Effect of New Synthesized Copper Complex of 4-azomalononitrile antipyrine with Superoxide Dismutase Activity on Ehrlich Ascites Carcinoma in Mice

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ABSTRACT

Background: *A number of Cu(II) chelate complexes that exhibit cytotoxic activity through cell apoptosis or enzyme inhibition was reported to have numerous biologic activities including antibacterial, antifungal, antiviral and anti-tumor properties. The present work aimed to study the effect of new synthesized Cu complex of 4-azomalononitrile antipyrine [CuL(OH)(ClO_4)] which exhibits superoxide dismutase (SOD)-mimetic activity on tumor in mice induced by Ehrlich ascites carcinoma (EAC) cell line.* ***Results:*** *The administration of 10 mg/kg body weight [CuL(OH)(ClO_4)] 24 hours after intraperitoneal injection of EAC, effectively inhibited tumor growth and the proliferation of EAC cells. Cu complex ameliorated the increase in serum aspartate transaminase (AST) and alanine transaminase (ALT) activities after implantation of EAC cells. On the other hand, the level of creatinine was increased. Moreover, Cu complex of 4-azomalononitrile antipyrine significantly improved the hepatic and erythrocytes SOD and GRX activities. The glutathione content of hepatic tissues and erythrocytes was restored in EAC tumor bearing mice. Furthermore, it also, inhibited the formation of nitric oxide and lipid peroxidation products (thiobarbituric acid reactive substance, TBARS) in EAC tumor bearing mice. This effect was associated with inhibition of cell cycle progression and induction of apoptosis. Administration of [CuL(OH)(ClO_4)] complex 24 hours after injection of EAC for 3 weeks arrested cells in G0/G1 phase and resulted in a decrease in the viability.* ***Conclusions:*** *Cu complex [CuL(OH)(ClO_4)] has a strong inhibitory activity against growth of tumors. The anti-tumor mechanism may be mediated by preventing oxidative damage and induction of apoptosis.*

Keywords: *Antitumor activity; Copper complex; Oxidative stress; Flow cytometry; Ehrlich ascites carcinoma cells*

INTRODUCTION

It is well known that metal ions have a great importance in biological systems where many enzymes, hormones and antibiotics contain metal ions in their structures and their actions are inhibited if the metal ion is lost. The relationship between the antioxidant systems and growth of malignant cells is a feature observed in several studies. However, low activity of antioxidant system was

found in cancer cases **(Anand et al., 2008)[1].**

The reactive oxygen species produced in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and free radicals such as the hydroxyl radical ($OH^{\cdot}$) and the superoxide anion(O_2^{-}) **(Valko et al., 2006)[2]**. The hydroxyl radical is particularly unstable and will react rapidly and non-specifically with most biological molecules. This species is produced from hydrogen peroxide in metal-catalyzed redox reactions such as the Fenton reaction **(Valko et al., 2005) [3]**. These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation, or by oxidizing DNA or proteins **(Lecour et al., 2006)[4]**. Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms **(Cesaratto et al., 2004)[5]** while damage to proteins causes enzyme inhibition, denaturation and protein degradation (**Kc et al., 2006) [6]**.

Superoxide dismutase (SOD) is an enzyme currently thought to provide a defense against toxic products of oxygen metabolism and to be necessary in all oxygen utilizing cells. SOD is the antioxidant enzyme that catalyzes the dismutation of $O^{2\bullet-}$ to O^2 and to the less-reactive species H_2O_2 **(Barondeau et al., 2004) [7]**. Two forms of SOD have been found in the cells of higher animals. A copper and zinc-containing form (CuZn.SOD) is found largely in the cytosol and a manganese containing form (Mn.SOD) is found primarily in the mitochondria **(Landis and Tower, 2005) [8]**. Both forms of the enzyme are generally found in all normal cells of higher animals with exception of the red cell which do not contain mitochondria and thus have no Mn.SOD **(Behrend et al., 2003) [9]**. Since DNA is a potent target of cytostatic drugs, the effect of copper compounds on DNA functionality is very important. The ability of Cu(II) complexes to bind to DNA and exhibit nuclease activity in the presence of reducing agents is well established **(Wang et al., 2010) [10]**.

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent leading to formation of free radicals. In turn, these radicals can start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions **(Sies, 1997) [11].**

Copper exhibits considerable biochemical action either as an essential trace metal or as a constituent of various exogenously administered compounds in humans **(Suntres and Lui, 2006)[12]**. In its former role it is bound to ceruloplasmin, albumin, and other proteins, while in its latter it is bound to ligands of various types forming complexes that interact with biomolecules, mainly proteins and nucleic acids **(Brewer, 2009) [13]**. It is an essential component of several endogenous antioxidant enzymes, and that free radicals have been proposed to play a role in the process of carcinogenesis, the effects of dietary copper levels on the development of cancer have been investigated **(Daniel et al., 2004) [14]**. Although copper

homeostatic mechanisms play an important role in the prevention of copper toxicity, exposure to excessive levels of copper can result in a number of adverse health effects including liver and kidney damage, anemia, immunotoxicity, and developmental toxicity **(Bonham et al. 2002)** [15]. It was shown that copper proteins are associated with metabolic changes in cancer cells **(Tisato et al., 2010)** [16] and most importantly play a significant role in angiogenesis by stimulating proliferation and migration of human endothelial cells. In this context, we examined the inhibitory activities of new synthesized Cu complex with 4-azomalononitrile antipyrine $[CuL(OH)(ClO_4)]$ which exhibits superoxide dismutase (SOD)-mimetic activity against Ehrlich carcinoma cells implanted intraperitoneally in female mice.

MATERIALS & METHODS

Materials:

All chemicals used in the present study were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diagnostic kits for assaying alanine transaminase (ALT); aspartate transaminase (AST) and creatinine were purchased from Diamond Diagnostics Company, Cairo, Egypt.

The copper perchlorate $[CuL(OH)(ClO_4)]$ Complex was prepared according to methods of **El-Said et al.(2000)** [17].

Experimental animals:

All experiments were performed using adult female Swiss albino mice, with an average body weight of 25 g purchased from Theodore Bilharz Research Institute, Giza, Egypt. The mice were housed in steel mech cage and provided with commercial standard diet and tap water ad libitum. A total of 40 Swiss albino mice were divided into 4 groups, 10 mice each, according to the following scheme: group 1: control (untreated) mice; group 2: mice injected by 10 mg/kg copper complex intraperitoneally (i.p.) for three weeks, 3 times/week; group 3: mice inoculated intraperitoneally with $1x10^6$ EAC tumor cells; group 4: mice were treated by 10 mg/kg Cu complex after 24 hours of implantation of $1x10^6$ EAC tumor cells. After two days of the last treatment, the animals were sacrificed by decapitation. Ascetic fluid, blood and livers were immediately obtained after the animals were sacrificed. Blood samples allowed to clot for 10 to 15 minutes, centrifuged and the serum was separated and then the erythrocytes were washed with saline solution three times and hemolysed by diluting with deionized water. The serum and hemolysate were kept in -70°C. An accurately weighed piece of each of livers was homogenized in ice-cold 0.9% saline using a Teflon pestle connected to a homogenizer motor. The homogenates were adjusted at a concentration of 5% (w/v), centrifuged at 5000 rpm for 30 minutes at 4ºC to remove cell debris and nuclei. The resulting supernatant was used for biochemical analysis.

Determination of viability in vitro:

Seven days after implantation, Ehrlich ascites carcinoma cells were harvested, and viability of living cells using Trypan Blue (0.23%) was

checked according to the method of **Boyse et al. (1964)** [18].

Determination of survival time:

Two groups of mice (n=12 each) were implanted with Ehrlich carcinoma cells. One of them was daily treated intraperitoneally by 10 mg/kg body weight Cu complex. Each time Cu complex was given between 11.00 and 11.30 AM during the day, firstly to avoid circadian interference and secondly, to increase its level at the time Cu complex level in circulation is minimal **(Tapiero et al., 2003)** [19].

Enzyme assay:

Reduced glutathione (GSH) content was estimated as described by **Beutler et al. (1963)** [20]. ALT and AST activities and creatinine level were measured using Diagnostic kits (Diamond Diagnostics Company, Cairo, Egypt) according to the manufacturer's instructions. Glutathione peroxidase (GSH-PX) was measured by the method of **Beulter (1975)** [21]. Superoxide dismutase activity (SOD) was assayed as described by **Nishikimi et al. (1972)** [22]. Nitric oxide (NO) was assayed by the method of **Schmidt et al. (1995)** [23], Thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation, was determined as described by **Satoh (1978)** [24]. Protein in the homogenates was estimated by the method of **Lowry et al.(1951)** [25]. Hemoglobin (Hb) contents of the samples were measured as described by **Wintrobe et al. (1965)** [26].

Flow Cytometry:

Single cell suspension was prepared from fresh biopsy samples in RPMI-1640 medium. Cells were permeabilized with Triton X-100 followed by staining using propidium iodide as a DNA- specific fluorochrome. Flow cytometric analysis was performed on FACS Calibur Flow Cytometer, Becton Dickinson, Heidelberg, Germany; excitation of fluorescence occurred at 488 nm (15 mw air cooled argon ion lazer) and optimized for linear fluorescence single detection with the use of fluorochrome-labeled microspheres (DNA Checked Beads, Coulter Corp.), Chicken red blood cells and human peripheral blood. Non-linear events were excluded from flow cytometric DNA analysis by grading on histogram of 90^{o} light scatter versus forward angle light scatter. Data analysis was performed with DNA analysis software program using a linear S-fit method for cell cycle kinetic analysis. A minimum of 5000 cells (range, 5000-25000) cells; mean, 15000 were analyzed for each case **(Baisch et al., 1975)** [27]. Interpretation of DNA histograms was analyzed using cytoloic software (Coulter Corp.).

Statistical Analysis:

The results are expressed as mean ± SD. Statistical analysis was performed according to the method of **Murray (1982)** [28]. Data were analyzed using unpaired student's t-test. P values of <0.05 were considered to be statistically significant

RESULTS

SOD-mimetic activity of Cu complex of 4-azomalononitrile antipyrine was measured as percent of inhibition of reduction of nitro blue tetrazolium (NBT). As shown in

figure (1), the copper complex showed higher SOD-like activity at dose dependent manner and reached to maximum inhibition of NBT reduction (86%) at 200µM concentration of this complex. Fig. 2 shows the effect of different concentrations of $CuL(OH)(ClO_4)$ (25, 50, 100 and 200 µmole) on the viability of EAC cells *in vitro*. It is observed that there was gradual decrease in the viability with increasing the Cu complex concentration in a dose dependent type. Fig.3 shows the effect of daily treatment with $CuL(OH)(ClO_4)$ complex on the survival time and the percentage of survivals were followed for 30 days. An increase in the survival time of $CuL(OH)(ClO_4)$ complex treated mice compared to untreated group. Serum transaminases (ALT and AST) activity and creatinine level in different studied groups are presented in table 1. A significant reduction in the activities of AST and ALT in tumor bearing mice treated with $CuL(OH)(ClO_4)$ complex compared to untreated mice group, whereas serum level of creatinine was significantly elevated after treatment by Cu complex compared to untreated mice group. Superoxide dismutase (SOD) and GPX activities of liver tissues and erythrocytes are shown in tables (1-2). SOD, GPX activities were significantly increased by administering tumor bearing mice group by 10 mg/kg Cu complex. Moreover, the level of GSH of liver tissues and erythrocytes significantly increased at respective doses of 10 mg/kg Cu complex. The effect of intraperitoneal administration of $CuL(OH)(ClO_4)$ complex on hepatic nitric oxide (NO) level is presented in table 2. A significant increase was observed in the level of hepatic nitric oxide in tumor bearing mice group. This level was suppressed in EAC-bearing mice treated by $CuL(OH)(ClO_4)$ complex. A significant decrease in the level of TBARS was obtained in the liver tissues and erythrocytes of tumor bearing mice group after treatment by 10 mg/kg of Cu complex of 4-azomalononitrile antipyrine. Fig.4 shows EAC cells proliferation measured by flow cytometer to analyze the component of the cell cycle. It is evident that EAC cells from Cu complex treated mice exhibited significantly higher G0/G1% and lower S-phase and G2/M% as compared with those obtained from untreated mice. In addition, DNA index which indicates aneuploidy cells is significantly lower in EAC cells aspirated from Cu complex treated mice than cells obtained from untreated mice. G2/G1% is insignificantly different in both groups.

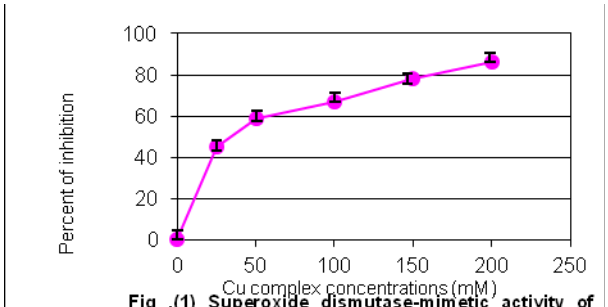

Fig .(1) Superoxide dismutase-mimetic activity of Cu complex of 4-azomalononitrile antipyrine. Values are mean plus standard deviation n=6). P 0.05 compared with the values without complexes.

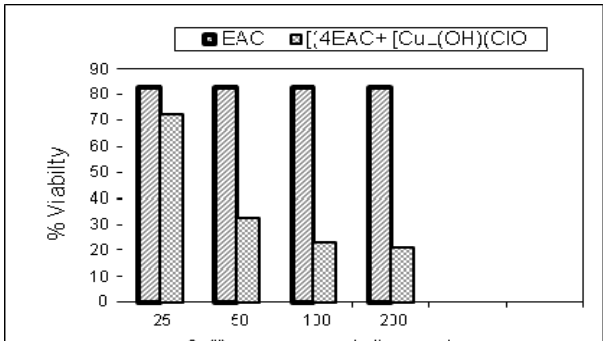

Fig.(2): Effect of different concertrations of $[CuL(OH)(ClO_4)]$ complex on the percentage v ability of EAC cells *in vitro*

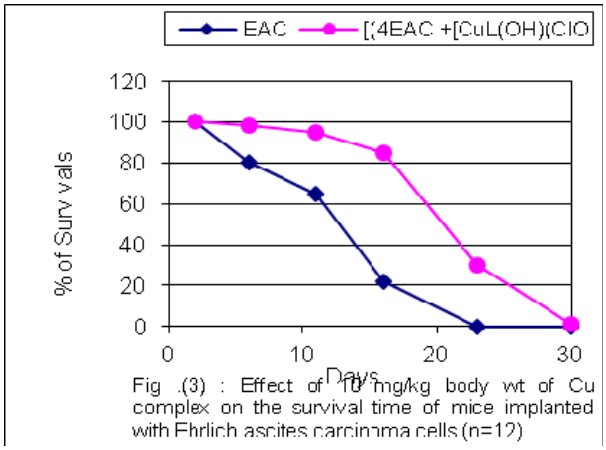

Fig .(3) : Effect of 10 mg/kg body wt of Cu complex on the survival time of mice implanted with Ehrlich ascites carcinoma cells (n=12)

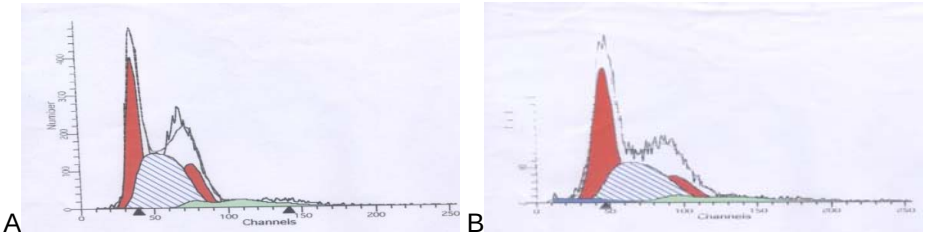

Fig. 4: DNA histogram of EAC cells: A: histogram of EAC cells isolated without treatment, B: histogram of EAC cells isolated from [CuL(OH)(ClO_4)] – treated mice

Table 1: Liver and Kidney Function tests in different groups studied

Parameter	**Control (healthy mice)**	**[CuL(OH)(ClO_4)]-treated mice**	**EAC bearing mice**	**[CuL(OH)(ClO_4)]-treated EAC bearing mice**
ALT (U/L)	21.44 ± 0.62	42.74 ± 0.33*	151.88 ± 0.1	107.30 ± 0.22+
AST (U/L)	30.00 ± 0.16	23.40 ± 0.78*	192.84 ± 0.2	119.48 ± 0.40+
Creatinine (mg/dl)	0.26 ± 0.04	0.24 ± 0.03*	0.22 ± 0.01	0.23 ± 0.02+
Hb, g./dl	14.51 ± 0.18	13.43 ± 0.19*	10.24 ± 0.21	11.453 ± 0.27+

Results are expressed as mean ± SD of mice (n= 10). *Significant (p> 0.05) compared to normal control group. + Significant (p> 0.05) compared to tumor- bearing group

Table 2: Mean and standard deviation of SOD and GPX activities as well as NO, MDA and GSH contents in liver of different groups studied.

Parameter	**Control (healthy mice)**	**[CuL(OH)(ClO_4)]-treated mice**	**EAC bearing mice**	**[CuL(OH)(ClO_4)]-treated EAC bearing mice**
SOD(U/mg protein)	474.35 ±	480.55 ± 0.80*	292.94 ± 0.42	539.06 ± 0.76+
GPX(U/mg protein)	43.82 ± 0.52	51.00 ± 8.09*	36.17 ± 2.01	63.83 ±1.92+
GSH(µmol/mg	10.62 ± 0.23	13.00 ± 0.27*	7.31 ± 0.81	15.04 ± 0.30+
NO (µmole)	10.59± 0.29	10.88 ± 0.41*	12.59 ± 0.29	10.92 ± 0.41+
TBARS (µmol/mg	6.44 ± 0.21	6.53 ± 0.25*	11.31 ± 0.85	8.44 ± 0.32+

Results are expressed as mean ± SD of mice (n= 10). * Significant (p>0.05) compared to normal control group. + Significant (p>0.05) compared to tumor- bearing group

Table 3: Mean and standard deviation of SOD and GPX activities as well as MDA and GSH contents in erythrocytes of different groups studied.

Parameter	Control (healthy mice)	[CuL(OH)(ClO_4)]-treated mice	EAC bearing mice	[CuL(OH)(ClO_4)] -treated EAC bearing mice
SOD(U/g.Hb)	408.80 ± 2.00	435.27 ± 2.15*	361.76 ± 1.34	530.97 ±0.98+
GPX(U/g.Hb)	35.51± 0.65	48.44 ± 0.98*	28.00 ± 1.12	54.88 ± 0.87+
GSH(µmol/g. Hb)	6.01± 0.28	7.20 ± 0.81*	3.11 ± 0.17	7.14± 0.18+
TBARS (µmol/g.	4.25 ± 0.27	5.53± 0.32*	10.69 ± 0.33	7.44 ± 0.32+

Results are expressed as mean ± SD of mice (n= 10). * Significant (p< 0.05) compared to normal control group. + Significant (p>0.05) compared to tumor- bearing group

DISCUSSION

Cancer is a pathological state involving uncontrolled proliferation of tumor cells **(Psomas et al., 2006)** [29]. The present study demonstrated that Cu complex markedly reduced the growth of Ehrlich ascites carcinoma cells. This is evident from decreased cell viability and increased survival time of mice treated daily with CuL(OH)(ClO_4) complex. The present investigation revealed that ALT and AST activities exhibited a significant decrease in EAC group treated with Cu complex compared to untreated EAC mice group. Creatinine level exhibited a significant increase in EAC group treated with Cu complex compared to untreated EAC mice group. These results are in agreement with those of **Khanam et al. (1997)**[30]. CuL(OH)(ClO_4) treatment of EAC tumor bearing mice also restored ALT and AST levels to almost normal values and thereby rescued them from liver failure.

The concurrent study revealed that TBARS levels in both liver homogenate and erythrocyte lysate and hepatic NO exhibited significant decrease in EAC group treated with Cu complex compared to EAC group without treatment. These results are in agreement with those of **kern and kehrer (2005)**[31]. Interestingly, the present work showed that the activity of SOD, GPX and as well as the level of GSH in both liver homogenates and erythrocytes were decreased in EAC group treated with Cu complex compared to EAC group without treatment and this finding is comparable to that of **Qiao et al. (2011)**[32]. So, it could be suggested that the studied Cu complex modulates SOD and GPX to reduce oxidative stress in liver and erythrocytes. Therefore, the present finding, in part, is consistent with the suggestion that Cu complex might induce apoptosis as evidenced by decreased EAC cells viability and increased survival time of mice might suggest the delaying impact of Cu complex on cell division **(Tripathi, et al. 2007)** [33].

The present flow cytometric study supported these findings and showed that Cu complex not only retarded EAC cells growth by delaying their progression from G0/G1 to the DNA synthetic phase of the cell cycle but also significantly slowed DNA synthesis during the cell cycle in Cu complex treated mice as compared with that in untreated mice. These results are in accordance with those of other investigators **(Elo, 2004)**[34]. Furthermore, the DNA index, which is the ratio of abnormal to normal cellular DNA, indicated a decrease in the aneuploidy status of EAC cells obtained from mice treated with Cu complex (Fig.4). Considering these cell cycle results with reduced viability and increased SOD, GPX and GSH levels, it appears that Cu complex might produce apoptotic cells which might be phagocytosed either by macrophages or by adjacent viable cells (**Osinsky et al., 2004)**[35]. It could also, be concluded that in addition to the direct effect of Cu complex on specific receptors in tumor cells **(Iakovidis et al., 2011)**[36]**,** Cu complex $[CuL(OH)(ClO_4)]$ can induce, in apart, apoptosis of EAC cells and phase delay of the cell cycle.

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تأثير متراكب النحاس مع ٤-ازومالونونيتريل انتيبيرين الذى له نشاط مماثل لإنزيم السوبر أكسيد ديسميوتيز على خلايا ايرلش السرطانية فى الفئران

أم على يوسف الخواجة [١] – إبراهيم حلمي السيد [٢]

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يستخدم عدد من متراكبات النحاس ذات التأثير السام للخلايا من خلال الموت المبرمج وتثبيط الإنزيمات كمضادات لكل من البكتيريا والفطريات والفيروسات والأورام. لذلك فان الدراسة الحالية تهدف إلى دراسة تأثير متراكب النحاس مع ٤- أزومالونونيتريل انتيبيرين على خلايا ايرلش السرطانية بعد أن ثبت علميا أن له نشاط مشابه لنشاط إنزيم السوبر أكسيد ديسميوتيز الذي يتناقص في الخلايا السرطانية .

استخدمت فى الدراسة الحالية الفئران الصغيرة الحاملة لأورام ايرلش الاستسقائية كنموذج تجريبي لدراسة تأثير هذا المتراكب على بعض التغيرات الكيميائية فى الفئران. وقد أظهرت النتائج انخفاضا ملحوظا فى نمو وانقسام الخلايا السرطانية كذلك وجد انخفاض ملحوظ في نشاط إنزيمي الناقل لمجموعة الامين من حمض الالانين ومن حمض الاسبارتيك ، بينما لوحظت زيادة ذات دلالة احصائية فى نشاط كل من SOD, GRX و مستوى الجلوتاثيون المختزل فى أكباد الفئران الحاملة للورم والمعالجة بمتراكب النحاس . كما وجد أيضا ارتفاع معنوي فى مستوى كل من الكرياتينين وأكسيد النيتريك والمواد المتفاعله مع حمض الثيوباربتيورك فى مجموعات الفئران الحاملة للورم مقارنه بالمجموعة الضابطة. وعلى الجانب الأخر وجد أن إعطاء الفئران متراكب النحاس لمدة أسابيع ثلاث بعد زرع الخلايا السرطانية فى الفئران أدى الى ارتفاع ملحوظ فى نشاط الإنزيمات المضادة للأكسدة ومستوى الجلوتاثيون المختزل ووجد نقص ملحوظ فى مستوى أكسيد النيتريك وحمض الثيوباربتيورك مقارنة بالفئران الحاملة للورم. كما أوضحت الدراسة أن متراكب النحاس موضع الدراسة عمل على تثبيط نمو الخلايا السرطانية فى مراحل محدودة وكذلك إيقاف التكوين الخلوي للحامض النووي DNA للخلايا السرطانية مما أدى إلى حدوث موت مبرمج للخلايا السرطانية. ومن النتائج التي توصلنا إليها نستنتج أن متركب النحاس له تأثير مثبط لنمو الخلايا السرطانية عن طريق تثبيط التكسير التأكسدى وتحفيز الموت المبرمج للخلايا السرطانية.