Evaluation of the Antioxidant Effect of Doxorubicin as a Treatment for Mammary Gland Carcinoma in Rats

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Abstract: Background: Despite of using doxorubicin (DOX) over a period of several months was found to result in the development of cardiomyopathy and congestive heart failure (CHF) in humans; it has great therapeutic effects on several types of cancers. The aim of this study was to evaluate the antioxidant effect of DOX on the mammary gland tissue and the mammary gland tumors as a treatment for mammary gland carcinoma and confirmation of this data by biochemical investigations. Materials and methods: This study was carried out on 46 female *Sprague-Dawley* rats divided into 3 groups. Group 1 (10 rats) was the negative control and injected 0.9% saline. Group 2 (18 rats) was injected N-Methyl-N-Nitrosourea (MNU). Group 3 (18 rats) was injected MNU then treated with DOX. Results: DOX could decrease the oxidative stress marker MDA and increased the antioxidant enzymes trying to face the mammary gland tumours. Besides that, there was a great different between the study groups in the liver functions and hormones analysis while, the kidney functions and lipid profiles certificated differences between the experimental groups with no statistical significance. Conclusion: DOX has well therapeutic effect on mammary gland carcinoma by decreasing the oxidative stress and increasing the activity of antioxidant enzymes in the mammary gland tissues.

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1. Introduction

Doxorubicin (DOX) is an anthracycline drug with a wide antineoplastic activity. It is one of the most widely and successfully used antitumour drugs (Tong *et al.,* 2015). It acts by forming a stable complex with DNA and interfering with the synthesis of nucleic acids (Martindale, 2011). It is active in all phases of the cell cycle with maximal activity in S phase. It has several modes of action including intercalation between DNA base pairs, inhibition of DNA and RNA synthesis by template disordering and steric obstruction, production of oxygen-free radicals which cause damage to DNA and cell membrane (Khaleel *et al.,* 2016).

Overproduction of reactive oxygen species (ROS) results in oxidative stress, a state in which tissue and cellular redox balance is altered towards a more oxidizing environment (Medina *et al.,* 2005). ROS has an important role in carcinogenesis because of induction of DNA damage such as including base modifications, strand breakage and DNA-protein cross-links. These types of mutation are reported in genes whose dysfunction is involved in the genesis of cancer (Frederiks *et al.,* 2009). ROS also can lead to a cumulative damage to protein, lipids, carbohydrates, and membranes. The prime functions of antioxidative defenses are suppressors of the generation of ROS, scavenging them, besides repairing and promoting reconstitution of damage, and inducing the expression of antioxidant proteins and enzymes (Naik *et al.,* 2011).

Antioxidant enzymes are compartmentalized, and the activity is controlled by genetic regulation. These enzymes include superoxide dismutase (SOD), catalase (CAT) and glutathione-s-transferase (GST). Main biochemical reactions produce and scavenge ROS. Superoxide dismutase is a key antioxidant enzyme present in the cells as a first line of defense against the accumulation of superoxide radicals. It can catalyze the superoxide anion to hydrogen peroxide and molecular oxygen (Khan *et al.,* 2010). Catalase (CAT) found extensively in living organisms that use oxygen in their metabolism. Catalase can prevent the accumulation of and protects cellular organelles and tissues from damage by peroxide, which is continuously produced by numerous metabolic reactions (Ighodaro and Akinloye, 2017). Glutathione-S-transferase (GST) has the major role of enzymatic detoxification in many species by conjugation of the activated xenobiotics to reduced glutathione (GSH) to protect cells from toxins produced by biotransformation reactions in the cytoplasm (Ramsay and Dilda, 2014) and reduce lipid hydroperoxides through their Se-independent glutathione-peroxidase activity and that these enzymes can also detoxify LPO end-products (Singhal *et al.,* 2015).

2. Materials and Methods

Healthy female Sprague-Dawley rats were obtained from the Holding Company for Biological Products & Vaccines (VACCERA), Helwan-Giza, Egypt. Rats were allocated to a plastic cage covered with metal grids with dry husk padding at temperature and relative humidity about 22 ± 5 ºC and 55 ± 5% respectively, and natural light-dark (day/night) cycle. Animals were to acclimate for 10 days in the animal facility conditions before being divided into groups for experimentation when reached the age of 46 days.

Chemicals

N-Methyl N-Nitrosourea (MNU) was obtained from Sigma Aldrich, St. Louis, MO, USA. Doxorubicin is manufactured in India by KHANDELWAL LABORATORIES PVT. LTD. and marketed by ROYAL MEDICAL PVT. LTD.

Experimental design

A total of 46 female rats were divided into 3 groups as follows:

- Group1 (10 rats): Injected by 0.9% NaCl only (-ve control).
- Group2 (18 rats): Administrated by MNU only (+ve control).
- Group3 (18 rats): Administrated by MNU then DOX. The whole experiment duration was 23 weeks.

Experimental induction of mammary tumours

MNU was dissolved in 0.9% acidic NaCl (PH 4.0). After thawing, MNU was injected after 15 minutes. 36 female rats received two intraperitoneal (i.p.) MNU doses of 50 mg/kg b. w., at 46 and 52 days of age. The injection was given along the abdominal middle line of the animal, between the 3rd and 4th pair of mammary glands (Thompson and Singh, 2000). Control animals had two similar injections but with 0.9% saline. After 10 weeks from MNU administration, group 3 was received a dose of 12mg/kg b.wt. of DOX twice a week for 4 weeks (Todorova et al., 2011). In all rats, an injection volume of 0.5 ml was used.

Biochemical investigations and hormone analysis

Blood samples were collected from the abdominal aorta from rat at autopsy using 5 ml syringes. 0.5 ml of whole blood samples used for complete blood picture (CBC) analysis by automatic methods. The remaining amounts of collected blood samples were left to clot in a centrifuge tube and centrifuged at 3000 xg for 15 min to separate the sera.

Complete blood count was counted by the automated method using Dirui BCC-3600, MA, USA automated hematology analyzer. By using commercial Kit supplied by DIALAB GmbH-Vienna/Austria, AST, ALT, ALP, Total protein, albumin, urea and creatinine were assessed according the methods of Rei (1984), Thomas (1998), Belfield and Goldberge (1971), Thomas (1998), Sternberg (1977), Thomas (1998) and Newman and Price, (1999) respectively. Serum total cholesterol and triglycerides levels were determined using quantitative kit (Linear Chemicals, S.L., Spain) according to the method of Allain et al. (1974) and Fossari and Principe (1982) respectively. Biochemical hormonal analyses were established using a commercially available radioimmunoassay (RIA) kits to measure serum concentrations of estrogen and progesterone hormones (Diagnostic Products Corp., Los Angeles, CA) (Huang *et al.,* 1978).

Tissue sampling for measurements of antioxidant enzymes activities and oxidative stress markers:

Small pieces of mammary gland tissues and solid tumours were carefully collected from all the experimental groups, placed in liquid nitrogen to avoid squeezing the tissues. The tissues were homogenized in ice-cold phosphate buffer (50 mM, pH 7.4) containing triton X-100 (1%) using Omni international homogenizer (USA) at 22.000 rpm for 20 s each with 10 s intervals. The supernatant was freeze-thawed thrice to completely disrupt mitochondria. Then, the supernatant was centrifuged at 6000xg in cooling centrifuged at 4^{o}C for 15 min and the yielded supernatant which contains the cytosolic and mitochondrial enzymes was used for immediate enzyme assays. The UV/VIS Spectrophotometer (JENWAY 6505, UK) used for the measurements of enzyme activities and oxidative stress parameters at 25^{o}C.

Total SOD activity

The activity assayed according to the method of Paoletti and Mocali, (1990), which monitors NADH oxidation. The SOD activity was assayed by assessing the inhibition of NADH oxidation by β-mercaptoethanol in the presence of EDTA and Mn as substrate.

CAT activity

CAT was assayed according to the method of Xu et al (1997). It was based on two molecules of hydrogen peroxide (2H2O2) in the presence of catalase to yield 2H2O and O2.

GST activity

The determination of GST activity was assayed through the comjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) according to the method of Habig et al., (1974).

MDA levels

Lipid peroxidation level was assayed according to the method of Buege and Aust, (1978).

Statistical analysis

The data were expressed as means ± S.D. were analyzed using t-test analyses while the data were analyzed using the Graph Pad Prism version 7.0, USA.

P<0.05 was considered as significant for all statistical analyses in this study.

3. Results

Final Body Weight

In general, the final body weights in the tumour-induced groups 2 & 3 were significantly lower than the control group 1 with some intergroup differences **Figure 1.** Also, the relative organ weights of the liver and spleen showed generally a significant decrease in MNU and MNU+ DOX treated groups as compared with the control group while, the relative organ weights of the right and left kidney showed no significant changes in MNU and MNU+DOX treated groups as compared with the control group.

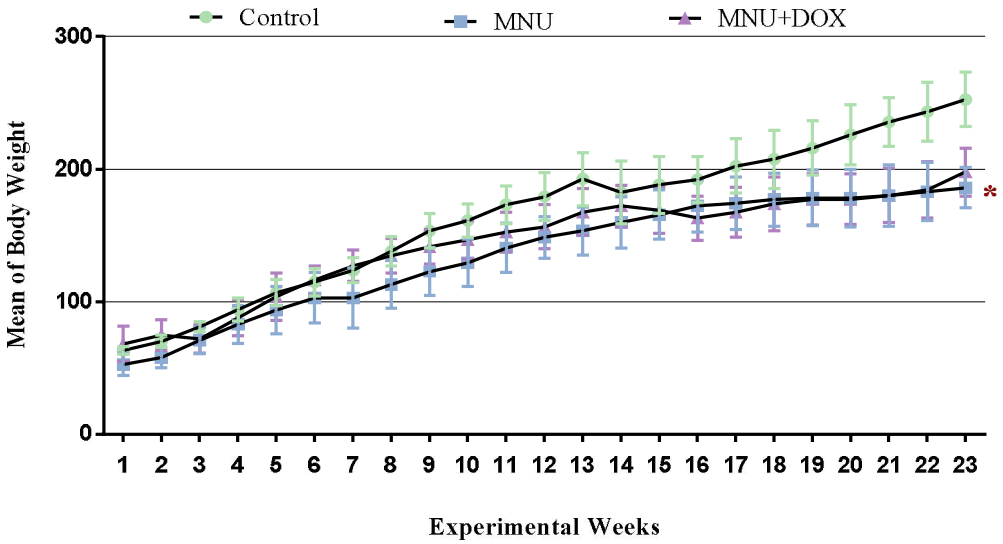

Figure 1: Growth curves of rats in all experimental groups. MNU: N-Methyl-N Nitrosourea; DOX: Doxorubicin; *: Significant vs. group 1 at *P*<0.05.

Blood biochemistry

Complete Blood Count (CBC)

MNU-injected rats showed significant decrease in RBCs compared with the normal control while group 3 treated with DOX showed slightly increasing in RBCs count as compared with group 2. On the other hand, the hemoglobin and hematocrit in group 2 showed slightly decreasing compared with the normal control while, DOX normalizing them to the control. Moreover, WBCs in group 2 showed significantly increased compared with the normal control while, group 3 showed slightly increased as compared with the control group. As WBCs, the platelets in group 2 showed significantly increasing as compared with the normal control while, platelets in group 3 was slightly decreased as compared with the positive control **(Table 1).**

Table 1: Complete Blood Count (CBC) Data.

Group	Treatment	RBC (10^6/µL)	HB g/dL	HCT (%)	WBC (10^3/µL)	PLT (10^3/µL)
1	**Normal Control**	11.3±1.9[a]	13.1±0.52	41±1.7	10.4±2.4	531.7±45.3
2	**MNU**	6.5±0.2*	10.6±3.1	39.3±3.5	20.1±1.3*	790±21.1*
3	**MNU+DOX**	7.1±0.15	13.1±0.46	41±1.0	14.4±2.8	709±21.9

a: Values are expressed as means ± S.D.; RBC: Red blood cells; HB: Hemoglobin; HCT: Hematocrit; WBC: White blood cell; PLT: Platelet. *: Significant vs. group 1 at *P*<0.05.

Liver function analysis

Table 2 showing liver functions of the all experimental groups, where the serum levels of alanine aminotransferase SGPT (ALT) showed significant increase in group 2 compared with the normal group, while its levels in group 3 showed significant decrease versus group 2. The serum levels of aspartate aminotransferase SGOT (AST) showed significant increase in group 2 compared with the normal control. Group 3 treated with DOX after MNU administration has significantly decreased the levels of SGOT compared with group 2. The serum level of alkaline phosphatase (ALP) was elevated significantly in MNU-treated groups compared with the normal

control, while DOX decreased its level in group 3 compared with group 2. The serum level of total protein in group 2 was almost similar to the normal control values. After the treatment with DOX, group 3 showed significant increasing compared with group 2. Serum levels of albumin were almost similar in all experimental groups with no significant changes **(Figure 2).**

Kidney function analysis

Blood urea concentration levels showed slightly decreasing in MNU-treated groups compared with its concentrations in the normal control with no statistical differences. Moreover, creatinine concentration levels in group 2 showed slightly decreasing compared with its concentration in the normal group while, group 3 treated with DOX recorded a small increasing compared with the negative and the positive control with no statistical significance **(Table 3).**

Lipid profile analysis

Triglycerides concentration levels in the serum of group 2 were almost similar to the normal control. On the other hand, group 3 treated with DOX after MNU injection showed significant increase in the concentration of serum triglycerides compared with the negative and the positive groups. There was no significant difference in the level of Cholesterol between the experimental groups but it elevated in group 3 **(Table 3).**

Table 2: Blood Concentration Levels of Liver Functions (ALT, AST, ALP, Total Protein, Albumin)

Group	Treatment	ALT (U/L)	AST (U/L)	ALP (U/L)	TP (g/L)	Alb (g/dl)
1	Normal control	25 ±3	192.3±3.5	135 ±2	7.3±0.6	3.5±0.4
2	MNU	46.3±4.04*	246±7*	264.3±5.5*	6.7 ± 0.2	3.4±0.1
3	MNU+ DOX	32.3 ±2.5*,**	210.7±4*,**	130.3±2.3**	7.9 ±0.4**	3.4 ±0.4

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; TP: Total protein; Alb: Albumin. *: Significant vs. group 1; **: Significant vs. group 2 at $P<0.05$.

Table 3: Kidney Functions and Lipid Profile Analysis

Group	Treatment	*n*	Blood urea (mmole/l)	Creatinine (mmole/l)	Cholesterol (mg/dl)	Triglycerides (mg/dl)
1	Normal control	5	44±2.7	0.82±0.05	79±9.5	92.3±3.1
2	MNU	5	40.3±2.1	0.81±0.04	75±6.6	91±3.6
3	MNU+DOX	5	41.7±3.8	0.86±0.05	95.3±12.9	214.7±4.2**

n: Number of samples analyzed; **: Significant vs. group 2 at $P<0.05$.

Serumestrogen and progesterone levels

The mean serum estrogen levels in all rats injected with only MNU (group 2) has significantly elevated compared with the normal control, while the treatment with DOX after MNU-administration in group 3 showed decrease in the mean serum estrogen levels if compared with group 2 but these values still higher than those of normal rats **(Table 4).**

The mean of serum progesterone levels in group 2 injected with MNU only was significantly increased compared with the normal control. On the other hand, the rats injected with DOX after MNU administration showed slightly decrease in serum estrogen levels **(Table 4) (Figure 3).**

Table 4: Serum Estrogen and Progesterone Levels

Group	Treatment	*n*	Estrogen	Progesterone
1	Normal control	5	9.9±4.8[a]	8.8±1.9
2	MNU	5	47.3±11.2*	24.0±0.85*
3	MNU+ DOX	5	22.4±12.9	18.5±2.7

a: Values are expressed as means ± S.D.; *n*: number of samples analyzed; *: Significant vs. group 1 at $P<0.05$.

Oxidative stress levels

Table 5shows the concentration of MDA in the normally-appearing mammary tissues of group 2 was significantly increased compared with the normal control. Treatment with DOX was significantly decreased MDA concentration compared with group 2. On the other hand, the concentration of MDA in tumours of group 2 was significantly increased compared with the mammary tissues in the same group, while the tumours treated with DOX showed increased in MDA concentration compared the

normally-appearing mammary tissues in the same group but showed no statistical significance.

Antioxidant enzymes

Glutathione-s-transferase activity

The activity levels of GST was increased in group 2 compared with the normal control and also increased in group 3 treated with DOX compared with group 2 treated with MNU only but without any statistical significance, while the activity levels of GST in the tumours collected from groups 2 and 3 treated with MNU was decreased in both of the two groups compared with its activity levels in the mammary tissue collected from the same groups **Table 6**.

Table 5: Average Oxidative Stress Marker of Mammary Glands Tissues and Tumours from All Experimental Groups.

Group	Treatment	n	MDA (nmole/g/ tissue)
1	Normal control	5	1963±332.1
2	MNU	5	5561±185.9*
3	MNU+DOX	5	1400±146.1**
4	MNU (Tumour)	5	3109±199.3**
5	MNU+DOX (Tumour)	5	2001±757.2

n: number of samples analyzed; MDA: malondialdhyde*: Significant vs. group 1 at $P<0.05$; **: Significant vs. group 2 at $P<0.05$.

Catalase activity

Table 6 shows CAT activity levels in the normally-appearing mammary tissue of group 2 were slightly decreased compared with the normal control, while showed significant increase in group 3 compared with the positive control. CAT activity levels in tumours collected from groups 2 and 3 showed slightly decrease compared with the mammary tissue collected from the same groups.

Superoxide dismutase activity

The activity levels of SOD in the mammary tissue showed slightly decrease compared with the normal control, but its activity levels in group 3 treated with DOX showed significantly decrease compared with the group treated with MNU only. The activity levels of SOD in the tumour collected from groups 2 and 3 were showed very slightly decrease compared with the normally-appearing mammary tissues collected from the same groups **table 6**.

Table 6: Average Oxidative Stress Marker and Antioxidant Activity Levels of Mammary Glands Tissues and Tumours from All Experimental Groups.

Group	Treatment	n	GST (nmole/min/ g tissue)	CAT (nmole/min/g tissue)	SOD (nmole/min/g tissue)
1	Normal control	5	1.94±0.57	0.27±0.04	0.15±0.03[a]
2	MNU	5	2.03±0.18	0.25±0.04	0.13±0.02
3	MNU+DOX	5	3.26±0.9**	0.82±0.08**	0.06±0.01**
4	MNU (Tumor)	5	1.33±0.43**	0.24 ±0.05	0.14±0.02
5	MNU+DOX (Tumor)	5	0.93±0.22	0.46±0.07	0.06±0.01**

n: number of samples analyzed; MDA: malondialdhyde; GST: glutathione-s-transferase; CAT: catalase; SOD: superoxide dismutase; *: Significant vs. group 1 at $P<0.05$; **: Significant vs. group 2 at $P<0.05$.

4. Discussion

The body weight of the female rats of all the experimental groups was recorded weekly to follow up and access their health. After MNU induction the weight of group 2 treated with MNU only was significantly decreased compared with the normal control. This result was compatible with many studies that explained this suppression in the body weight (Emoto *et al.,* 2016). The growth of the tumours decreasing the mobility of rats, resulting in difficulty grooming and interfere with the rat's ability to feed itself (Saminathan *et al.,* 2014).

Biochemical analyses were carried out to investigate the female rats. CBC test showed decrease in RBC count, HB and hematocrit, while there was significant increasing in WBC count and platelets in group 2 compared with the normal control. Decreasing RBC and increasing WBC counts have indication of developing anemia and lymphocytosis respectively. These data are in line with other studies which demonstrated these inflammatory responses

(Sivakumar *et al.,* 2016). DOX-treated rats showed improved in their CBC compared with group 2 indicating its therapeutic effect.

Aspartate transaminase (AST) and alanine transaminase (ALT) are the enzymes which are involved in the conversion of amino acids to ketoacids. They are pathophysiological marker enzymes used to assess liver tissue damage because they are produced mainly from liver (El-Demerdash *et al.,* 2005). The increase in the activities of ALT, AST and ALP may be primarily due to leakage of these enzymes from liver cytosol into the blood stream as a result of tissue damage. Tissue damage is a critical feature in cancerous environment (Saravanan *et al.,* 2014).

In this study, there were significant elevations in ALT and AST levels in group 2 compared with the control. This elevation of the liver specific marker enzymes is a possible indicator of liver damage occurrence. Several studies have been reported the increased levels of ALT and AST due to hepatotoxicity induced by various carcinogens in different experimental animals (Saravanan *et al.,* 2014). The elevated serum ALP levels observed could be due to the toxic effect of MNU in the liver. Also, the increased activity of this enzyme was noticed in different types of cancer as in subjects who may also be due to osteolytic bone metastases in breast cancer leading to increased osteoclastic activity and bone resorption (Abdel-Moein *et al.,* 2013).

ALT, AST and ALP levels in MNU-induced female rats treated with DOX was showed significant decreasing if compared with MNU-treated rats which could restore liver functions by probably interfering with metabolic activations in MNU-treated rats.

Urea and creatinine are metabolic by-products of the excess protein, creatine phosphate and purine in the liver and muscle, which are passed into the bloodstream and excreted by kidneys (Yaacob *et al.,* 2015). In this experiment urea and creatinine levels showed decreasing in group 2 compared with the normal control but with no statistical significance. This data was in line with another study which illustrated this decreasing by the hyperactivity of the kidneys in removing excess fluids and wastes from the blood stream (Yaacob *et al.,* 2015).

Treating with DOX slightly increased their levels to be close to the normal values. The increase in serum creatinine and urea could be related with catabolic action of chemotherapeutic agent.

Serum estrogen and progesterone levels after MNU administration in group 2 showed significantly increasing as compared with its level in the normal control. Treating with DOX decreasing those levels compared with group 2. This result was in matching with another study which showed that estrogen induces breast cancer through stimulation of cellular proliferation, resulting in more opportunities for accumulation of genetic damages leading to carcinogenesis (St-Hilaire *et al.,* 2011). Another possible mechanism of action may be through the metabolism of estrogen, which may induce oxidative stress and playa key role in mammary cancer development (Mense *et al.,* 2008). Estrogen metabolites may exert DNA mutations from ROS or DNA mutations which may lead to the accumulation of genomic alterations essential for mammary tumor genesis (St-Hilaire *et al.,* 2011).

It is well studied that estrogen is genotoxic agents causing oxidative DNA damage (Chang and Singh, 2017). Oxidative stress has been suggested to contribute to the pathogenesis of carcinogenicity (Klaunig *et al.,* 2010). In this experiment, we demonstrated the activities of CAT, GST and SOD also, we demonstrated the oxidative stress marker MDA in the control tissue and the normally-appeared mammary tissue and the mammary tumours from group 2 and group 3. MDA levels in normally-appeared mammary tissue and tumour collected from group 2 showed significant increasing compared with the normal tissue while, the tissue and tumours collected from group 3 showed increasing in MDA levels comparing with group 2.

The increased level of MDA is consistent with elevated peroxidation of lipids in cell membranes (Selamoglu *et al.,* 2009). The previous studies reported higher MDA level in breast cancer (Badr *et al.,* 2015). Hence, it is of interest to assess MDA as a marker of oxidative stress and the role played by lipid peroxidation and the modulation of antioxidants during the progression of breast cancer (Klaunig *et al.,* 2010). Treating with DOX has significantly recovered the oxidative stress in group 3 by decreasing the lipid peroxidation of the cancer-mammary gland tissue.

GST is an enzyme involved in antioxidant defense and involved in detoxification. GST perform function ranging from catalyzing the detoxification of electrophilic species including the metabolites of genotoxic and non-genotoxic compounds via a spontaneous enzyme catalyzing conjugation reaction to protect the cells against peroxidative damage (Kalaiselvi *et al.,* 2013). Activity of GST was found to be increased in group 2 compared with its activity in the normal control. This result was incompatible with the previous studies which showed that GST decreased in mammary cancer (Badr *et al.,* 2015). DOX increased the activity of GST in mammary gland tissue.

The activity of CAT and SOD were decreased in group 2 compared with the normal control. These results were in line with the study of Badr *et al.* (2015). This decreasing may be related to the elevation

in circulating lipid peroxides which generated during metabolic activation of DMBA. This can result in accumulation of O^{2-} which is a highly diffusible and potent oxidizing radical capable of traversing membranes causing deleterious effects at site far from the tumour. The levels of free radicals overcome the saturation level of SOD resulting in super saturation of SOD with a high concentration of ROS which in turn causes inhibition of the enzyme. Reduction of CAT may be related to the reduction of SOD altered antioxidant status caused by carcinogenesis (El Kholy *et al.,* 2013).

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