

2.1 Material

Reagent: NaOH (AR), HCL (AR), Active carbon (The No. 3 Chemistry Reagent Factory of Tianjin City); Fresh activated sludge; Reactive brilliant red X-3B (C. I. Reactive Red 2); Acid lake blue A (C. I. Acid Blue 7) (The No. 3 Reagent Factory of Shanghai).

Apparatus: TL-5.0 desk style centrifugal machine (Shanghai Centrifugal Machine Graduate School, Shanghai, China); 79-1 magnetic force heating blender (Jintan Medical Treatment Apparatus Factory, Jintan, China); BDL-B surface potential particle diameter apparatus (Shanghai Shangli Survey Apparatus Factory, Shanghai, China); pH-pXFL:G1 acidity meter (Fanlong Instrument Corporation, Shanghai, China); 721 spectral pho-

tometer (The No. 3 Analysis Apparatus Factory of Shanghai, Shanghai, China).

2.2 Method

(1) Pretreatment of sludge: Remove some water of the fresh activated sludge by centrifugal (2000 rpm, 3 min), and wash it four times with distilled water. Then get the abluent wet sludge (containing water 93%).

(2) Effect of pH value on zeta potential of dissociative bacteria in activated sludge: Get 20 g wet sludge every share, place in 100 cm^3 distilled water, and adjust the pH to needed value. Then separate it by centrifugal and measure pH value of the clear liquid above, and determine respectively the surface zeta potential of six kinds of dissociative bacteria, that is staphylococcus, micrococcus, diplococcus, spirillum, bacillus and capsul-micrococcus[6].

(3) Effect of pH on adsorbability of activated sludge: Separately place 2 g wet sludge in 50 cm^3, 100 mg/L reactive brilliant red solution and acid lake blue solution. And adjust the pH value using 0.5 mol/L HCl. When the system reaches adsorption equilibration, determine pH and absorbency of the solution at the most adsorption wavelength of the corresponding dyestuff with 721 spectral photometer. Then calculate the adsorption value of activated sludge and elimination rate of reactive brilliant red and acid lake blue at different pH.

(4) Compare of adsorbability of activated sludge and active carbon: Dry some active carbon (−200 mesh) at 105℃ for 4 hours in oven. Take 0.15 g dried active carbon every share, at pH = 6 and 3, separately place in 50 cm^3 reactive brilliant red solution (250 mg/L) and acid lake blue solution (300 mg/L). When it comes to adsorption equilibration, determine its absorbency. Simultaneously, at the same condition determine the adsorption of 1.5 g dried activated sludge at pH = 3. Calculate respectively the equilibrium concentration and adsorption value.

3 Results and Discussion

3.1 Effect of pH on zeta potential of dissociative bacteria in activated sludge

With alteration of pH, the changing circs of surface zeta potential of the six kinds of bacteria are shown in Figure 1.

We can see from Figure 1 that when pH reduces, zeta potential (minus means the surface of bacteria take negative charge) of the six kinds of bacteria are all decreasing. At low pH, the changing trend is similar to that of surface potential of activated sludge granule reported[3]. And the differences between them just prove that zoogloea is not entirely equal to dissociative bacteria in character.

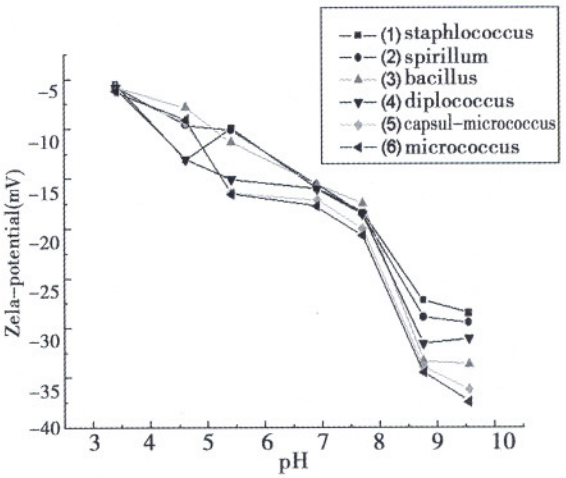

Figure 1. Effect of pH on zeta potential of dissociative bacteria in activated sludge

The changing trend of zeta potential of the dissociative bacteria in Figure 1 can be explained by the chemical reaction between H^+ and peptidoglycan. When add acid to the dissociative system of bacteria, on the one hand, amido and imido group in peptidoglycan are protonized and then take positive charge. On the other hand, ionization equilibration of carboxyl moves to the reverse direction and thus the amount of carboxyl negative ion is reduced. Therefore, while pH of the system is minshing, the negative charge of cellular surface is decreasing, and bacterial velocity of electrophoresis is also decreasing with the function of electric field. It results in reduction of bacterial surface zeta potential.

3.2 Effect of pH on adsorbability of activated sludge

Alteration of pH value makes change of the adsorbability of activated sludge. Figure 2 shows adsorption value of reactive brilliant red and acid lake blue by activated sludge at different pH value, and the change of elimination rate of the two dyestuffs in the experimental condition.

It can be seen from Figure 2 that the adsorption value is increased when pH value minishes. And the elimination rate takes on the same changing trend. Take reactive brilliant red for example, when pH reduces to about 4.7, the adsorption of activated sludge increases promptly. And when pH reduces to about 3.0, the adsorption goes on to increase but the change is not obvious. At pH = 3.0,

the adsorption adds to 29.47 mg/g, and the elimination rate adds to above 95%. The changing status of acid lake blue is similar. Therefore pH=3 is an appropriate critical point of adsorption.

Comparing Figure 2 and Figure 3, we can know that with the change of pH, the changing trend of adsorption of activated sludge is in accord with that of dissociative bacterial surface zeta potential. Reactive brilliant red and acid lake blue are negative ion dyestuff. In water solution, electronegative sulfonic group ($-SO_3^-$) they ionized links with electropositive amido ($-NH_3^+$) in peptidoglycan by ionic bond[7]. When pH decrease, the zeta potential decrease, that is the negative charge of bacterial surface reduces. And it is advantageous for it to contact closely with the molecule of dyestuff, and for adsorption to happen. So the adsorption value is increased.

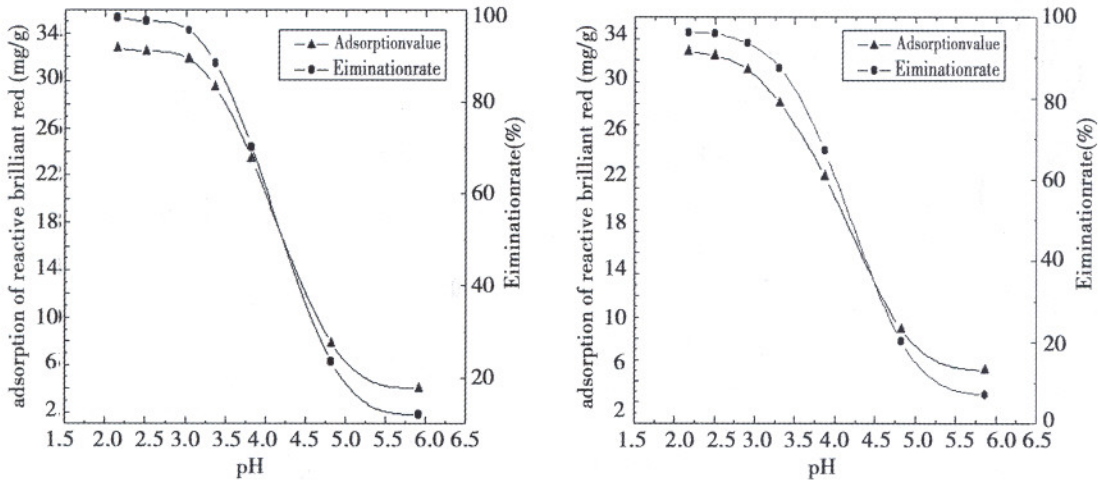

Figure 2. Effects of pH on adsorption of activated sludge on reactive brilliant red and acid lake blue

Table 1. Compare of adsorbability of activated sludge and active carbon

Dyestuffs and Their Concentrations	Active carbon		Activated sludge	
	PH=6	PH=3	PH=3	
	Balance Concentration mg/l	Balance Concentration mg/l	Balance Concentration mg/l	Balance Concentration mg/l
Reactive Brilliant Red (250 mg/l)	31.4	11.5	79.5	11.6	91.7
Acid Lake Blue (300 mg/l)	79.1	31.8	89.4	17.5	108.7

3.3 Compare of adsorbability of activated sludge and active carbon

As show in Table 1, the adsorption of active carbon is better at rather low pH condition. However, the adsorption of activated sludge is much higher than that of active carbon when pH = 3. This suggests that the adsorption of acidic activated sludge on dyestuffs is more rapid in pace, better in capability and effect.

4 Conclusion

To sum up from above, the change of pH condition makes surface zeta potential of bacteria change, and so does the adsorbability of activated sludge. Thereby, we can let activated sludge have better adsorbability by adjusting the pH.

Remanent activated sludge is the object for disposing in City Sewage Disposal Factory. If it can be used as sorbent after simple treatment, then not only the cost is very cheap but the effect is rather good. And this nicely accords with the research and development direction of sorbent for dyestuffs—sorbent with high effect and low cost.

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Simvastatin Improved Matrix Metalloproteinase Mediating Ventricular Remodeling in Rats after Myocardial Infarction

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Abstract: Objectives. It was observed that effect of simvastatin on both matrix metalloproteinase (MMP)-2,9 and type I collagen mediating ventricular remodeling in rats after acute myocardial infarction (AMI). **Methods.** The AMI model of rat was made by ligature of left anterior descending coronary artery, and the animals were divided into three groups: simvastatin treatment group (MI-S), myocardial infarction control group (MI-C) and sham group (Sham). The animals were feed four weeks. The mRNA expression of MMP-2 and MMP-9 in noninfarcted zone of left ventricle (LV) were determined by reverse transcription-polymerase chain reaction (RT-PCR), and cardiac type I collagen in noninfarcted zone were measured by immunohistochemistry, cardiac function was determined by echocardiography and hemodynamics analysis. **Results.** After four weeks, mRNA expression of MMP-2 and -9 and type I collagen in LV post-myocardial infarction (MI) groups were more than Sham group ($P<0.05$), and the indices in MI-S group were significantly lowered than those in MI-C group ($P<0.05$), while higher than Sham group ($P<0.05$). Compared with Sham group, hemodynamics analysis showed that left ventricular end-diastolic pressure (LVEDP) significantly increased ($P<0.01$), while systolic blood pressure (SBP), diastolic blood pressure (DBP), left ventricular systolic pressure (LVSP) and LV pressure $\pm dp/dt_{max}$ significantly decreased ($P<0.05$), and there was no change for heart rate (HR) in MI-C group. Compared with MI-C group, the LVEDP significantly lowered ($P<0.05$), and LV pressure $\pm dp/dt_{max}$ obviously increased ($P<0.05$), while HR, SBP, DBP and LVSP did not alter in MI-S group. Compared with Sham group, echocardiography showed that left ventricular end-diastolic diameter (LVEDd) significantly increased, and both fractional shortening (FS) and ejection fraction (EF) significantly reduced ($P<0.05$, respectively) in MI-C group. Compared with MI-C group, simvastatin significantly decreased LV dilatation and improved LV function ($P<0.05$). **Conclusion.** Simvastatin could attenuate mRNA expression of MMP-2 and MMP-9 in LV 4 weeks after MI-rats, reduced collagen synthesis, and improved cardiac function. [Life Science Journal. 2005;2(1):72-76] (ISSN: 1097-8135).

Keywords: simvastatin; acute myocardial infarction; matrix metalloproteinase-2,9; RT-PCR; immunohistochemistry; hemodynamics; echocardiography; rat

1 Introduction

The extent of primary ischemic necrosis as well as the later effects of distending forces and the cardiac tissue healing process influenced ventricular dilatation after myocardial infarction (MI)[1-3]. The activity and dynamic expression of matrix metalloproteinases (MMPs) may affect lots of the morphological changes that happen after MI at both infracted and peri-infarcted zones[4,5]. MMPs are members of a family of enzymes that degrade specific extracellular matrix (ECM) components; the activity of MMPs is increased in both experimental MI and clinical dilated cardiomyopathy[6]. As extracellular matrix degradation may play an important role in LV remodeling, MMP inhibiting has emerged as a potential therapeutic strategy for patients at risk for development of congestive heart failure. Preliminary data proposed that administration of an MMP inhibitor may decrease left ventricular enlargement in pacing-induced models of congestive heart failure and in spontaneous heart failure in rats[7]. The effects of MMP inhibition in the post-MI period are incompletely understood. The present study evaluated the effects of administration

of oral simvastatin in early left ventricular remodeling as assessed by transthoracic echocardiography and hemodynamics after experimental MI in rats.

2 Materials and Methods

2.1 Infarction model and experimental groups

Wistar rats weighing 200 − 240 g were anesthetized by intraperitoneally injection of sodium pentobarbital (30 mg/kg) intubated, and ventilated with a small-animal respirator. The left anterior descending coronary artery was ligated proximately with a 7 − 0 silk suture after a left anterior thoracotomy. Sham-operated rats underwent the identical procedure without ligation of coronary artery. The following experimental groups were studied: ① Sham-operated (Sham) ($n = 10$); ②MI control (MI-C) ($n = 12$); ③MI simvastatin (MI-S) ($n = 12$). Simvastatin (40 mg/kg body weight) was given by gastric gavage 24 hours after the anesthesia, then continued for 4 weeks (40 mg/kg per day). An equal amount of normal saline was given to the other two groups every day.

2.2 Measurement of MMP-2, 9 mRNA expression by RT-PCR

The infarcted heart was sectioned into noninfarcted zone by visual inspections. Total RNA was reversely transcribed into first-strand cDNA after isolation by using TRIzol reagent. MMP-2, 9 and GAPDH gene expression were analyzed in noninfarcted area. The sense primer (S) and the antisense primer (A) for MMP-2, 9 were as follows:
MMP-2 S, 5′-ACCATCGCCCATCATCAAGT -3′, A, 5′-CGAGCAAAAGCATCATCCAC -3′(348bp-production).
MMP-9 S, 5′-AACTTTGTAGGGTCGGTTCTG-3′, A, 5′-CCCTGTGAGTGGGTTGGATT-3′ (469bp-production).
GAPDH S, 5′-TATGATGACATCAAGAAGGTGG -3′, A 5′-CACCACCCTGTTGCTGTA-3′ (213bp-production).

PCR amplification was performed by adding each cDNA sample 2 μl to 20.5 μl of reaction mixture. Each cycle consisted of denaturation at 94°C for 40 seconds, annealing for 40 seconds (MMP − 2, 9 at 60°C, GAPDH at 56°C), extension at 72°C for 1 min, and final extension at 72°C for 5 min. Each PCR product was separated by electrophoresis on a 1.5% agarose gel and tested by a digital image analysis system (GSD8000, UVP, England). Each amplified cDNA fragment was counted for semiquantitative evaluation by normalization with the GAPDH band.

2.3 Measurement of type I collagen by immunohistochemistry

Paraffin-embedded myocardium specimens in noninfarcted zone were serial sectioned into a thickness of 3 μm. The section was incubated with primary antibodies [anti-rat type I collagen (Monoson) (1 : 100)] which were stored at 4℃ overnight. Incubation with biotinylated second antibody was performed at room temperature for 30 min. Immunoreactivity was evaluated under the microscope using the HPIAS-2000 software.

2.4 Echocardiography and hemodynamics analysis

Two-dimensional echocardiography was performed on each rat before surgery and 4 weeks after surgery with a 10 MHz (short focus) transducer. Long-axis, short-axis, and apical four-chamber images were measured. The LV end-diastolic and end-systolic volumes (EDV and ESV, respectively) were calculated by the modified Simpson's method[8]. Cardiac output was calculated as (EDV-ESV)/1000 × heart rate; the LV ejection fraction (LVEF) was determined as (EDV-ESV)/EDV × 100%. The fractional shortening (FS) was measured at the short-axis image. Hemodynamic studies were performed after the animals were anesthetized with an intraperitoneal injection of 1.0 g/kg urethan. Through the right common carotid artery, a catheter filled with heparin solution was inserted into LV and hemodynamic data recorded.

2.5 Statistic analyses

The data are given as mean ± SD. The multivariate ANOVA was used to determine the overall difference between the three independent groups. The statistical significance between groups was determined using a post hoc Bonferroni/Dunn test. A value of $P<0.05$ was considered significant.

3 Results

3.1 RT-PCR analysis of MMP-2, 9 gene

Compared with Sham operation group, the mRNA expression of MMP-2 and MMP-9 significantly increased in noninfarcted zones of MI-C group ($P<0.05$). MMP-2 and MMP-9 expression were obviously decreased in MI-S group ($P<0.05$), but still higher than those in Sham operation group ($P<0.05$) (Figure 1).

3.2 Immunohistochemistry analysis of type I collagen

Compared with Sham operation group, type I collagen was markedly increased in noninfarcted zone of MI-C group ($P<0.01$). Compared with MI-C group, type I collagen was significantly lowered in MI-S group ($P<0.05$) (Figure 2).

3.3 Hemodynamics analysis

Table 1 displays hemodynamic data at 4 weeks after operation. In the MI group, LV end-diastolic

pressure (LVEDP) significantly increased ($P<0.01$), systolic blood pressure (SBP), diastolic blood pressure (DBP), LV systolic pressure (LVSP) and LV pressure maximal rate of rise and fall ($\pm dp/dt_{max}$) noticeably decreased ($P<0.05$), while heart rate (HR) did not change compared with the Sham operation group. LVEDP decreased ($P<0.05$) and $\pm dp/dt_{max}$ significantly increased ($P<0.05$) in the MI-S group compared with the MI-C group. Other variables were not different between both MI groups.

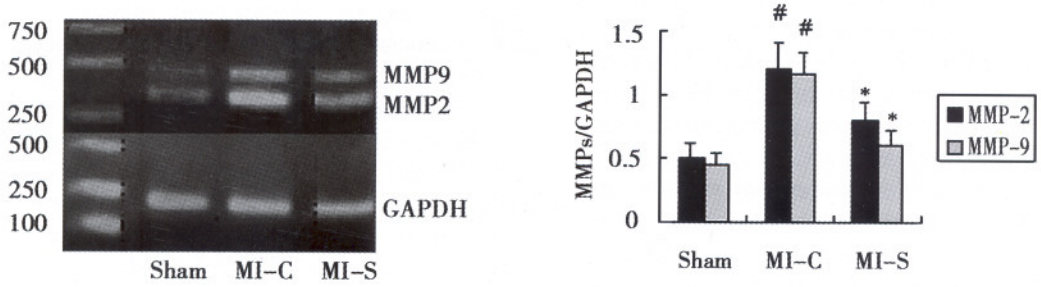

Figure 1. Effect of simvastatin treatment on mRNA expression of MMP-2,9 in Sham group, non-infacted LV myocardium of MI-C group and MI-S group 4 weeks post-MI. Quantitative analyses mRNA of MMP-2,9, * $P<0.01$ vs. Sham group, # $P<0.05$ vs. MI-C group.

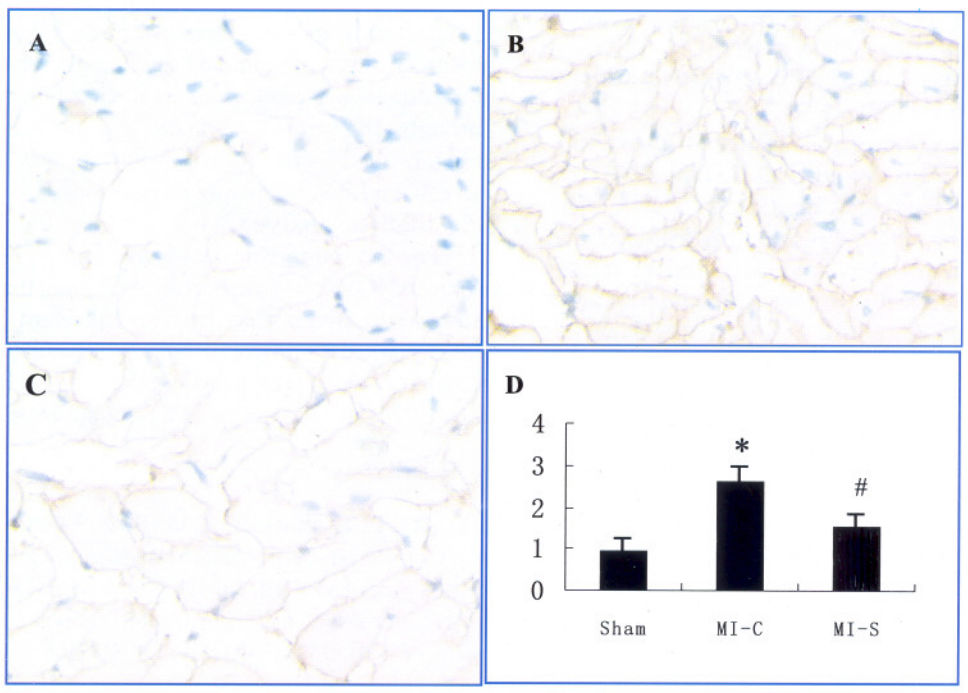

Figure 2. Effect of simvastatin greatment on protein production of type I collagen in Sham group (A) non-infacted LV myocardium of MI-C group; (B) and MI-S group; (C) 4 weeks post-MI (magnification × 200); (D) Quantitative image analyses of type I collagen production, * $P<0.01$ vs. Sham group, # $P<0.05$ vs. MI-C group.

Table 1. The effects of simvastatin treatment on hemodynamics

Groups	*n*	HR (bpm)	SBP (mmHg)	DBP (mmHg)	LVSP (mmHg)	LVEDP (mmHg)	$+dp/dt_{max}$ (mmHg/s)	$-dp/dt_{max}$ (mmHg/s)
Sham	10	361 ± 23	125.7 ± 8.3	99.1 ± 9.0	131.9 ± 8.3	2.2 ± .0.3	6546 ± 631	5477 ± 485
MI − C	12	383 ± 25	109.4 ± 9.1*	89.7 ± 7.8*	113.3 ± 10.5*	22.5 ± 4.7*	4084 ± 449*	2837 ± 251*
MI − S	12	377 ± 19	110.1 ± 9.6*	90.3 ± 7.1*	112.8 ± 11.1*	12.6 ± 1.5*#	4951 ± 381*#	3435 ± 358*#

Compared to Sham group, * $P<0.05$; Compared to MI-C group, # $P<0.05$

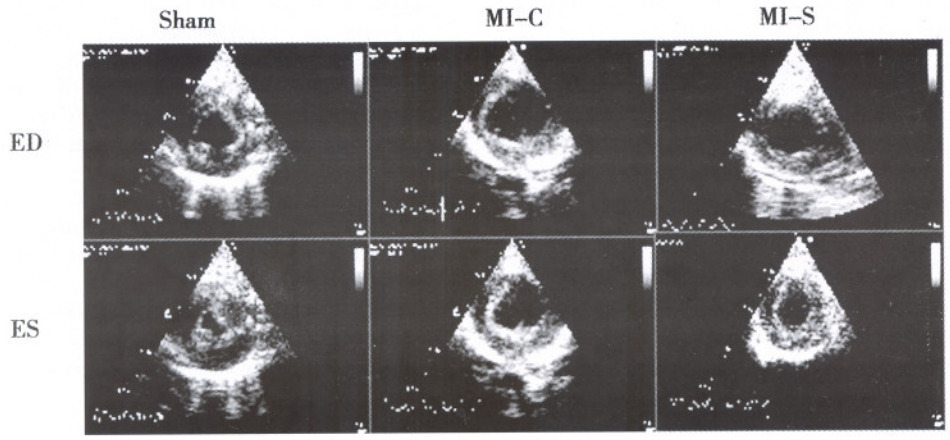

Figure 3. Echocardiographic vews of LV from a sham-operated rat (Sham), an untreated MI rat (MI－C) and a simvastatin-treated MI rat (MI-S) at end-diastolic (ED) and end-systolic (ES) dimension. The MI-S rat has decreased left ventricular diastolic dimensions compared with the untreated MI-C rat.

3.4 Echocardiographic studies

Figure 3 shows representative short-axis echocardiographic images from a Sham-operated animal, an untreated MI rat and a simvastatin-treated MI rat. Compared with Sham operation group, there were significantly increased LVEDd ($P<0.01$), markedly decreased FS ($P<0.01$) and EF ($P<0.01$) in the MI-C group. Although these parameters also differed from the Sham operation group in the MI-S group, simvastatin significantly attenuated LV dilatation and improved LV function compared with the MI-C group ($P<0.05$) (Table 2).

Table 2. The effects of simvastatin treatment on echocardiographic measurements

Groups	n	LVEDd (mm)	EF (%)	FS (%)
Sham	10	4.3±0.2	87.2±3.5	53.4±3.3
MI－C	12	7.8±0.4*	42.1±3.9*	21.2±2.1*
MI－S	12	5.6±0.3#	49.7±4.1#	27.5±2.2#

Compared to Sham group, * $P<0.05$; Compared to MI-C group, # $P<0.05$

4 Discussions

MMPs are a family of zinc-depending endoproteinases that specifically degrade ECM components. They are important enzymes which degrade matrix in the cardiac remodeling process after MI. The activity increase of MMPs leads to reduction of ECM, destruction of cardiac supporting structure and ventricular dilatation[9]. MMPs modulate synthesis of collagen, the rise of MMPs activity enhances fibrosis, and fall of MMPs activity decreases fibrosis[10]. Clinical research confirmed[11] that MMPs were obviously associated with stability of atherosclerotic plaque, the plasm MMPs markedly rose in patients with coronary heart disease, and inhibition of MMPs activity may increase plaque stability. The experimental study proposed that MMPs participated in the remodeling process after MI, and were activated 1 day after MI. Activity and gene expression of MMP-2, 9 markedly increased during remodeling process after MI[12,13], and application of MMPs inhibitors could decrease ventricular dilatation in rats after MI and improve cardiac performance[14－17]. It was further showed that the rat with MMP-9 deficiency existed residual necrosis expansion, the healing process retard after MI[17,18].

Statins are potent inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, which are capable of lowering the serum cholesterol level and are successfully used to treat hypercholesterolemia and atherosclerosis. Moreover, the ability of statins to lower the mortality and morbidity of cardiovascular diseases has been ascribed not only to their cholesterol-lowering activities but also to a number of additional effects, including improving endothelial cell function, enhancing fibrinolysis, and antithrombotic activity. In addition, a number of important anti-inflammatory effects of statins have been reported.

This study confirmed that mRNA expression of MMP-2, 9 was significantly higher in MI rats, and simvastatin could obviously decrease mRNA expression of MMP-2, 9. It was assumed that simvastatin could not only stabilize atherosclerotic plaque, lower the incidence of unstable angina and MI, but also alleviate cardiac fibrosis, restrict development of ventricular remodeling and heart fail-

ure and improve prognosis patient with MI by inhibiting expression of MMPs. However, it is unknown whether statins directly or indirectly inhibited expression of MMPs.

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EGF Receptor Tyrosine Kinase Inhibitor Tyrphostin AG1487 Induce Human Tongue Cancer Cells Tca8113 Cell Cycle at G1 Phase and Apoptosis

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Abstract: Objective. This study was to investigate the effects of tyrphostin AG1487 on expression of activated ERK1/2, distribution of cell cycle and apoptosis in Tca8113 cells. **Materials and Methods.** Tca8113 cells were exposed to different concentrations of phosphotyrosine kinase inhibitor tyrphostin AG1487, and then immunocytochemistry, Western blot, flow cytometry, and electron transmission microscopy were employed to investigate the effects of AG1487 on expression of activated ERK1/2, distribution of cell cycle and apoptosis of Tca8113 cells. **Results.** The results of immunocytochemistry showed intensity of activated ERK1/2 reactivity in treated cells was marked decreased. These results were corresponded with the expression of activated ERK1/2 in Western blot: expressions of activated ERK1/2 gradually reduced along with the increasing of concentration of tyrphostin AG1487. Cell cycle kinetic analysis demonstrated that AG1487 induced a delay in cell cycle progression and arrested at G1 phase. Furthermore, AG1487 could induce a marked apoptosis of Tca8113 cells. All these effects were in a dose-dependent pattern. **Conclusion.** EGF receptor tyrosine kinase inhibitor tyrphostin AG1487 can inhibit the expression of activated ERK1/2, induce cell cycle arrest at G1 phase and apoptosis. [Life Science Journal. 2005;2(1):77-84] (ISSN: 1097-8135).

Keywords: Tca8113 cell; EGF receptor; ERK1/2; cell cycle; apoptosis

Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated kinase; ERK, extracellular signal- regulated kinase

1 Introduction

The EGF receptor belongs to the erbB family of four closely related cell member receptors: EGF receptor (erbB1), erbB2, erbB3 and erbB4. All these receptors are transmembrane glycoproteins that consist of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity for signal transduction[1-3]. After ligand binding, the tyrosine kinase of the receptor is activated, and initiates a cascade of intracellular events[4]. These events lead to recruitment and phosphorylation of several intracellular substrates, such as extracellular signal-regulated kinase (ERK), which lead to the mitogenic signaling and other cellular activities[1,4]. Many studies have shown EGF receptor is highly expressed in malignent tumors, and is associated with progression of malignant tumors and poor prognostic features. EGF receptor and its downstream signaling pathways are, therefore, becoming the promising antitumor targets for cancer therapy.

A variety of different approaches are currently being used to target the EGF receptor, the most promising strategies include to prevent ligand binding and to inhibit autophosphorylation of EGF receptor. Therapeutic mAbs targeting the extracellular domains of EGF receptor have similar affinity binding to EGF receptor as EGF and TGF-α, compete with these ligands for receptor binding, and block activation of receptor tyrosine kinase induced by EGF and TGF-α[5,6]. Small molecule inhibitors are generally reversible competitors with respect to ATP for binding to the intracellular catalytic domain of the tyrosine kinase[5]. Some studies had shown that small molecule inhibitors not only compete with ATP in the classical mode of action but also induce the formation of inactive, unphosphorylated EGFR dimmers[5,7].

Both *in vitro* and *in vivo* studies have demonstrated that EGF receptor targeted agents could in-

hibit the processes involved in tumor growth and progression, including proliferation, apoptosis, metastasis and angiogenesis[4,5,8]. All these effects are correlated with the blockade of EGF receptor signaling. ERKs, as one of the important signaling molecule that lies downstream of EGF receptor, usually be highly expressed in malignant tumor cells[9]. Some studies demonstrated EGF receptor targeted agents could inhibit the activation of EGF receptor and ERKs in malignant tumor cells[10-12].

For further study the EGF receptor agents effects on malignant tumor cells, we adopted small molecule inhibitor of EGF receptor tyrphostin AG1487 to treat human tongue cancer cells (Tca8113), and then to investigate the effects of AG1487 on expression of activated ERK1/2, distribution of cell cycle and apoptosis in Tca8113 cells.

2 Material and Methods

2.1 Tumor cell lines and regents

The cell line of human squamous cell carcinoma of tongue, Tca8113 cells, were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences and cultured in PRMI medium 1640 (GIBCO) containing 10% heat-inactivated FBS, 100 units/ml penicillin G and 100 unites/ml streptomycin at 37℃ in humidified air containing 5% CO_2. Polyclonal anti-EGF receptor antibodies obtained from Boster Biological Technology Co. (Boster, Wuhan, China), anti-total ERK1/2 antibodies and anti-activated ERK1/2 antibody obtained from Sigma Chem. Co. (St. Louis, MO, USA), Tyrphostin AG 1487 obtained from Calbiochem (San Diego, CA, USA).

2.2 Immunocytochemistry

Cells were seeded on coverslips coated with poly-L-lysine placed in six culture plates under the same conditions. On the next day, PRMI medium 1640 was refreshed and tyrphostin AG 1487 was added at indicated concentration (100 nM) to the cultures for 2 h. Then the slides were removed from the culture plates, and cells on coverslips were fixed in 2% paraformaldehyde solution with 0.1% Triton X-100 for 30 min at room temperature. Nonspecific protein reactivity was blocked with 10% goat serum (SABC) in PBS for 10 min. And endogenous peroxidase reactivity was blocked in 3% hydrogen peroxide solution for 10 min. Incubation with primary antibodies were made at room temperature for 1 h at following dilutions: EGF receptor (1:100), activated ERK1/2 (1:1000) and total ERK1/2 (1:1000). After being washed with PBS three times, the coverslips were incubated with goat anit-mouse IgG-HRP or goat anti-rabbit IgG-HRP antibodies (Boster, Wuhan, China) for 1 h at room temperature. Coverslips were visualized using 3-3′-diaminobenzidine as a chromogen for 5 min. Then the specimens were dehydrated and coverslips were mounted on the glass slides using mounting media. Positive and negative controls were included in each staining run.

2.3 Western blot

Western blot was performed as reported previously[13]. Cells were seeded in parallel and under the same conditions. When cells grew to monolayers, PRMI medium was refreshed and phosphotyrosine kinase inhibitor tyrphostin AG1487 was added at the indicated concentrations (0 nM, 50 nM, 100 nM, 200 nM) and incubated for 2 h. Then the cells were scraped into 0.5 ml of cold lysis buffer [50 mM Tris-HCI (pH8.0), 150 mM NaCI, 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml Aprotinin, 1% NP-40, 0.5% sodium deoxycholate], and protein was extracted. The concentration of protein was determined by Bradford method. Western blots were performed to detect the expression of total ERK1/2 (Sigma 1:10,000) and activated ERK1/2 (Sigma 1:10,000).

2.4 Flow cytometry

Tca8113 cells were cultured at same condition and then exposed to indicated concentrations of tyrphostin AG 1487 (0 nM, 50 nM, 100 nM, 200 nM) and treated for 24 h, 48 h and 72 h respectively. Cells were washed with PBS, trypsinized and harvested, and then fixed with pre-cold (−20℃) ethanol for 2 h. The cells were centrifuged for 5 min at 1500 rmp and quickly removed the supernate, washed with PBS and added RNase 50 μl (20 μg/ml) for 10 min at room temperature, then stained with propidium iodide (PI, sigma) 200 μl (5 ng/ml) for 20 min, washed with PBS and resuspended. After filtering cells through 100 μm pore size mesh, cell cycle distribution was analyzed by a flow cytometer (Becton Dickinson FAC Sort).

2.5 Electron transmission microscopy

Tca8113 cells were cultured as above and exposed to different concentrations of tyrphostin AG1487 (0 nM, 50 nM, 100 nM, 200 nM) for 48 h, then washed with PBS, trypsinized and harvested. Cells were fixed with 2% glutaraldehyde solution. Ultrathin Epon plastic sections were prepared and stained with uranyl acetate followed by lead citrate and stabilized for transmission electron microscopy (HITACHI. H-300). Changes of Tca8113 cells after exposing to tyrphostin AG 1487 were clearly identified by its characteristics.

3 Results

3.1 Expression of EGFR

The results of immunocytochemical staining demonstrated EGF receptor protein of Tca8113 cells was mainly in the cytoplasm and on the plasma membrane. There was no significant difference about expression of EGF receptor in before and after treated cells (Figure 1a, b). Unfortunately, we did not detect the expression of activated EGF receptor. But in a few of studies shown AG1478 and other EGFR inhibitors could inhibit EGFR phosphorylation without reduced expression of EGFR protein[2,14].

3.2 Expression of activated ERK1/2 and total ERK

To study the effects of tyrosine kinase inhibitor tyrphostin AG1487 on EGF receptor signaling of Tca8113 cells, we adopted immunocyotchemistry and Western blot methods to detect the expression of total ERK1/2 and activated ERK1/2. The results of immunocytochemistry showed that there was no significant difference in intensity of total ERK1/2 reactivity in Tca8113 cells before and after treated with AG1487 (100 nM) (Figure 1c, d). As for activated ERK1/2, it was marked decreased of Intensity of reactivity in nucleus and made nucleus to be a shadow (Figure 1e, f). These results were corresponded with the expression of total ERK1/2 and activated ERK1/2 in Western blot (Figure 2): Along with the increasing of concentration of tyrphostin AG-1487, expression of activated ERK1/2 was reduced or abolished in Western blot. But there was no significant difference in expression of total ERK1/2.

3.3 Distribution of cell cycle after treatment with AG1487

In order to investigate the effects of AG1487 on cell cycle in Tca8113 cells, we employed different concentrations of AG1487 to treat the cells for different time (0 h, 24 h, 48 h, 72 h). The results suggested that the percentage of cells in G1 phase was correlated with the concentration of tyrphostin AG1487 and treating time. Along with the increasing of concentration of tyrphostin AG1487 and treating time, the percentage of cells in G1 phase also increased (Figure 3). When exposing Tca8113 cells to 100 nM AG1487 for 72 h, the percentage of cells in G1 phase reached to maximum (77.57%, Table 1).

3.4 Effect of AG1487 on apoptosis of Tca8113 cells

Some studies demonstrated EGF receptor targeted agents could induce tumor cells apoptosis *in vivo* or *in vitro*, and this effect was correlated with inhibition of EGF receptor signaling pathway. In the present study, effect of tyrphostin AG1487 on apoptosis of Tca8113 cells was also studied. The results showed EGF receptor tyrosine kinase AG1487 could induce apoptosis of Tca8113 cells, and the effect was correlated to the concentration of tyrphostin AG1487. Along with the increase of concentrations of AG1487, the number of apoptotic cells gradually increased (Figure 4a, b). Comparing with untreated cells, the number of apoptotic cells was nearly 7 fold in Tca8113 cells treated with high concentration of AG1487 (200 nM) (Figure 5).

Table 1. Distribution of cell cycles of Tca8113 cells exposed to different concentrations of AG1487 (nM) and treated for different times

Time	Cell cycle (%)											
	G1				S				G2			
(hour)	0 (nM)	50 (nM)	100 (nM)	200 (nM)	0 (nM)	50 (nM)	100 (nM)	200 (nM)	0 (nM)	50 (nM)	100 (nM)	200 (nM)
24	32.35	34.65	38.81	47.70	54.89	50.78	49.17	45.64	12.76	14.57	12.02	6.67
48	32.15	45.50	60.67	74.04	55.20	48.34	33.48	21.07	11.90	6.16	5.85	4.89
72	32.35	75.64	77.57	54.04	54.85	18.06	17.19	38.05	10.90	6.30	5.23	7.91

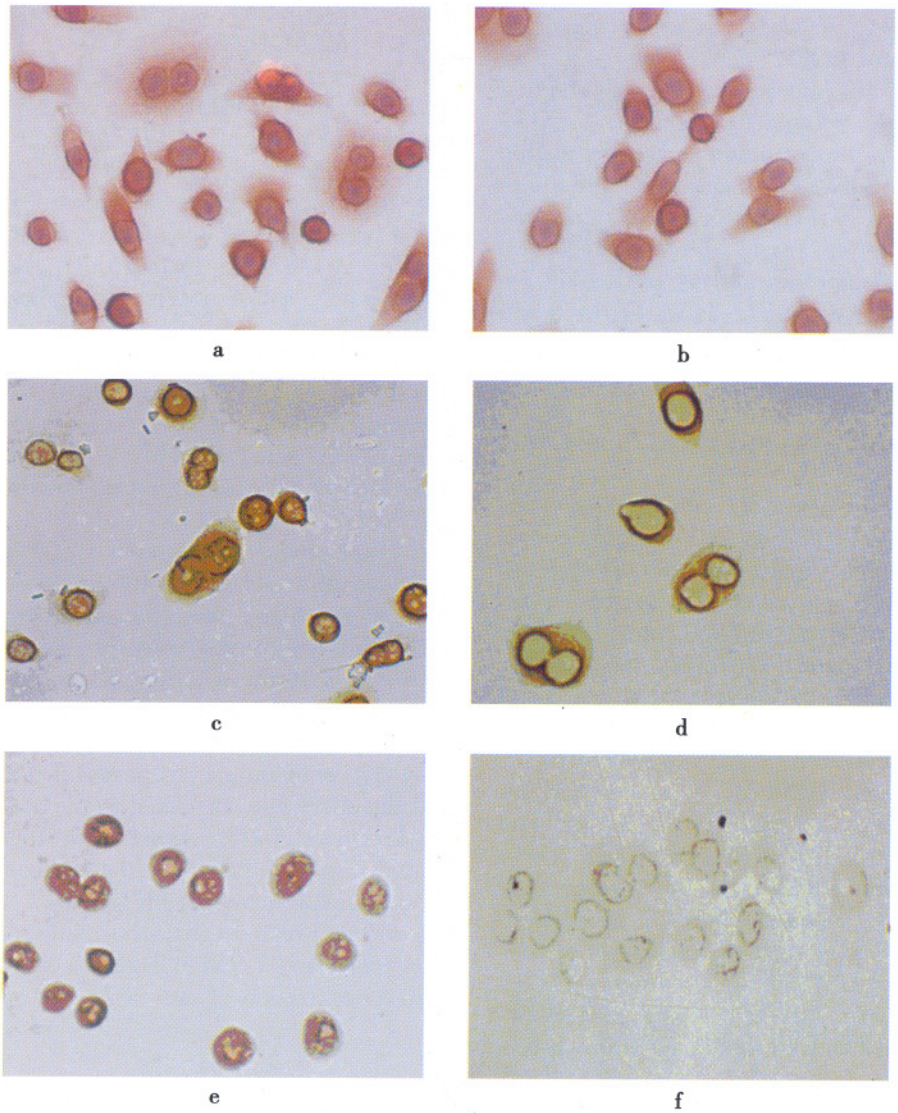

Figure 1. Immunochemistry for EGF receptor, total ERK1/2 and activated ERK1/2 in Tca8113 cells treated or untreated with AG1487 (×400). a. EGF receptor immunochemical staining in untreated cells. b. EGF receptor immunochemical staining in treated cells. c. Total ERK1/2 immunochemical staining in untreated cells. d. Total ERK1/2 immunochemical staining in treated cells. e. Activated ERK1/2 immunochemical staining in untreated cells. f. Activated ERK1/2 immunochemical staining in treated cells.

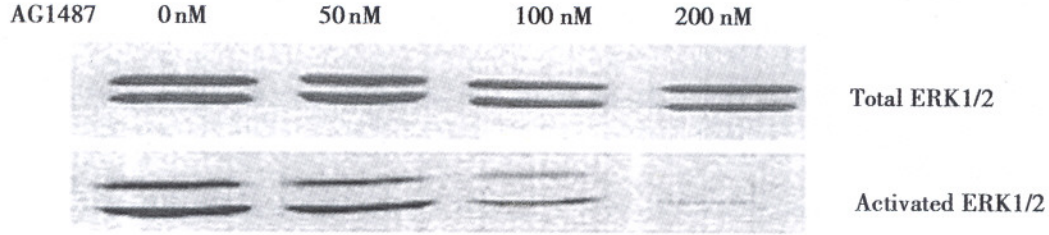

Figure 2. Expression of total ERK1/2 and activated ERK1/2 in Tca8113 cells treated or untreated with AG1487. Tca8113 cells pretreated with 0, 50, 100 and 200 nM AG1487 in an equal amount of DMSO (final concentration 0.5%) for 2 h at 37℃. Cell lysates were prepared and Western blot analyses were performed with antibodies to total ERK1/2 and activated ERK1/2.

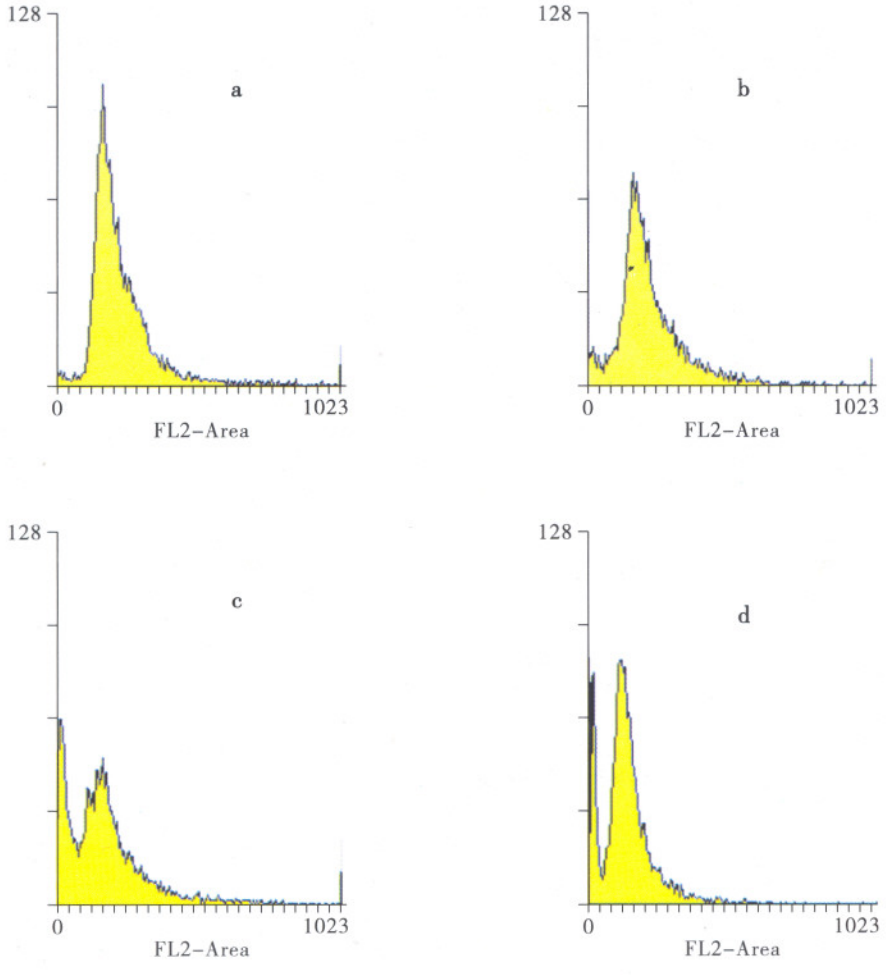

Figure 3. Effects of AG1487 on cell cycle distribution in Tca8113 cells. Tca8113 cells were cultured with PRMI medium 1640 and exposing to different concentrations (0 nM, 50 nM, 100 nM, 200 nM) of AG1487 and for different time (24 h, 48 h, 72 h), and cells were then harvested and stained with propidium iodide as described under Materials and Methods. Their cell cycle distribution was analyzed by flow cytometer. a. Cell cycle distribution in Tca8113 cells untreated with AG1487. b. Cell cycle distribution in Tca8113 cells treated with AG1487 in concentration 50 nM for 24 h. c. Cell cycle distribution in Tca8113 cells treated with AG1487 in concentration 100nM for 48 h. d. Cell cycle distribution in Tca8113 cells treated with AG1487 in concentration 200 nM for 72 h.

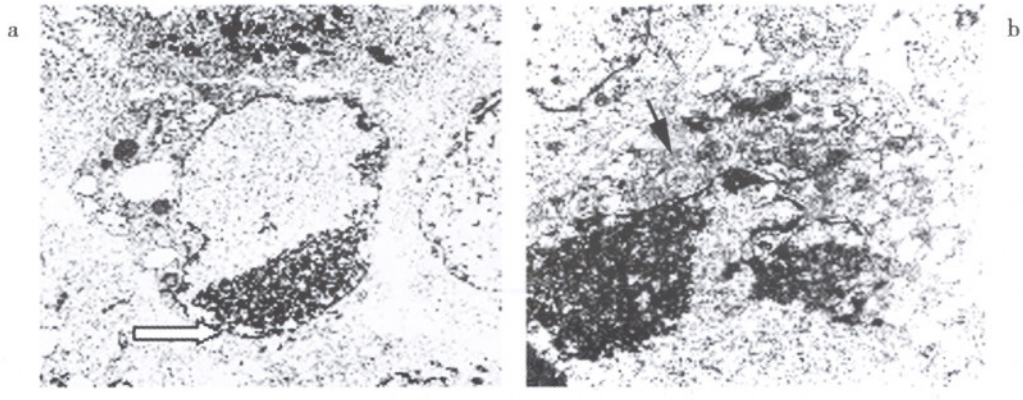

Figure 4. Effects of AG1487 on apoptosis of Tca8113 cells. a. Nuclear condensation (⇨), vesicle formation (→) occurring during apoptosis of Tca8113 cells exposed to AG1487 at concentration of 100 nM (×7,000). b. Apoptotic cell exhibiting swelling of endoplasmic reticulum (→) of Tca8113 cells exposed to AG1487 at concentration of 200 nM (×15,000).

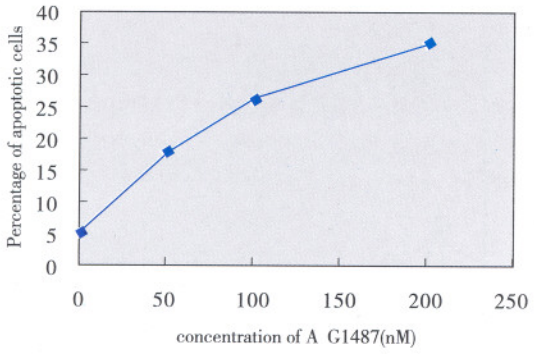

Figure 5. The relationship between percentage of apoptotic cells and different concentrations of AG1487 in Tca8113 cells.

4 Discussion

Dimerization and phosphorylation of the epidermal growth factor (EGF) receptor are the initial and essential events of EGF or TGF-α-induced signal transduction, so selective inhibition of tyrosine kinase activity is one of promising strategy for cancer treatment. Tyrphostin AG1487, as one of the phosphotyrosine kinase inhibitors, has been used in a few of studies and showed it can induce inactive, unphosphorylated EGF receptor – erbB-2 heterodimers, thereby sequester EGF receptor from signaling interactions with other erbB coreceptors[15]. These effects lead to the activation of ERKs-MAPK (mitogen-activated kinase) be inhibited[12,16]. Biochemical and cell fractionation studies have indicated that activation of ERKs and translocation from the cytoplasm to the nucleus are required for growth factor-induced gene expression and cell cycle entry[17]. At present study, we demonstrated expression of activated ERK1/2 could be effectively inhibited by tyrphostin AG1487. And this inhibitory effect was in a does-dependant pattern. The result of immunochemical staining showed intensity of activated ERK1/2 reactivity in nucleus marked decreased, it means translocation process of activated ERK1/2 from cytoplasm to nucleus was inhibited by tyrphostin AG1487. All these results suggested small molecule inhibitor of EGF receptor tyrosine kinase, AG1487, could effectively inhibit the ERK-MAP kinase pathway and translocation process of activated ERK1/2 in Tca8113 cells.

Besides EGF receptor regulating the growth and progression of human oral squamous cell carcinoma, the link between EGFR signaling and the cell cycle has also been identified[18]. A few of studies demonstrated both therapeutic mAbs and small molecule inhibitors of EGF receptor tyrosine kinase induced a remarkable G1 cell cycle arrest, accompanied by a decrease in the percentage of cells in the S phase in malignant tumor cells[13,14], and this was associated with increased expression of p27 (KIP1), which binds to and inactivates cyclin-dependet kinase-2 activity and produces cell cycle arrest in G1 phase[14]. At present study, the percentage of Tca8113 cells in G1 phase was studied after treated with tyrphostin AG1487 at different concentrations and for different time, the results showed the percentage of cells in G1 phase was correlated to the concentrations and treating time. Along with the increasing of concentrations and prolonging of treating time, the percentage of G1 cells gradually increased. When exposing Tca8113 cells to 100 nM AG1487 for 72 h, the percentage of cells in G1 phase reached to maximum (77.57%). Further analysis suggested these results were in dose- and time-dependent patterns.

Apoptosis is a physiological mechanism of cell death that occurs during normal development and under certain pathological conditions[19,20]. Several types of regulatory pathway can contribute to the cellular decision to enter apoptosis. These include DNA damage, dominant death signals from "death receptors" such as Fas, tumor necrosis factor receptor, and block the signaling pathways from growth factor receptor-activated kinase cascades[21]. Activation of the EGF receptor provides a potent survival signal in many cell types. PI3K and ERK1/2 are signaling molecules on two major downstream pathways initiated by the activation of EGF receptor[22,23]. A few of studies demonstrated small molecule inhibitor of the EGF receptor tyrosine kinase, for example ZD-1839 and AG1487, could induce apoptosis of malignant tumor cells *in vivo* or *in vitro*[16,24]. And this effect was also dose dependent. At present study, the percentage of apoptotic cells were calculated after exposing Tca8113 cells to different concentrations of AG1487. The results showed along with the increasing of concentrations of AG1487, the percentage of cells proceeding apoptosis also increased. And the morphologic changes of apoptotic cells, such as vesicle formation in the cytoplasm, nuclear condensation were also influenced by the concentration of tyrphostin AG1487. All these results suggested tyrphostin AG1487 could induce apoptosis of Tca8113 cells and the effect was in a dose dependent pattern.

In summary, EGF receptor signaling pathways play an important role in regulating cellular processes in Tca8113 cells. Small molecule inhibitor of EGF receptor tyrosine kinase, AG1487, could effectively inhibit ERK1/2-MAPK pathway and re-

duce the expression of activated ERK1/2 in a does-dependent manner. Cell cycle kinetic analyses demonstrate AG1487 induce a remarkable G1 cell cycle arrest, followed by a modest apoptotic response. All these results suggest the EGFR kinase specific inhibitor is of potential to be developed into drugs for squamous cell carcinoma treatment.

Acknowledgments

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Phylogenetic Relationship of *Tetraogallus* Inferred from Sequences of Cytochrome b Gene

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Abstract: A phylogenetic tree of Neighbor-joining (NJ) for the sequences of cytochrome b (Cyto b) gene was constructed to study the phylogenetic relationship of the genus *Tetraogallus*. Numbers near the branches were bootstrap probability (BP) values coming from 1000 replications. Some bootstrap probability values were 92% (*Coturnix chinensis* / *Coturnix japonica*), 100% (*Tetraogallus altaicus* / *Tetraogallus himalayensis*), and 100% (*Tetraogallus altaicus* / *Tetraogallus himalayensis* / *Tetraogallus tibetanus*). The overall average distance was 0.112, and average genetic distance among *Tetralgallus* was 0.042. The genera *Alectoris*, *Coturnix*, *Tetraogallus* and *Pucrasia* formed a monophyletic group. The two genera, *Alectoris* and *Coturnix*, should have a common ancestor, which was the sister taxon of the ancestor of the genus *Tetraogallus*. The interspecific genetic distances of the genus Tetraogallus were 0.012 (*T. altaicus* vs *T. himalayensis*), 0.067 (*T. himalayensis* vs *T. tibetanus*) and 0.068 (*T. altaicus* vs *T. tibetanus*) respectively. By combining the geographical distribution pattern, morphological characteristics and the genetic distance among these species of the genus *Tetraogallus*. It can be inferred preliminarily that Tibetan snow cocks were the primitive species among the three breeds; and that 1.7 million years ago, one subspecies of Tibetan snow cocks, *tibetanus* with a deep body color, distributing in the southwest of Xinjiang and the midwest of Tibet, migrated towards the west and entered the Himalayan Mountain to become the present Himalayan snow cocks; and that later, another subspecies of Himalayan snow cocks, *koslowi*, owning a light body color and often emerging in the Altai Mountain and the east of Kunlun Mountain, evolved into Altai snow cocks. [Life Science Journal. 2005;2(1):85-89] (ISSN: 1097-8135).

Keywords: snow cock; cytochrome b gene; phylogeny; mitochondrial DNA

1 Introduction

Snow cocks, the herbivore birds with high medicinal value inhabiting in the highest sea level in the world, are the children of the *Tetraogallus* under *Galliform*, *Phasianidae*. Nowadays, five species of snow cock have been found in Eurasia only. Except the foreign species, caspian snow cock (*T. caspius*) and Caucasian snow cock, the other three species including Altai snow cock (*T. altaicus*), Himalayan snow cock (*T. himalayensis*) and Tibetan snow cock (*T. tibetanus*), can all be seen in Xinjiang Uygur autonomous region of China (*T. caucasicus*) (Zheng, 2002). All of them had been listed as the international first-grade endangered and national second-protected wildlife. Presently, foreigners have little research about them except some researchers of Soviet Union who once had a shallow study on them at the beginning of 20th century. In the 1990s, people began to research their domestication and reproduction in Xinjiang, Gansu and Qinghai. In China, researches were promoted at the beginning of 1960s, and have been limited by the domains of their ecological distribution and process. Now, we know that snow cocks are an excellent group for biological studies. However, the phylogenetic relationships among the snow cock species, their phylogenetic position and evolution process are still a secret that impedes our further researches of snow cocks.

Mitochondrial DNA can be used as a molecular marker since people have paid overwhelming attention to its traditional characteristics such as rigorous maternal inheritance (Gyllensten, 1985), rapid evolution (Vawter, 1986) and non-rearrangement (Cann, 1987). In recent years, the polymorphic analyses of mitochondrial DNA have become the effectual means for phylogeny reconstruction and taxonomy (Kirchman, 2000; Meyer, 1990). For example, cytochrome b (Cyto b) gene of mitochon-

drial DNA, is widely used for avian molecular phylogenetics at the levels of species and genus (Tuinen, 2000), which can be used to resolve the problems bequeathed by morphological taxa (Avise, 1994). In this study, snow cocks and their close related genera were selected as the experimental materials in order to study the phylogenetic position of the genus *Tetraogallus*, understand the phylogenetic relationships among the available snow cock species (the foreign species of *T. caspius* and *T. caucasicus* are very difficult to find and sample, and the related sequence data can not be found in other literatures), and infer the evolution process of the *Tetraogallus* by combining the molecular data, distribution patterns and morphological traits.

2 Materials and Methods

2.1 Materials and nucleotide sequences

Altai snow cocks and Himalayan snow cocks were captured in the Altai Mountain and the areas of Tianshan, respectively. The blood samples were collected. The other sequence data were retrieved from GenBank as shown in Table 1.

Table1. The sequence data used in this study[a]

Code	Latin name	English name	Accession No
AG	*Alectoris graeca*	Rock Partridge	ZA8772
CA[b]	*Crossoptilon auritum*	Blue Eared-Pheasant	AF534552
CC	*Coturnix chinensis*	Blue-breasted Quail	NC004575
CJ	*Coturnix japonica*	Japanese Quail	NC003408
FF	*Francolinus francolinus*	Black Francolin	AF013762
PM	*Pucrasia macrolopha*	Koklass Pheasant	AF028800
TT1[c]	*Tetraogallus tibetanus*	Tibetan snow cock	AY563128
TT2[c]	*Tetraogallus tibetanus*	Tibetan snow cock	AY563130
TT3[c]	*Tetraogallus tibetanus*	Tibetan snow cock	AY563133

Notes: [a] The information included the supplementary materials' Latin names, English names and sequence accession numbers in the GenBank, and, at the same time, the codes for these supplementary materials were formed by the two first letters of their Latin names in this study. [b] This species was selected as outgroup in this study. [c] The digitals behind the codes represent the individuals we selected in this study.

2.2 Extraction of total genomic DNA

The blood samples were digested with SDS/Protease K. Then the supernatant was extracted with phenol/chloroform. Finally, ethanol precipitation would be preformed to condense the total genomic DNA. The concentrated DNAs were dissolved in TE (pH 8.0) and stored at −20℃ for later use (Ausubel, 1995).

2.3 PCR amplification

Total genomic DNA was extracted from blood using a modified protocol, and used as the template in the polymerase chain reaction (PCR) amplifications. The primers were designed using DNAMAN software according to the published Himalayan snow cock Cyto b sequence (Accession No. AY678108). The upstream primer (5′-CTATACTACggCTCCTACCTg-3′) and downstream primer (5′-gTTTgggATTgAgCgTA ggATg-3′) were used in this study. Then, reactions were conducted in volumes of 50 μl, typically with a amplification profile as follows: 3 min at 95℃, 35 cycles of 45 sec at 95℃, 45 sec at 52℃, and 3 min at 72℃, followed by a final extension at 72℃ for 10 min using the HiFi Ready-to-use PCR kit (Sangon, Cat. #SK 2073, USA) on PTC-100™ Thermocycler.

2.4 Purification, cloning, and sequencing of PCR products

PCR products were purified from agarose gels using the EZ-10 spin column PCR product purification kit (BBI, Cat. #BS363, USA) and cloned directly into pGEM- T-easy vector (Promega, Cat. #A3600, USA). Sequencing was carried out with an ABI 3100 Genetic Analyzer, using the BigDye Terminator Cycle Sequencing Kit according to the manufacturer's protocols.

2.5 Sequence analysis and phylogenetic tree construction

Mitochondrial Cyto b gene sequences from Altai and Himalayan snow cocks were generated in this study. The other data was obtained from GenBank or from the literatures (Table 1). All nucleotide sequences were aligned with the CLUSTAL X (version 1.8) multiple alignment program and refined manually. Phylogenetic analysis was performed with MEGA version 2.1 (Kumar, 2001) and TREE PUZZLE version 5.0. We applied two different methods of phylogenetic analysis, Maximum parsimony (MP) and Neighbor joining (NJ), to ensure that our analyses were fit enough for the reality. Phylogenetic trees were inferred from the ML distances calculated with Kimura 2-parameter with *Crossoptilon auritum* as outgroup.

3 Results and Discussion

Snow cocks are an excellent kind of birds for biological studies. There are five species of this genus living in Eurasia only, and *T. altaicus*, *T. himalayensis* and *T. tibetanus* can be seen in China except *T. caspius* and *T. caucasicus*. Traditionally, *Tetraogallus* were classified into different species according to their morphological characteristics and geographical distributions. Tibetan snow cocks, one of these species, can be divided into six subspecies whose distribution areas are composed of Himalayan Mountain, Parmir Plateau, Mishmi Mountain and Tibetan Plateau. Nowadays, it is said that there are 0.2 million Tibetan snow cocks in Tibet. Altai snow cocks are composed of two subspecies, which can be discovered from Altai Mountain to the Northwest Territories of Mongolia. Himalayan snow cocks include four subspecies, which have a wide distribution including Afghanistan, Turkey to Nepal, and the northwest territories of China (Zheng, 2002). Nowadays, only shallow researches have been done. However, the evolutional relationship among the Snow cocks still remain unclear, and even the evolution process of the whole genus is sometimes in doubt.

The overall average distance was 0.112, and average genetic distance among *Tetralgallus* was 0.042. The genetic distances between *C. auritum* and all the other species was the biggest one of all the genetic distances in the same lines. The interspecific genetic distances of the genus *Tetraogallus* were 0.012 (*T. altaicus* vs *T. himalayensis*), 0.067 (*T. himalayensisc* vs *T. tibetanus*) and 0.068 (*T. altaicus* vs *T. tibetanus*), respectively (Table 2). It is well known that phylogenetic reconstruction benefited from increased character inclusion, which provided all characters sharing a common evolutional history. In recent avian genetic evolutionary researches, people propose that the genetic distance of Cyto b gene is bigger than 0.01 (>0.01) between species and smaller than 0.01 (<0.01) between subspecies (Krajewski, 1996). So, the result suggested that Altai snow cocks and Himalayan snow cocks had an intimate relationship, and Altai snow cocks and Tibetan snow cocks had a distant relationship among the three species. The length of mtDNA Cyto b gene sequence in our sampled species was 535 base pairs, and the average base composition of *Tetraogallus* was 25% thymine (T), 35% cystine (C), 27.5% adenosine (A), and 13% guanine (G), and the T and A contents (52%) was higher than that (47.7%) of C and G. This composition was very similar to that of other Snow cocks. At the same time, we found that no gaps were introduced into the Cyto b gene alignment, and there were 227 sites that were variable ones, and conservative sequence composing of 113 base pairs was discovered.

Table 2. Genetic distance (Lower-left) and standard error (Upper-right) analyzed by MEGA version 2.1 with Kimura 2-parameter[a]

	AG	CC	CJ	FF	PM	TA[b]	TH1[c]	TH2[c]	TH3[c]	TT1[c]	TT2[c]	TT3[c]	CA
AG		[0.018]	[0.018]	[0.017]	[0.017]	[0.018]	[0.017]	[0.018]	[0.017]	[0.017]	[0.017]	[0.017]	[0.017]
CC	0.141		[0.016]	[0.018]	[0.018]	[0.018]	[0.018]	[0.018]	[0.018]	[0.018]	[0.018]	[0.018]	[0.019]
CJ	0.139	0.121		[0.019]	[0.018]	[0.018]	[0.018]	[0.018]	[0.018]	[0.018]	[0.017]	[0.018]	[0.019]
FF	0.136	0.150	0.161		[0.016]	[0.017]	[0.017]	[0.017]	[0.017]	[0.017]	[0.017]	[0.017]	[0.016]
PM	0.129	0.149	0.151	0.112		[0.017]	[0.017]	[0.017]	[0.017]	[0.017]	[0.017]	[0.016]	[0.016]
TA	0.135	0.143	0.138	0.127	0.133		[0.005]	[0.005]	[0.005]	[0.012]	[0.012]	[0.012]	[0.019]
TH1	0.131	0.143	0.143	0.129	0.133	0.011		[0.003]	[0.003]	[0.012]	[0.012]	[0.012]	[0.019]
TH2	0.135	0.145	0.145	0.132	0.136	0.013	0.006		[0.003]	[0.012]	[0.012]	[0.012]	[0.019]
TH3	0.133	0.143	0.143	0.127	0.133	0.011	0.004	0.006		[0.012]	[0.012]	[0.012]	[0.019]
TT1	0.126	0.139	0.138	0.134	0.129	0.068	0.067	0.070	0.068		[0.002]	[0.003]	[0.019]
TT2	0.124	0.137	0.136	0.132	0.127	0.066	0.065	0.068	0.066	0.002		[0.002]	[0.019]
TT3	0.126	0.139	0.139	0.129	0.124	0.068	0.068	0.070	0.068	0.004	0.002		[0.018]
CA	0.143	0.162	0.164	0.135	0.140	0.160	0.159	0.162	0.160	0.153	0.150	0.148	

Notes: [a] The genetic distances (lower-left) and standard errors (upper-right) were analyzed using MEGA version 2.1 with Kimura 2-parameter. The genetic distances between CA and all the other species were the biggest ones in the same line. The overall average distance was 0.112 and average genetic distance among *Tetralgallus* was 0.042. The interspecific genetic distances of the genus *Tetraogallus* were 0.012 (*T. altaicus* vs *T. himalayensis*), 0.068 (*T. altaicus* vs *T. tibetanus*) and 0.067 (*T. himalayensis* vs *T. tibetanus*) respectively. [b] The species of Altai snow cock (*T. altaicus*). [c] The species of Himalayan snow cock (*T. himalayensis*) and Tibetan snow cock (*T. tibetanus*), and the digitals were the code of individuals used in this study.

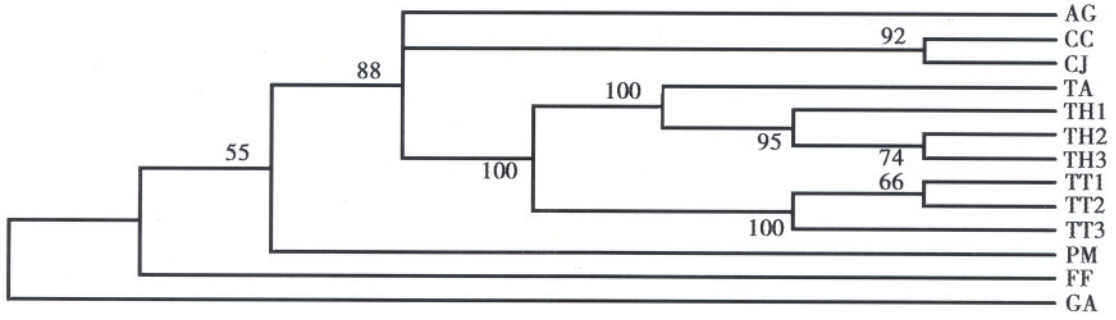

Figure 1. Phylogeny of *Tetraogallus* and its close related genera inferred from mitochondrial cytochrome b gene sequences in the analysis. The tree topology was inferred from a Neighbor-joining (NJ) distance matrix calculated with Kimura 2-parameter. Numbers near the branches were bootstrap probability values coming from 1000 replications. Some bootstrap values were 92% (*Coturnix chinensis*/*Coturnix japonica*), 100% (*Tetraogallus altaicus* / *Tetraogallus himalayensis*), and 100% [(*Tetraogallus altaicus* / *Tetraogallus himalayensis*) / *Tetraogallus tibetanus*]. *Crossoptilon auritum* was used as outgroup. The tree topology was also supported by Maximum-Parsimony (MP) analyses.

As the NJ tree shown that the genus *Tetraogallus* formed a sole clade, and which was well resolved with bootstrap percentage value 100%. The final results based on the molecular data supported that the genus *Tetraogallus* encompassed three (except two species, *T. caspius* and *T. caucasicus*) named groups given the rank of species: *T. altaicus*, *T. himalayensis* and *T. tibetanus*. And, our data suggested that the genera *Alectoris*, *Coturnix*, *Tetraogallus* and *Pucrasia* formed a monophyletic group. The bootstrap percentage value of the branch composed of the genera *Tetraogallus*, *Alectoris* and *Coturnix* was 88%. Therefore, the two genera, *Alectoris* and *Coturnix*, should have a common ancestor, which was the sister taxon of the ancestor of the genus *Tetraogallus* (Figure 1). Both the phylogenetic trees and the genetic distances suggested that the *Francolinus francolinus* was older than other studied species in its evolutionary history.

The NJ tree, at the same time, suggested that the genus *Tetraogallus* had evolved into two branches with bootstrap percentage (100%): one branch was composed of the Altai and Himalayan snow cock with bootstrap percentage (100%), and the other one was the Tibetan snow cock. This was also supported by the data of MP analysis. On the other hand, there are also several lines of evidence including distribution patterns and morphological traits to support our point of view for the evolutionary pattern of the three snow cock species. i) Both the Altai and Himalayan snow cocks have a similar body form, about 60 centimeters in length, while the Tibetan snow cocks have a body form about 53 centimeters in length; ii) The two subspecies of Altai snow cocks can be discovered from Altai Mountain to the Northwest Territories of Mongolia, and have an offwhite breast and belly. *Himalayensis*, one subspecies owning a deepest body color of Himalayan snow cocks, can be detected in the areas of Tianshan and northwest of Xinjiang Uygur autonomous region, while another subspecies, *koslowi*, owning a light body color, often emerge in the Altai Mountain and the east of Kunlun Mountain. There are two subspecies holding a light body color, *Przewalskii* and *henrici*, can be discovered in the east of their distribution area, and one subspecies holding a deep body color, *tibetanus*, distributes in the southwest of Xinjiang and the midwest of Tibet.

Shields and Wilson pointed out that the divergence rate of avian mitochondrial DNA Cyto b gene is about 0.02 per million years (Shields, 1987). Therefore, according to the conclusion, we can deduce the divergence time of snow cocks was 1.7 million years ago when the terrain and environment of Hengduan mountains region had a greatly changes in the Pliocene. Considering its molecular phylogeny and geographical distribution patterns, and the evidences of the genetic distances between species are all bigger than 0.01, and the results of genetic distance (0.12) between Altai snow cocks and Himalayan snow cocks reveal that the two species have a closer relationship. It is possible that Tibetan snow cocks might be the primitive species among the three breeds and originate in the Heng-

duan mountains region according to the witness of geographical distribution pattern and morphological character combining the genetic distances among them. So we could propose that one subspecies of Tibetan snow cocks, *tibetanus*, holding a deep body color, migrated towards the West and entered the Himalayan Mountain to evolve into the present Himalayan snow cocks. Later, another subspecies of Himalayan snow cocks, *koslowi*, owning a light body color, often emerge in the Altai Mountain and the east of Kunlun Mountain to evolve into Altai snow cocks.

Although the present study provided a good start towards our understanding of the relationships of the snow cock breeds found in China, detailed interrelationships and limits of the subgroups still need further study. But in all our trees Tibetan snow cocks occupy a relatively basal position. As for Caspian snow cocks and Caucasian snow cocks, we consider that our sample of *Tetraogallus* taxa is insufficient to resolve the evolutional processes of the foreign snow cock breeds, and that solid conclusions on the status of the total *Tetraogallus* awaited inclusion of additional related taxa, and require further investigation. But, we can give a hypothesis that, according to the geographical distribution range and morphological character, Tibetan snow cocks may be the most primitive breed among the five snow cock species, and Caucasian snow cock derived from Altai snow cocks. As to Caspian snow cocks, they might derive from Himalayan snow cocks. This hypothesis needs further investigation and study.

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Anti-tumor of ILTT Combined with Cisplatin in a Rat Glioma Model

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Abstract: Objective. This study is to evaluate the therapeutic effect of interstitial laser thermotherapy (ILTT) combined with cisplatin chemotherapy on rat glioma model with deeply sited tumors. **Methods.** C_6 glioma cells were injected into the nucleus caudatus by stereotactic technique to induce transplanted gliomas in the rat brains. The rats bearing glioma were treated with ILTT in combination with cisplatin. The survival time of rats was observed up to 40 days after ILTT, and the tumor size of rats at 7th day after ILTT was measured. The data in different groups were statistically analysed. **Results.** The mean survival period of rats was (26.1± 3.6) days in the control group, (27.3±3.9) days in the cisplatin treated alone group, (30.4± 5.3) days in the ILTT alone group and (34.4±4.0) days in the ILTT combined with cisplatin chemotherapy group. At the 7th postoperative day, the maximum diameter of tumors measured in coronal sections of the brains were different between control group and experimental groups: (6.2±0.2) mm of the control group, (6.2±0.1) mm of the cisplatin treated alone groups, (4.8±0.2) mm of ILTT group and (4.9±0.1) mm of the ILTT combined with cisplatin chemotherapy group. **Conclusions.** Based on the above described results, we would conclude that the combination of ILTT and cisplatin chemotherapy might provide a significantly greater antitumor effect in the treatment of glioma. [Life Science Journal. 2005; 2(1): 90-93] (ISSN: 1097-8135).

Keywords: anti-tumor; cisplatin; interstitial laser thermotherapy; rat glioma

1 Introduction

Interstitial laser thermotherapy is a new invasive method for treating cerebral tumors, especially suitable for deep brain tumors[1]. Cerebral glioma is one of the most common tumors in central nerve system. Glioma is difficult to be completely excised by operation for its infiltrative growth, and those located in deep or functional regions are more restricted with operations. The chemotherapy effect is unable to be ensured because most chemotherapy drugs are difficult to penetrate into blood-brain barrier which exists in central nerve system. It is necessary to explore novel treatments because the conventional therapy effect on cerebral glioma is unideal. So we performed the experimental study on treating rat cerebral glioma with ILTT combined with cisplatin. The experimental findings are reported as follows.

2 Materials and Methods

2.1 Cerebral glioma model

C_6 cell line of rat cerebral glioma (presented kindly by Graduate School of Neurosurgery in Shanghai) were cultured for passages with culturing method of monolayer cells in complete culture solution RP-1640 with 10% bovine serum. During phase of logarithmic growth, the cells which were dealt with digestive solution of 0.24% pancretin and then washed with Hanks solution twice were made into suspended solution for inoculation. 64 healthy rats (offered by animal laboratory of Tongji Medical University) weighed 300-350 g were fastened after anesthesia to head arc of Jiangwan type, stereotactic apparatus for rat brain. The indications of rat skull were exposed by operation and the inoculated target was selected: at a point 1.0 mm to the front side of bregmatic midpoint, 3.0-3.5 mm to the right side of sagital sutura, and to a depth of 5 mm under dura. Hole in skull was drilled and $2.0\times10^6/15\mu l$ C_6 cells (livability>90%) were inoculated into each rat with micro-injector directed by stereotactic apparatus. The injection time was over 10 minutes and the injector was slowly withdrawn after being kept for 5 minutes, then the scalp was stitched. The rats fed on food and water freely in cages. The 14-days rats after inoculation were selected as cerebral glioma model in this experiment.

2.2 Treatment method

At 14 days after inoculation of C_6 cells, the rats were randomly divided into 4 groups: 16 rats in each group (10 of 16 were kept for observing survival time, 6 for measuring tumor diameter), Group A acted as control group (without treatment); Group B as cisplatin treatment group; Group C as ILTT group; Group D as ILTT combined with cisplatin group. 1 μg/g cisplatin was given to rats of group B by intravenous injection of rat tail, and afterwards each time equivalent dosage of cisplatin was given every 48 hours up to 3 times. 1μg/g cisplatin was given to rats of group D by intravenous injection of tail 30 minutes before ILTT, and each time equivalent dosage of cisplatin was given every 48 hours up to 3 times. Dexamethason (7 mg/kg) was given by intramuscular injection at 24 hours, 30 minutes before ILTT, and at 24 hours after ILTT, respectively, to prevent cerebral edema. After ILTT, 40,000 U of penicillin was administered by intramuscular injection each day for 3 days in sequence.

2.3 Interstitial laser thermotherapy technique

At 14 days after inoculation of C_6 cells, all rats were fastened to head arc of Jiangwan type, stereotactic apparatus (products of Shanghai) for rat brain after anesthesia with intra-abdominal injection of 2% pentobarbital sodium. Dura was excided along injector pathway after skull was exposed by operation. One side of optic fiber with a core diameter of 0.5 mm was connected with laser and another side (naked optic fiber) implanted into the selected target by stereotactic apparatus. Continuous Nd: YAG laser with wave-length of 1.06 μm and a power of 1.5 W was given for irradiation for 120 seconds, and equivalent quantity of laser was given again after an interval of 120 seconds, then treatment was finished.

2.4 The measurement of largest transverse diameter of tumors

At 21 days after inoculation of tumor, rats were perfused with alcoholic solution containing 0.5% methylene blue through aorta of left ventricle, complete brain was taken out and fixed with 10% formaldehyde, and then coronary section was made along the center of tumor (the center of optic fiber pathway). The largest transverse diameter of each tumor was measured on horizontal direction (including pathological tissues such as necrosis etc.).

2.5 Survival time

After treatment rats were recovered and raised continuously. Rats in each group were observed each day till rats died and the survival times (day) were written down. The survival time of rats without death was counted as 40 days (observation duration was 40 days). Two-sample test of nonparametric tests for multiple samples (Nemenyi method) was applied to analyze the survival time of each group.

3 Results

3.1 Survival time

Rats in all groups had no signs of nerve function deficiency such as hemiplegia et al. and there was no death in late period after treatment. The average survival time is: Group A(26.14 ± 3.58) days, Group B (27.26 ± 3.90) days, Group C (30.40 ± 5.27) days, Group D (34.42 ± 4.05) days. By comparison, there is a significant difference between group D and A ($P<0.01$) and a difference between group D and C($P<0.05$). There is no significant difference among other groups.

3.2 The largest transverse diameter of tumors

Group A (6.2 ± 0.2) mm, Group B (6.2 ± 0.1) mm, Group C (4.8 ± 0.2) mm, Group D(4.9 ± 0.1) mm.

4 Discussion

In recent several decades, the therapeutic effect of malignant glioma is still unsatisfactory. It is difficult to eradicate glioma for its infiltrative growth. Only biopsy can be usually performed and relapse will occur shortly after operation if tumor is situated in deep position or functional region of brain, the average survival time is 9 months[2]. It is necessary to explore novel methods to enhance therapy effect, so we carried out the experimental study on cerebral glioma treated with ILTT combined with cisplatin. The biological behavior of C_6 cells of rat with cerebral glioma are similar to that of human glioma, in recent years C_6 cells are widely applied to establish rat glioma model to expand experimental research. C_6 cell which was inoculated into caudatum could be acted as glioma model in deep position of brain[3].

Nd: YAG laser is a laser that can transmit through a flectional optic fiber which can be implanted into brain tumor tissue with brain tridimentional direction-finder. Its character of striking penetration to tissue ensures to develop more potent thermal effect by contacting irradiation to tissues with less scatter[4], so the laser can be a thermal resource for thermotherapy of brain tumors. It indicated in one research that thermal effect of Nd: YAG laser with ILTT developed an globose coagulated necrotic region surrounded by a band of edema zone[1]. It presented in our research that thermal

effect in edema zone also could induce apoptosis of brain glioma (pending materials for publication). The effect on coagulating tumor with ILTT may kill out an absolute majority of glioma tissue as possible as can, but a small quantity of tumor cells may survive in peripheral zone around effective focus for the infiltrative growth of glioma. If further corresponded treatments such as irradiation or chemotherapy are not applied, the effect of ILTT will be influenced because the residual tumor tissues may grow quickly to develop a relapse of tumor. It shows in this experiment that at 1 week after ILTT the largest transverse diameter of tumors is less than 5 mm in treatment group, and more than 6 mm in control group and cisplatin group; the average survival time of rats loaded with tumor in single ILTT group is longer than that of control group or single cisplatin group, but shorter than that of group of ILTT combined with cisplatin.

Presently the chemotherapy of brain tumors is a hard nut to crack relatively, most chemotherapy drugs are restricted to penetrated into central nerve system for blood-cerebral barrier[5,6], only those drugs with small molecular weight and with lipid-solubility can enter into tumor tissue across blood-cerebral barrier. Although *in vitro* studies proved that many chemotherapy drugs could influence the proliferation of glioma cells, they were not still used for therapy of brain glioma mainly because the drugs can't overpass blood-cerebral barrier[7]. Researches have proved that thermotherapy could open blood-cerebral barrier and promote the transportation of chemotherapy drugs between blood and tissues[8]. The experiment has showed the distribution of Evans Blue in thermal effect focus and its surrounded tissues[9]. Depending on the results we deduced that permeability of blood-cerebral barrier in effective focus and its surrounded tissues enhanced, so cisplatin can enter effective focus and its surrounded glioma tissues and accumulate to a definite concentration on which cisplatin can induce cell apoptosis to kill tumor cells. So we applied cisplatin to kill residual tumor cells right after ILTT. Cisplatin is a kind of non-specific cell-cycle drugs which can be activated in cells with light or heat and its effect is not restricted by cell cycle[10].

At present the reports on therapy of brain glioma with ILTT published increasingly, the animal experiment of EI－Ouahabi[11] presented that the average survival time of rats with brain glioma treated with single ILTT was a little longer than that of control group but there was no difference in statistics. Just as the analysis given by the author, the remained tumor tissue after ILTT and relapse of tumor may be the main cause for animal death. Reports from each clinical research differed[12,13]. Single ILTT was mostly applied for treatments in which temperature in therapeutic zones was controlled by computer system or was monitored by real-time imaging. Our experiment shows that although average survival time can be prolonged with single ILTT, there is no statistic difference by comparison with average survival time of control group. The average survival time of rats loaded with tumor can be more prolonged if cerebral glioma is treated with ILTT combined with cisplatin. There is a significant difference of average survival time between treatment group with ILTT combined with cisplatin and control group or single cisplatin group ($P<0.01$ and $P<0.05$). So the therapeutic effect of treatment group with ILTT combined with cisplatin is markedly superior to that of single ILTT group.

The mechanism of tumor therapy with ILTT may include several aspects as follows: (1) Coagulating tumor tissue: it mainly develops in therapeutic zones with higher temperature nearby optic fiber. Tumor cells in a state of nutrition deficiency acquires energy mainly by anaerobic glycolysis, and lactic acid accumulates in tumor cells; Vessels in tumor tissue which is short of smooth muscle can't dilate in accordance with the increase of temperature; and the vessel structure is not intact and has a low tolerance of heat. All these characters enhance the sensitivity of thermotherapy to tumor cells. (2) Inducing apoptosis of tumor tissue. (3) Activating cytokines, inflammatory media and vascular active substances to influence blood supply for tumor and/or develop a direct killing effect. Even so, the effect of single ILYY is still not ideal. Although average survival time on treatment group with ILTT is longer than that of control group, there is no statistic difference. It may be because that glioma grows with infiltration state but ILTT creates a globose effective focus, so residual tumor cells in some position lead to tumor relapse after the end of treatment. The opening of blood-cerebral barrier enables chemotherapy drugs to enter tumor tissue to kill remained tumor cells.

ILTT is a palliative therapy for glioma. After ILTT the relapse of glioma is inevitable for residual tumor cells. If ILTT is combined with chemotherapy and/or photodynamic therapy, it will helpful to raise the therapeutic effect of malignant cerebral glioma.

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