In vitro and *in vivo* studies for Evaluation the Genotoxicity of Mancopper

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Abstract: The genotoxic effects of Mancopper, a widely used fungicide of the ethylene-bis-dithiocarbamate (EBDC) group, were studied. The cytogenetic parameters, chromosome aberrations and sister chromatid exchanges (SCEs) were the main cytogenetic assays used in this study *in vitro* and *in vivo* on Swiss albino male mice. Mice splenocytes were cultured *in vitro* after 24 h exposure to Mancopper (Conc. 10^{-3} to 10^{-7} M/ml). The percentage of cell viability was increased gradually with concentration decreasing from 10^{-3} to 10^{-7}. It reached 82.48% in the cultures treated with 10^{-7} M Mancopper /ml medium compared to 91.5% in the non- treated cultures. The concentrations 10^{-7}, 10^{-6}, 10^{-5} M and $5x10^{-5}$ M Mancopper/ ml medium induced chromosome aberrations and (SCEs) in cultured splenocytes in a concentration dependent manner ($p<0.01$). For in vivo studies, mice were orally treated by gavage with 10, 20 and 40 mg Mancopper/ kg b. wt for 24 h, the chromosome aberrations and SCEs were observed to be increased gradually with dose increasing. Multiple injections with 40 mg Mancopper/ kg b. wt for another 3 and 7 days consecutively, increased the percentages of chromosome aberrations in bone- marrow cells and spermatocytes. The results indicate that the herbicide Mancopper is genotoxic in mice somatic and germ cells under the circumstances tested. We recommend using natural compounds in agriculture rather than these fungicides. [Nature and Science 2011;9(12):50-56]. (ISSN: 1545-0740).

Key words: Mancopper, *in vitro, in vivo*, Mice, Chromosome aberrations, SCE's

1. Introduction

Ethylenebisdithiocarbamate (EBDCs) are widely used in agriculture as fungicides, mainly on fruit, vineyard, and potato crops. Their extensive world-wide consumption can be ascribed to their low acute toxicity and their short environmental persistence (Lombardi et al., 1991, Padget et al., 1992 and Pruett et al., 1992). EBDCs are absorbed primarily dermally and are metabolized to ethylene thiourea (ETU). Dermal absorption of EBDCs range from 1 to 10%, and approximately 7.5% of the absorbed dose is converted to ETU. EBDCs and ETU are mildly genotoxic in bacterial and animal systems (U.S. EPA, 1992). The EPA classifies ETU as a carcinogen, based on animal data showing that it causes thyroid and other cancers in rodents. Because of concern about carcinogenicity, in 1992 the EPA canceled the use of EBDCs on 11 crops, but EBDCs are used in the United States on a wide variety of fruit, nut, and vegetable crops such as apples, almonds, and tomatoes (U.S. EPA, 1992). Nowadays, EBDCs are used extensively in the Egyptian agricultures especially Mancopper (Abdel-Shafy et al., 2005).

Different EBDCs share a common molecular structure, but differ by metal cation (Maneb, Mancozeb and Zineb). Environmental exposure to pesticides, including EBDCs fungicides, may contribute to neuronal toxicity and subsequent pathologies. Mancozeb (MZ), a Mn/Zn-containing EBDC fungicide, is widely utilized on golf courses, residential lawns, and agricultural lands throughout the United States. The adverse effects of MZ in humans and other living organisms have not been widely studied and there are few studies to evaluate the neurotoxic action of MZ in experimental models (Soleo et al., 1996, Vaccari et al., 1999 and Domico et al., 2006).

Based on the similarities of the chemical structure of Mancopper with other EBDCs pesticides, it is observed that Mancopper is composed of EBDCs mixed metal complex containing manganese and copper. Copper is used extensively in pesticide formulations as a fungicide and antimicrobial agent, particularly for the treatment of wood and water supplies for drinking water and recreational use (Browning, 1969 and Matheson, 1975).

There are no reports on the effects of Mancopper in somatic and germ cells of animals to evaluate the genotoxicity of this fungicide. Hence, the present investigation is undertaken as Mancopper is one of the main fungicides used in the Egyptian agriculture fields. The cytogenetics parameters chromosome aberrations and sister chromatid exchanges were studied *in vitro* in splenocytes and *in vivo* in bone-marrow cells and spermatocytes.

2. Materials and methods

2.1. Chemicals:

The herbicide Mancopper is purchased from Kafr-El-Zayat Company for Pesticides and Chemicals-Egypt. Mancopper (commercial grade 69.5% wettable powder) composed of the active ingredient Mancozeb (manganese ethylene bis dithio carbamate polymeric

complex with zinc salt; 52% w/w), purchased from Room and House, Italy, and Copper oxy-chloride 17.5% w/w.

2.2. Animals:

Laboratory-bred strain Swiss albino male mice of 9-12 weeks old with an average weight of 27.5±2.5g obtained from the National Research Center, Cairo, Egypt, were used. Animals were housed in groups (5animals/ group) and maintained under standard food and water *ad libitium.*

***2.3. In vitro* studies:**

The spleen cells cultures were prepared according to Moorhead et al. (1960) with modifications (Amer et al., 1993).

2.3.1. Cell Viability:

Spleen cells cultured for 24h were exposed to a wide range of Mancopper concentrations from 10^{-7} to 10^{-3}M/ ml medium. The number of viable cells was estimated using trypan blue and a Neubauer chamer. The percentage of viable cells was determined from five separate plates for each Mancopper concentration.

2.3.2. Chromosome Aberrations:

Spleen cells cultures were exposed to the concentrations 10^{-7}, 10^{-6}, 10^{-5} and $5x10^{-5}$ M Mancopper/ ml medium for 24h the full culture period. Colchicine was added to the cell cultures 2h prior to harvest. Five separate plates were conducted using each Mancopper concentration. 500 metaphases were analyzed for chromosome aberrations for each concentration.

2.3.3. Sister Chromatid Exchanges (SCE's):

The used concentrations were the same as those used for chromosome aberrations. Bromodeoxyuridine (BrdU, Sigma) was added at the time of culturing the cells at 10µg/ ml medium. The cell cultures were treated with Mancopper solution for 24h and colchicines was added 2h before harvesting. Hypotonic treatment, fixation of the cells and chromosome preparations were made according to Goto et al. (1978). Five separate plates for each Mancopper concentration were conducted. At least 200 well spread metaphases were analyzed for SCE's/ each concentration.

Separate experiments were conducted using distilled water (negative control) and Mitomycin C (MMC) at a final concentration of 0.15µM was used as a positive control groups.

***2.4. In vivo* studies:**

Male Swiss mice were orally treated by gavage with 10, 20 and 40mg Mancopper/ kg b. wt. Both bone-marrow cells and spermatocytes were taken after 24h for single treatments. For the repeated treatments the animals were orally treated with the highest dose 40mg Mancopper/ kg b. wt and the samples were collected after 24h, 3 and 7days after the last treatment. Mitomycin C (MMC; 1mg/kg b. wt) was used as a positive control group.

2.4.1. Chromosome Aberrations:

Bone-marrow metaphases were prepared following the method of Yosida and Amano (1965). 100 well spread metaphases/ animal were analyzed for the different types of chromosome aberrations including gaps, fragments, breaks, deletions, centric fusion and polyploidy. Five animals were taken for each treatment.

Meiotic chromosome preparations were made according to the air-drying technique of Evans et al. (1964). Slides were stained with 7% Giemsa in phosphate buffer (pH 6.8). About 100 well spread diakinasis- metaphase I cells/ animal were analyzed for abnormal chromosome associations including XY univalents, autosomal univalents and translocations in the form of chain IV. Five animals were taken for each treatment.

2.4.2. Sister Chromatid Exchanges (SCE's):

A subcutaneous implantation of BrdU tablets (55mg) was initially made in each mouse 30 minutes later after injecting the animals with the different doses of Mancopper. The animals were injected intraperitoneal with colchicine 22h (McFee et al., 1983) after the treatment with Mancopper. Bone-marrow cells were fixed and stained with fluorescence plus Giemsa (Perry and Wolff, 1974). At least 40 well spread metaphases were analyzed/ mouse, i.e. 200 cells were analyzed for SCE's for each dose.

2.5. Statistical evaluation:

The significance of differences between experimental and the control data was calculated using the student's t-test. The level of 0.05 was taken as significant and that of 0.01 was taken as highly significant.

3. Results

***3.1. In vitro* studies**

3.1.1. Cell viability

A gradual decrease in the percentage of viable cells was observed after treatment of cultured mouse spleen cells with the concentrations 10^{-7}- 10^{-3} M Mancopper/ ml medium. It reached 82.48% and 23.14% after treatment with 10^{-7} and 10^{-3} M Mancopper/ ml medium respectively (Figure 1).

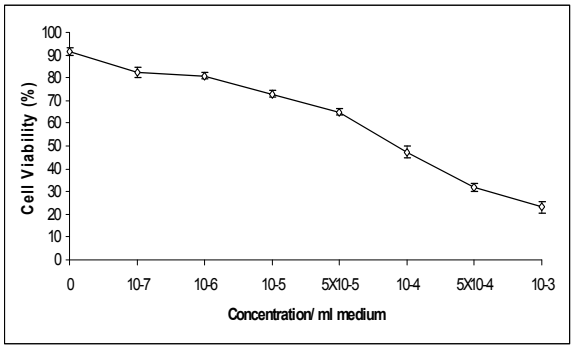

Figure 1: Percentage of viable cells in cultured mouse spleen cells treated with different concentrations of Mancopper.

3.1.2. Chromosome aberrations

The concentrations 10^{-6}, 10^{-5} and $5x10^{-5}$ M Mancopper/ ml medium caused a significant increase in the percentage of chromosome aberrations in the cell cultures. It reached 10.20±0.70, 14.0±0.73 and 18.60±0.66 ($p<0.01$), respectively, as compared with 5.20±0.66 in the non- treated cultures, and 27.60±0.83 in the cultures treated with Mitomycin C. The main types of chromosome aberrations observed were breaks, fragments and deletions (Table 1).

3.1.3. Sister chromatid exchanges

The tested concentrations of Mancopper induced a dose- dependant increase of SCE values. The frequency of SCE's/ cell reached 8.90±0.48/ cell ($p<0.01$) after treatment with the concentration 5x10-5 M Mancopper/ ml medium compared with 4.96±0.50/ cell in the non- treated cell cultures and 13.90±0.58/ cell ($p<0.01$) in the cultures treated with Mitomycin C (Table 1).

Table 1: The number and percentage of chromosomal aberrations and SCE's induced in cultured mouse spleen cells after treatment with different concentrations of Mancopper *in vitro*.

Treatment Conc. M/ml	Abnormal Metaphases[a]			No. of different types of metaphases						SCE's[b]	
	No.	Mean (%)±SE Including Gaps	Mean (%)±SE Excluding Gaps	G.	Frag. and/or Br.	Del.	C.F.	M.A.	Polyp.	No.	SCE's/Cell Mean (%)±SE
I. Control	26	5.20±0.66	3.80±0.70	7	11	8	0	0	0	992	4.96±0.50
II. MMC (0.15µM/ml)	138	27.60±0.83**	20.20±0.85**	37	40	20	10	28	3	2781	13.90±0.58**
III. Mancopper											
10^{-7}	37	7.40±0.58	5.60±0.64	9	20	8	0	0	0	1172	5.86±0.83
10^{-6}	51	10.20±0.70**	7.60±0.83*	13	26	7	1	2	2	1435	7.17±0.55*
10^{-5}	70	14.0±0.73**	11.0±0.64**	15	35	11	3	4	2	1632	8.16±0.63**
$5x10^{-5}$	93	18.60±0.66**	14.20±0.83**	22	38	15	4	10	4	1781	8.90±0.48**

(a) Total number of examined metaphases for chromosomal aberrations 500 (5 plates/treatment)
(b) No. of scored cells for SCE's 200 metaphases/ sample (5 plates/ treatment). *$p<0.05$; **$p<0.01$: Significance compared to Control. (t-test)
G.: Gap; Frag.: Fragments; Br. Breaks; Del.: Deletions; C. F.: Centric Fusions; M.A.: Multiple Aberrations; Polyp.: Polyploidy.

Table 2: The number and percentages of chromosomal aberrations and SCE's after treatment with different doses of Mancopper *in vivo*.

Dose mg/kg/day	Abnormal Metaphases[a]			No. of different types of metaphases						SCE's[b]	
	No.	Mean (%)±SE		G.	Frag. and/or Br.	Del.	C.F.	M.A.	Polyp.	No.	SCE's/Cell Mean (%)±SE
		Including Gaps	Excluding Gaps								
I. Control	21	4.20±0.45	2.80±0.40	7	9	5	0	0	0	971	4.85±0.51
II. MMC (1mg/kg b.wt.)	121	24.20±0.70**	18.0±0.92**	31	42	15	4	22	7	2601	13.00±0.60**
III. Mancopper											
10mg/1day	23	4.60±0.51	3.20±0.58	7	10	5	0	0	1	1015	5.07±0.61
20mg/1day	29	5.80±0.62	3.80±0.51	10	11	6	0	1	1	1142	5.71±0.55
40mg/1day	38	7.60±0.45**	5.40±0.54**	11	15	8	1	3	0	1581	7.90±0.51**
40mg/3days	51	10.20±0.70**	7.20±0.70**	15	21	7	1	5	2	ND	ND
40mg/7days	61	12.20±0.83**	7.80±0.83**	22	23	9	2	4	1	ND	ND

(a) Total number of examined metaphases 500 (5 animals/group)
(b) No. of scored cells 200 metaphases/ group (5 animals). *p<0.05; **p<0.01: Significance compared to Control. (t-test)
G.: Gap; Frag.: Fragments; Br. Breaks; Del.: Deletions; C. F.: Centric Fusions; M.A.: Multiple Aberrations; Polyp.: Polyploidy.

3.2. In vivo studies

3.2.1. Bone-marrow cells

3.2.1. a. Chromosome aberrations

A single i.p. injection with the dose 40mg Mancopper /kg b. wt. was the only treatment which induced a high effect on chromosome aberrations including gaps in mice bone- marrow cells. Its percentage reached to 7.60±0.45 ($p<0.05$) 24 h after treatments (Table 2). This percentage increased and reached to10.20±0.70 and 12.20±0.83 ($p<0.01$) in mice administered Mancopper for 3 and 7 successive days respectively compared with 4.20±0.45 in non- treated mice and 24.20±0.70 ($p<0.01$) in mice i.p. injected with Mitomycin C. The main types of chromosome aberrations observed were breaks, fragments and polyploidy (Figure 2a, b).

3.2.1. b. Sister chromatid exchanges

The number of SCE's/ cell induced by Mancopper at 10 and 20 mg /kg b. wt. was observed to have no effect in mice bone-marrow cells (Figure 2c, d). While, mice treated i.p. with 40 mg Mancopper /kg b. wt. were demonstrated to increase the number of SCE's/ cell (7.90±0.51; $p<0.01$) in comparing with 4.85±0.51 in non- treated mice and 13.00±0.60 ($p<0.01$) in mice treated with Mitomycin C (Table 2).

3.2.2. Spermatocytes

Chromosome aberrations

The significant effect of Mancopper on the induction of chromosome aberrations in mice spermatocytes was first observed to increase with dose response after i.p. injection with 10, 20 and 40mg Mancopper /kg b. wt. for 24h. Also, this percentage of chromosome aberrations was increased with time response (Table 3).

The main types of chromosome abnormalities in mice spermatocytes were the separation of univalents, however the frequency of XY univalents was higher than that of autosomal univalents (Figure 2e, f). Translocations in the form of chain (IV) were observed in the high concentration after 3 and 7 days of treatments with Mancopper (Table 3).

4. Discussion

Evaluating the genotoxic effect of Mancopper as one of the EBDCs is the main target of the present studies. It is demonstrated that Mancopper reduces the cell viability of the mouse splenocytes in vitro with dose response. These results agree with the studies carried out on other analoges of EBDCs such as zineb. High concentration-related cytotoxicity revealed by the absence of cells was observed in cultures harvested 24 h after treatment with concentrations >25.0 µg/ml zineb or 50.0 µg/ml azzurro, respectively (Soloneski et al., 2003). Also, they reported that complete cell death is achieved in CHO cells exposed to 50.0± 100.0 µg/ml of both compounds, when the culture period is extended up to 36 h after treatment (Soloneski et al., 2002a).

Table 3: The number and percentages of chromosomal aberrations after treatment with different doses of Mancopper in mice spermatocytes *in vivo*.

Dose mg/kg/day	Abnormal Metaphases[(a)]		No. of different types of metaphases				
	No.	Mean (%)±SE	XY Univ.	Au. Univ.	XY+Au. Univ.	Frag.	Chain IV
I. Control	16	3.20±0.48	12	4	0	0	0
II. MMC (1mg/kg b.wt.)	98	19.60±0.65**	49	40	3	2	4
III. Mancopper							
10mg/1day	18	3.60±0.55	12	6	0	0	0
20mg/1day	24	4.80±0.60	20	4	0	0	0
40mg/1day	33	6.60±0.52**	20	12	1	0	0
40mg/3days	44	8.80±0.71**	29	14	0	0	1
40mg/7days	57	11.40±0.54**	40	12	2	1	2

(a)Total number of examined metaphases 500 (5 animals/group)
**p<0.01: Significance compared to Control. (t-test)
XY univ.: XY univalents; Au. Univ.: Autosomal Univalents; Frag.: Fragments.

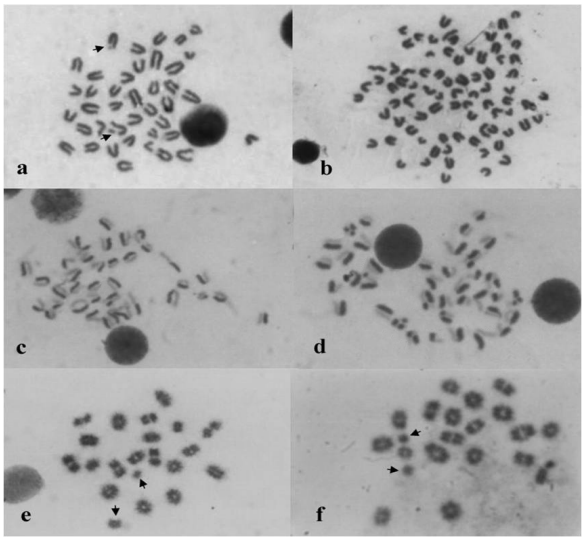

Figure 2: Metaphases spread from mice after treatment with Mancopper showing (a) break, (b) polyploidy, (c) low frequency of SCEs, (d) high frequency of SCEs from mice bone-marrow cells, (e) X-Y univalent, (f) autosomal univalent from mice spermatocytes.

A similar cytotoxic effect was observed in human lymphocytes cultured in an erythrocyte-free environment, such as plasma leukocyte cultures (Soloneski et al., 2002b), but not in lymphocytes exposed to 50.0 µg/ml of either pesticide in the presence of red blood cells (Soloneski et al., 2001).

Mancopper increases the frequency of SCE's/ cell and chromosome aberrations with dose increasing. This is in agreement with the studies carried out in Mexico workers exposed to EBDCs during application to tomatoes (Steenland et al., 1997). Their data on genotoxic outcomes indicated that the applicators had significantly increased SCE's, with or without adjustment for age and smoking. Besides, chromosome translocations were significantly elevated for both applicators and owners (p<0.001) before age adjustment, compared to nonexposed subjects.

Induction of chromosome damage represented with SCE's and chromosome aberrations in an indicator to the probability of induction of carcinogenesis with Mancopper. EBDCs pesticides, most probably lead to DNA damage via free radical reactions and production of active oxygen species and this indirectly lead to carcinogenesis (Soloneski et al., 2001 and 2002a, b). Reports from the early 1980s indicate that tumor promoters like 12-O-tetradecanoylphorbol- 13-acetate (TPA) can induce chromosomal aberrations and DNA damage by an indirect action, i.e. via free radical reactions and production of active oxygen species (Emerit and Cerutti, 1981and 1982).

Regarding to the mode of action of Mancopper, it was demonstrated that the metal ion copper which is conjugated with EBDCs causes DNA double strand breaks (Furuta et al., 2002). Also, they demonstrated that dithiocarbamate mediated accumulation of copper over the range of 25-40 µg/g of cellular protein increased the steady-state level of p53 in MCF-7 breast cancer cell lines. Stabilization of p53 is apparently the result of free-radical formation, which can promote DNA backbone strand breaks (Lloyd and Phillips, 1999). The low level of copper necessary for genotoxic stress-induced p53 activation is consistent with the fact that a p53 response can be elicited when less than ten DNA double-strand breaks occur per cell (Lloyd and Phillips, 1999). At levels of copper poisoning higher than 60 µg/g of cellular protein, such as those mediated

by pyrrolidine dithiocarbamate (PDTC), proteins with exposed thiol residues were oxidized (Furuta et al., 2002). Also, they demonstrated that at the concentrations used in their study, diethyldithiocarbamate (DEDTC) and ethylene (bis)dithiocarbamate (NABAM) and H_2O_2 created a sufficient amount of free radicals which had the ability to promote DNA-strand breaks and increased p53 protein concentration and PDTC was able to produce a sufficient amount of free radicals to oxidize p53 protein.

For *in vivo* studies, Mancopper induces genotoxic effects in mice somatic and germ cells. The frequencies of SCEs/ cell and chromosome aberrations in mice bone- marrow cell are increased highly significantly with dose increasing. Also, chromosome aberrations increased highly significantly with time exposure to Mancopper. Different EBDCs induces damages in different somatic. This is agree with the other previous studies when they demonstrated that exposure to mancozeb causes normocytic type of anemia, significant decrease in blood glucose and globulin levels and significant pathological changes in liver, kidney, spleen and heart congestion with slight enlargement and brain with few petechial hemorrhages (Hore et al., 1997). Also, there was significant decrease in the kidney, spleen and liver weight and protein, glycogen levels; however there was increase in thyroid, thymus weight and total lipids level of testis and liver in mice treated with mancozeb (Ksheerasagar and Kaliwal, 2003).

Studies on the reproductive effects of carbamate pesticides are of immense important in the field of toxicology. However, carbamates are chosen on the basis of their properties of biodegradable with low mammalian toxicity (O'Neil and Marshal, 1984), Mancopper induces genotoxic effects on mice spermatocytes highly significantly with dose and time dependant manner in the present studies. The clastogenic effects of Mancopper may be resulted from the carbon disulfide specially that carbon disulfide causes significant decreases in serum testosterone, marked degenerative changes in testicular tissue, affects spermatogenesis and also epididymal alterations (Patel kumud et al., 1999). Many EBDCs such as Mancozeb showed its biological effects through its metabolite ethylene thiourea (ETU) and carbon disulfide (O'Neil and Marshal, 1984). Also, it has been reported that mancozeb inhibits ovarian compensatory hypertrophy, ovarian follicular growth and biochemical constituents in hemicastrated and normal rats (Mahadevaswami et al., 2000 and Baligar and Kaliwal, 2001). Mancozeb shows anti-implantation activity in albino mice (Bindali and Kaliwal, 2002). Administration of mancozeb with lead acetate induces maternal toxicity embryotoxicity and characteristic teratogenic effects in rats (Varnagy et al., 2000). Also, Mancozeb decreases testes weight significantly in mice with oral administration at 800 mg/kg body (Ksheerasagar and Kaliwal, 2003). However, there was a significant decrease in the number of spermatogonia, diameter of spermatocytes and spermatids in 20 days and number of spermatids in 10 days mancozeb treated mice.

In conclusion, the present studies demonstrate the cytotoxic and genotoxic effects of the fungicide Mancopper in both in vitro and in vivo in somatic and germ cells of mice. Thus we recommend directing the attention towards using natural compounds have the ability to act as herbicides rather than the hazards of pesticides and fungicides.

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