BIOCHEMICAL EVALUATION OF CHEMOPREVENTION EFFICACY OF N-ACETYL CYSTEINE, ZINC OXIDE AND HIGH PH ASCORBATE ON MAMMARY GLAND CARCINOMA USING NANOTECHNOLOGY.

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A B S T R A C T

The present study was designed to evaluate the prevention efficacy of the novel prepared compound, high pH sodium ascorbate combined with N-acetyl cysteine / Zinc oxide nanocomposite in *vivo* on the incidence of 7, 12- dimethylbenz[a]anthracene-induced mammary tumor in female rats. The prevention efficacy of such treatment was evaluated by measuring the activity of caspase-3and caspase-9 in mammary gland homogenate in addition to the activities of the most important free radical scavengers of the antioxidant defense system in breast tissue including; reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), as well as malondialdehyde content (MAD) which is considered an indicator of lipid peroxidation. The result of the present study revealed that our novel tested compound, regarding their *in vivo* anti-tumor effect, so that combination between them as it could ameliorate or normalize most of the investigated parameters. In conclusion, these findings suggest that novel synthetic Nano composite and high pH ascorbate may potentially presents new hope for the development of breast cancer prevention, which should attract further scientific and pharmaceutical interest.

Key words: Nanotechnology; 7, 12- dimethylbenz[a]anthracene; N-acetyl cysteine; Zinc oxide

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1. INTRODUCTION

The susceptibility of the mammary gland to tumorigenesis is influenced by its normal development, particularly during stages of puberty and pregnancy that are characterized by marked alterations in breast cell proliferation and differentiation. Numerous epidemiologic studies have suggested that specific details in the development of the mammary gland play a critical role in breast cancer risk (Kelsy & Gammon 1990). Breast cancer is the second most common cancer world-wide, the fifth most common cause of cancer death, the global burden of breast cancer exceeds all other cancer types. (Jitender Monga *et al*., 2013). Polycyclic aromatic hydrocarbons (PAHs) including 7, 12-dimethylbenz[a]anthracene (DMBA) are environmental pollutants, which undergo metabolic activation to exert their carcinogenic effects (Szaefer H *et al*., 2008). Cancer prevention has become an important approach to control cancer; through avoiding exposure to known cancer-causing agents, enhancement of host defense mechanisms against cancer, lifestyle modifications, and chemoprevention. Cancer chemoprevention uses agents that slow the progression, reverse, or inhibit carcinogenesis in healthy subjects, there by lowering the risk of developing invasive or clinically significant disease (Sporn, M *et al*., 2005). Nanotechnology represents a

new and enabling platform that promises to provide a broad range of novel uses and improved technologies for biological and biomedical applications. One of the reasons behind the intense interest is that nanotechnology permits the controlled synthesis of materials where at least one dimension of the structure is less than 100 nm. This ultra-small size is comparable to naturally occurring proteins and biomolecules in the cell (McNeil SE., 2005). Recent studies have shown that ZnO nanoparticles exhibit a high degree of cancer cell selectivity with the ability surpass the therapeutic indices of some commonly used chemotherapeutic agents in similar *ex vivo* studies (Wang H. *et al*., 2009). *N*-acetyl-L-cysteine (NAC) is a synthetic cysteine derivative that has potential as a chemoprotective prophylaxis against toxic chemical exposures (Bobb, Arfsten, and Jederberg, 2004). Cyclodextrin commonly is used for improving the stability and solubility of molecules in drug delivery systems. Cyclodextrins are ordinary, broadly studied and cheaply accessible supramolecular hosts (Atwood *et al*., 1996) with a multiplicity of applications in the food, cosmetics and pharmaceutical industries (Del Valle, 2004). The extracellular pH (pHe) of malignant solid tumors is acidic, in the range of 6.5 to 6.9, whereas the pHe of normal tissues is significantly more alkaline, 7.2 to 7.5 (Griffiths JR., 1991). Acid pH was inhibited using oral NaHCO3, which has previously been shown to effectively reverse pH gradients in tumors and not affect the pHe of normal tissues (Raghunand N *et al.,* 1999). ascorbate has been examined in various epidemiologic studies as a potential chemopreventive agent for cancer (Lee *et al.*,2003). Accordingly, the purpose of the present study was to evaluating the role of N-Acetyl cysteine /zinc oxide and high pH sodium ascorbate prepare in nanoscale and administration as chemopreventive substances during breast carcinoma tumor.

2. MATERIALS AND METHOD

2.1. Experimental animals:

Sixty white adult female albino rats of weighting 100-120 gm were used in the experimental investigation of this study. The rats were obtained from the Laboratory Animals Research Center, Faculty of Veterinary Medicine, Benha University. Rats were housed in separated wire mesh cages and kept at constant environmental and nutritional conditions throughout the period of experiment. The animals were fed on constant ration, Fresh and clean drinking water was supplied ad- libitum. The animals were left 14 days for acclimatization before the beginning of the experiment.

2.2. Chemicals

- DMBA (7,12-dimethylbenz[a]anthracene) purchased from (Sigma, USA)
- N-acetyl cysteine purchased from (Sigma, USA).
- Zinc oxide nanoparticals purchased from (Sigma, USA).
- Vitamin C as Sodium ascorbate purchased from (Sigma, USA).
- Sodium bicarbonate purchased from El Nasr Pharm. Chem. Co. 'ADWIC'
- β-Cyclodextrin purchased from (Sigma, USA).

2.3. Preparations of N-acetyl cysteine, Zinc oxide, Sodium ascorbate and Sodium bicarbonate Loaded Cyclodextrin Complex Nanoparticles:

Formulation of drug N-acetyl cysteine (A), Zinc oxide (B), β-Cyclodextrin (C) , Sodium ascorbate (D) and Sodium bicarbonate (E) were mixed together as follows:

(A) = 2.25g & (B) = 2.25g & (C) = 3.5g & (D) = 1g & (E) = 1g.

The formula drug was mixed mechanically very well with the cyclodextrin oligosaccharide using vortex then both are grind to the nano sized

particles using ball mill (nano system unit) at 200 rpm for 10 hours.

2. 4. Experimental design:

The present studied was carried out on 60 Adult Albino female rats weighing (100–120gm) will be divided into 4 groups of 15 rats each:

Group I: Animals of this group are healthy (control group) received single oral dose of sesame oil at the beginning of the experiment.

Group II: Rats in this group received the vehicle oil at the beginning of the experiment then orally administrated with Nano composite (50 mg/kg b.w.) Dissolved in saline three times per week until the end of the experiment.

Group III: Animals received oral dose of DMBA diluted in Sesame oil (1 mL) injected orally by stomach tube 100 mg/kg body weight with two doses (one from the beginning of the experiment, second after 14th week from the beginning of the experiment) then animals left until mammary gland carcinoma tumor formed.

Group IV: Animals was treated with Nano composite, as in group II, during exposure to DMBA, as in the group III. Until the end of experiment.

2. 5. Sampling:

At the end of the treatment period (18 th ^{weeks}), animals were fasted overnight prior to sacrificing, and tissue specimens (mammary tissue) were collected.

*2. 5. 1. Tissue samples (*mammary *tissue):*

At the end of the experiment, rats of each group were sacrificed by cervical decapitation. The abdomen was opened and the mammary specimen was quickly removed and opened gently using a scrapper, cleaned by rinsing with ice-cold isotonic saline to remove any blood cells, then blotted between 2 filter papers and quickly stored in a deep freezer at (-20 °C) for subsequent biochemical analysis. Briefly, breast tissues were divided into appropriate portions, homogenized with a glass homogenizer in 9 volume of ice-cold 0.05 mM potassium phosphate buffer (pH7.4) to make 10% homogenates. The homogenates were centrifuged at 6000 r.p.m for 15 minutes at 4°C then the resultant supernatant were used for the determination of the following parameters: GPx, CAT, SOD, GSH, MDA and caspase -3, caspase -9.

2. 6. Biochemical analysis:

2.6.1. Antioxidant activity &Lipid peroxide determination: mammary tissue GPx, SOD, CAT, GSH, MDA activity were analyzed according to the methods described by (Gross et al., 1967; Necheles et al., 1968), (Minami and Yoshikawa, 1979), (Sinha, 1972), (Beutler *et al*., 1963) and (Yoshioka *et al*., 1979) respectively.

2.6.2. Molecular biology parameters: Analysis of caspase-3& caspase-9 activity using flowcytometery detection kit. (Tribukait, 1984)

2. 7. Statistical analysis:

Statistical analysis and correlations were performed using SPSS program version 16 (*Renno, et al.,* 2008). Data are presented as Mean ± standard error mean (SEM). Student "t" test and analysis of variance (ANOVA) followed by Bonferroni's post hoc analysis were used for comparisons between groups. The level of statistical significance was set at probability $P < 0.05$

3. RESULTS

3.1. Effect of DMBA- treatment on some tissue parameters mammary gland carcinoma cancer in female rats:

The obtained results in table (1) revealed that significant deleterious changes in antioxidant status. The results revealed a significant increase in MDA level, while GSH concentration

Table 1: Effect of DMBA and Nano composite and both on some mammary gland carcinoma tissue parameters of studied female rats.

Parameters \ Experiment groups	Control	Nano	DMBA	DMBA + Nano
CAT(mmol/g. tissue)	0.23 ± 0.01	0.24 ± 0.02	0.19 ± 0.01 [a,b]	0.24 ± 0.04 [c]
GSH (ng/g. tissue)	34.17 ± 0.25	35.77 ± 0.34 [a]	28.34 ± 0.72 [a,b]	35.32 ± 0.38 [a,c]
SOD (U/g. tissue)	5.28 ± 0.25	6.28 ± 0.41 [a]	2.92 ± 0.13 [a,b]	5.35 ± 0.25 [c]
GPx (ng/ g. tissue)	0.25 ± 0.01	0.26 ± 0.02 [a]	0.19 ± 0.03 [a,b]	0.26 ± 0.01 [c]
MAD(mmol/g. tissue)	25.31 ± 0.18	22.24 ± 0.34 [a]	37.95 ± 0.22 [a,b]	23.31 ± 0.29 [a,b,c]
Caspase3(Unit/g. tissue)	22.38 ± 0.31	37.83 ± 0.49 [a]	13.21 ± 0.45 [a,b]	26.17 ± 0.63 [a,b,c]
Caspase9(Unit/g. tissue)	23.97 ± 0.43	43.40 ± 2.96 [a]	11.86 ± 0.18 [a,b]	31.36 ± 0.92 [a,b,c]

Data are presented as (Mean ± S.E). S.E = Standard error.
Mean values with different superscript letters in the same row are significantly different at *(P<0.05).*

, GPx, CAT activity and SOD activity in rats mammary tissue showed a marked significant depletion, while decrease in the tissue levels of caspase-3and caspase- 9 activity as compared to the control group.

3.2. Effect of N-acetyl cysteine /Zinc oxide Nanocomposite and high pH ascorbate treatment on some tissue parameters mammary gland carcinoma in female rats:

The obtained results in table (1) revealed a significant increase in activities of SOD, GSH, GPx, and CAT and decrease in MDA level and increased in caspase-3 and caspase-9 Compared to control group.

3.3. Effect of N-acetyl cysteine /Zinc oxide Nanocomposite and high pH ascorbate treatment on some tissue parameters of DMBA- induction mammary gland carcinoma in female rats:

The obtained results in table (1) showed highly significant increase in activities of SOD, GSH, GPx and CAT compared to DMBA induction group , while significant decrease in MDA level was observed when compared to the control group, and significant increase compared to Nano composite supplement group and highly significant decrease compared to DMBA induction group at $P < 0.05$, and showed slightly increase in Caspase -3 &Caspase -9 compared to control group and very highly significant decrease compared to Nano supplement group and very highly significant increase compared to DMBA induction group at $P < 0.05$.

4. DISCUSSION

Antioxidants are the first source of protection of the body against free radicals and other oxidants, being the compounds that halt the attack and the formation of radical species within cells. The group of antioxidants inside the organism is known as the total antioxidant state (TAS) (Teixeira *et al*., 2013). The antioxidant

protection of human cells includes enzyme mediated and non-enzymatic defense mechanisms. Superoxide dismutase (SOD), catalase (CAT) and glutathione-peroxidase (GSH-px) are the most important antioxidant enzymes. SOD catalyses' the reaction of superoxide anion to hydrogen peroxide (H_2O_2); in turn, CAT converts H_2O_2 into water and oxygen. The affinity of CAT for H_2O_2 is relatively low, therefore, some H_2O_2 remains in the cell. GSH-px is capable of detoxifying the remaining H_2O_2 (Arrigoni O & De Tullio MC, 2002). The role of free radicals, oxidative stress, and lipid peroxidation in carcinogenesis and their contribution to the initiation and progression of the process are well documented (Himmetoglu *et al*., 2009). In recent years, using MDA as a marker of oxidative stress, there has been a growing interest in studying the role played by lipid peroxidation in cancer progression. MDA is low-molecular weight aldehyde that can be produced from free radical attack on polyunsaturated fatty acids. Increased plasma MDA levels have been reported in breast cancer (Kumaraguruparan *et al*., 2002). Our results showed increase in MDA level in mammary gland carcinoma as compared to controls thus agreeing with the previous studies that suggested increased lipid peroxidation in breast cancer patients. The present study reveals that the activity of SOD is depleted in the cancer-bearing animals, which may be due to altered antioxidant status caused by carcinogenesis. The presented data showed significant decrease in CAT activity This may be due to the utilization of antioxidant enzymes in the removal of H_2O_2 by DMBA. GPx is an important defense enzyme which catalyses' the oxidation of GSH to GSSG at the expense of H_2O_2, Decreased GPx activity was also observed in cancerous conditions (Bhuvaneshwari *et al.*,2001). The decrease in the tissue levels of caspase-3 activity and percentage of DNA fragmentation in the DMBA group as compared to the control group is in line with previous reports (Huigsloot *et al*., 2001) who reported that this reduction may be due to over expression of caspase-3 inhibitors and survivin in tumour cells (Krajewski *et al*., 1999). The obtained results in Table(1) showed significant increase in activities of SOD, GSH, GPx, and CAT and decrease in MDA level and increased in caspase-3 and caspase-9 Compared to control group in treatment of N-acetyl cysteine /Zinc oxide Nanocomposite and high pH ascorbate. Nano ZnO is able to protect cell membrane integrity against oxidative stress damage, increase antioxidant enzyme levels and decrease MDA level (Dawei *et al.,* 2009). NAC administration significantly raised the levels of GSH in kidney, which can effectively scavenge the ROS generated by cadmium. As a result, significant increase in activities of SOD, GPx, and CAT and decrease in MDA level showed the protective efficacy of NAC on Cd-induced oxidative damage (Wang L *et al*., 2009). The obtained results in Table(1) showed a significant showed that increased in caspase-3 and caspase-9 Compared to control group, our result in agreement with Mohd Javed Akhtar & Maqusood Ahamed et al., (2012**)** reported that the higher activity of caspase-3 enzyme along with the DNA fragmentation in liver cancer cells treated with ZnO NPs. Consequently, ZnO NPs were shown to selectively induce apoptosis in cancer cells, ZnO NPs show much promise as new anticancer agents, given the specific apoptotic response of cancer cells. supplementation of N-acetyl cysteine /Zinc oxide Nanocomposite and high pH ascorbate to carcinogenic rats show that highly significant increase in activities of SOD, GSH, GPx and CAT compared to DMBA induction group while showed slightly significant decrease in MDA level compared to the control group, and significant increase compared to Nano composite supplement group and highly significant decrease compared to DMBA induction group the at $P < 0.05$, and showed slightly increase in Caspase -3 &Caspase -9 compared to control group

and very highly significant decrease compared to Nano supplement group and very highly significant increase compared to DMBA induction group as in table (1). Zinc is one of the structural component of wide variety proteins and dependent enzymes like superoxide dismutase (SOD) that act as essential component of antioxidant defense system (Bao and choct, 2009). Ascorbic acid is a good scavenger of free radicals and it protect cellular membranes their by preventing degenerative disease like cancer (Gutteridge *et al*., 2000 &Vijayavel *et al*., 2006). Caspase-3 was activated on sodium ascorbate treatment, sodium ascorbate induced apoptosis via the mitochondria-dependent pathway in melanoma cells (Shuw-Yuan Lin *et al*., 2006).

CONCLUSION & RECOMMENDATIONS

In conclusion, the present study demonstrated that N-acetyl cysteine /Zinc oxide Nanocomposite and high pH ascorbate administration attenuates carcinogenic effects of DMBA on rat mammary glands and reverses DMBA-induced suppression of caspases -3 and -9 activity. Collectively, these observations suggested that novel synthetic Nano composite may potentially presents new hope for the development of breast cancer prevention.

We recommended that, administration of diet rich in the antioxidant and high pH is very important for protection of different body tissue against oxidative stress or even cancer.

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التقييم الكيميائي الحيوي لفعالية الأسيتايل سيستين واكسيد الزنك والأسكوربات عالى القلوية فى المنع الكيميائى لسرطان الغدداللبنية باستخدام النانوتكنولوجى

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1- قسم الكيمياء الحيوية- كلية الطب البيطري بمشتهر- جامعة بنها. 2 - قسم البتروكيماويات – معهد بحوث البترول- القاهرة.

الملخص العربي

في هذه الدراسة تم تقييم التأثير الوقائي للأسيتيل سستين واكسيد الزنك والأسكوربات عالى القلوية على التغيرات في مستوى الأكسدة الفوقية للدهون، مضادات الأكسدة الأنزيمية والغير انزيمية، ونشاط الكاسباس 3 جين، نشاط الكاسباس 9 جين في أنسجة الغدد اللبنية فى الفئران المستحدث فيها سرطان الغدد اللبنية بواسطة مادة الثنائى ميثيل بنزانثراثين. هذا وقد أستخدم لأجراء هذه الدراسة عدد 60 من اناث الفئران البيضاء أوزانها من100 -120جرام وقد قسمت إلى أربع مجموعات متساوية اشتملت كل مجموعة على عدد خمسة عشر فئران وتم توزيعها كالآتي: المجموعة الأولى: (المجموعة الضابطة): لم تعطى أي أدوية واستخدمت كمجموعة ضابطة للمجموعات الأخرى. المجموعة الثانية: (المجموعة التى تعطى عقار النانو التركيبى الجديد): تم اعطاء المركب النانو التركيبى عن طريق الفم بجرعة 50 مللى جرام / كيلوجرام للفئران 3 مرات فى الاسبوع. المجموعة الثالثة:(المجموعة المحدث بها سرطان الغدد اللبنية): تم تجريع الفئران بالثنائي ميثيل بنزانثراثين) دمبا) عن طريق الفم بجرعة مقدراها (100 مللى جرام/ كيلوجرام) وتتكررت نفس الجرعة بعد 14 اسبوع. المجموعة الرابعة:(المجموعة الوقائية): تم حقن الفئران بالثنائي ميثيل بنزانثراثين) دمبا) بجرعة (100 مللى جرام/ كيلوجرام) ثم تم تجريع الفئران بعقار النانو التركيبى 3 مرات فى الاسبوع عن طريق الفم بجرعة مقدراها (50 مللى جرام/ كيلوجرام). وقد تم تجميع عينات الانسجه فى الأسبوع الثامن عشر من بدايه التجربه. وقد أسفرت نتائج التحليل البيوكيميائى عن وجود انخفاض معنوى فى كلا من نشاط الجلوتاثايون بيروكسيديز، سوبر أكسيد ديسميوتيز، الكتاليز، الجلوتاثايون ريدكتاز، ونشاط الكاسباس 3 جين، نشاط الكاسباس 9 جين في نسيج الغدد اللبنية، من جهة اخرى اظهرت النتائج زيادة في ال -مالونديالدهيد فى المجموعه المحدث بها سرطان الغدد اللبنية. كما أن نتائج مجاميع الفئران والتي تم وقايتها وعلاجها بمركب النانو التركيبى الجديد (أسيتيل سيستين + اكسيد الزنك + الأسكوربات عالى القلوية) أظهرت زيادة في كلا من نشاط الجلوتاثايون بيروكسيديز، سوبر أكسيد ديسميوتيز، الكتاليز، في نسيج الغدد اللبنية، من جهة اخرى اظهرت النتائج البيوكيميائية الحيوية لمركب النانو التركيبى الجديد زيادة في نشاط الكاسباس 3 جين، نشاط الكاسباس 9 جين، بينما اظهرت انخفاض معنوى فى ال – مالونديالدهيد. وأوضحت الدراسة أن استخدام مركب النانو التركيبى الجديد (أسيتيل سيستين + اكسيد الزنك + الأسكوربات عالى القلوية) كماده واقية مضادة للأكسدة ومضادة للسرطان كان لها دور فعال في حماية انسجة الغدد اللبنية من التنكرز المحدث تجريبيا فى اناث الفئران باستخدام مادة الثنائي مثيل بنزانثراثين وأدى استخدامه كذلك الى الحفاظ على نسب القياسات البيوكيميائية فى الأنسجة لما يقارب النسب الطبيعية. لذلك توصى الدراسة بضرورة استغلال تلك المزايا الهائلة مركب النانو التركيبى كماده وقائية وعلاجية ومضادة للأكسدة وإدخاله كماده فعالة فى صناعة العقاقير الطبية المستخدمة فى الوقاية من سرطان الثدى.

(مجلة بنها للعلوم الطبية البيطرية: عدد 27(1):92- 99, سبتمبر 2014)