Antitumor Activity of Selenium in Ehrlich Ascites Carcinoma Bearing Mice

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The most common disease states of chronic liver illnesses include alcoholic liver disease (ALD), and viral hepatitis can progress to hepatocellular carcinoma (HCC). However, the role of selenium-associated tumor management angiogenesis in liver fibrosis and inflammation is yet unknown. As a result, in this current study, cytotoxicity of selenium (Se) was evaluated against hepatocellular carcinoma cells (HepG2) to determine IC50 (in vitro study) and we established a mouse model of Ehrlich Ascites Carcinoma (EAC) to explore the role of selenium in the processing of tumor angiogenesis in liver injury and inflammation (in vivo study). EAC cells was used to induce ascites tumor in albino mice and studied their consequence role on body weight gain and liver e. In EAC tumor-bearing mice, we discovered a substantial increase in body weight. Furthermore, mice with EAC tumors had higher levels of liver enzymes implicated in the etiology of liver inflammation, as well as biomarkers such as tumor necrosis factor a (TNF-), a fetoprotein (AFP), and caspase-3, Bcl2, and DNA damage.

Keywords: Caspase 3; DNA Damage; In Vitro; Inflammation; IC50.

The liver is the largest and one of the most essential organs in our bodies, serving as the primary regulatory organ for several physiological and biochemical processes ; angiogenesis is one of the fundamental physiological and pathological processes involved in carcinogenesis, progression, and metastasis, according to a large body of evidence[1]. Several growth hormones and cytokines are important in modulating the physiological process of angiogenesis. [2] In almost all malignancies, tumor angiogenesis, which is the growth of new blood vessels to feed nutrients to tumor cells that are dividing quickly, is a key step in the tumor metastasis route[3].

The damage and fibrosis of the liver caused by tumor angiogenesis is one of the characteristics of cancer that is connected to liver fibrosis and inflammation in chronic liver disease, and that it also promotesthe growth of hepatocellular carcinoma (HCC)[4]. However, the relationship between tumor angiogenesis and hepatitis remains unknown. As a result, a thorough understanding of the relationship between tumor angiogenesis and inflammatory liver illnesses is required for the development of cutting-edge therapeutic options for tumor-associated hepatitis[5]. This lethal disease-related syndrome is caused by a combination of growth factors and cytokines[5]. Hypoxia activates

numerous pro-angiogenic pathways, which promote capillary growth by secreting a variety of angiogenic growth factors, including the well-studied vascular endothelial growth factor (VEGF), placental growth factor (PIGF), fibrosis-associated transforming growth factor-beta (TGF-beta), and inflammation-associated tumor necrosis growth factor (TNF-beta), via the mitochondria[6]. In addition to these angiogenic growth factors, several inflammatory cytokines play an important role in liver inflammation and fibrosis[7]. Addressing tumor angiogenesis will be a promising therapeutic option for treating individuals with tumor-associated liver fibrosis and inflammation[2]. Several studies have revealed that angiogenesis is a key characteristic of several malignancies, including HCC[8]. Under normal physiological conditions, angiogenesis allows immune cells to migrate from one organ to another while delivering food and oxygen. It also stimulates the healing of tissue injury and the repair of damaged tissues during the course of tissue homeostasis[9]. However, the total tumor cell count and tumor volume increased as a result of the EAC cells' rapid multiplication in the peritoneal cavity, generating a hypoxic environment in the adjacent microenvironment. As a result, many angiogenic growth factors and cytokines are produced, including VEGF, PLGF, TNF, and TGF, stimulating endothelial cells[10]. These components have been found to have an important role in the angiogenesis of HCC[11, 12]. It has been proposed that EAC tumor-induced peritoneal angiogenesis and newly created capillaries can transport angiogenic and inflammatory markers to the liver and may promote the activation of stellate cells, Kupffer cells, and mast cells that are linked to the liver, resulting in liver damage. associated inflammation signalling[13]. Previous investigations indicated that angiogenesis pathways are crucial to the development of hepatitis, fibrosis, and HCC[14].

It has been demonstrated that selenium, an essential trace mineral present in both organic and inorganic chemical forms, is vital for sustaining mammalian cells in their ideal physiological state. It has demonstrated chemopreventive ability against the emergence of tumors as well as different types of environmental stress[15]. Recent studies suggested that the use of Se in combination with conventional chemotherapy medications and therapeutic hormones can disclose cancer, even though Se is in clinical trials for the chemoprevention of prostate, colon, and lung cancer[16]. Se' beneficial and harmful effects have a very narrow window; therefore its long-term supplementation for preventative and therapeutic reasons is constrained[16].

As a result, the current study assessed the impact of changing angiogenic regulators on the development of tumors and the indicators of inflammation and liver damage. Ehrlich cells were used as a tumor carcinoma model in mice to study the impact of selenium in vivo.

MATERIALS AND METHODS

Materials
• Doxorubicin (DOX) was purchased from the pharmacy.
• culture media was obtained from Invitrogen Life Technologies.
• selenite (Na_2SeO_3) was purchased from Merck Chemical Inc. (Darmstadt, Germany).
• All other using chemicals were HPLC grade and purchased from Sigma-Aldrich, Germany.

In vitro study
Cytotoxic effect on human cell lines
The mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan was used to determine cell viability.

Procedure
All procedures were carried out in a sterile environment with the use of a Laminar flow biosafety cabinet Class II A2.HepG2 cells were suspended in DMEM (Dulbecco's Modified Eagle Medium) with high glucose and stable glutamine, 1% antibiotic, and 5% fetal bovine serum at 37 °C in a CO2 incubator (Sartorius stedium,biotech).

Cells were seeded at a density of 10×10^3 cells/well in fresh complete growth medium in 96-well plastic plates at 37 °C for 24 h under 5% CO2 either alone (negative control) or with different concentrations of drugs and Doxorubicin (DOX) as a positive control group, yielding a final concentration of (50, 25, 12.5, 6.25, 3.125, 1.5625 mg/ml). After 48 hours, the medium was aspirated, and 20 ìl of MTT salt (2.5g/ml) was added to each well before incubating for another four hours at 37oC with 5% CO2. To dissolve the generated crystals and terminate the reaction, 200 ìl of 10% Sodium dodecyl sulphate (SDS) in 0.01 mole HCL

was added to each well, and the plate was then incubated overnight in a dark environment.[17, 18].

The absorbance was then measured using a microplate reader at 595nm and a reference wavelength of 620nm. Viability was calculated as follow:

Viability = absorbance of drug / absorbance of control x 100

Cytotoxicity = 100- viability

IC 50 was calculated from the relation between the different concentration of the drug and cell viability for each drug concentration.

The IC 50 was computed using the relationship between drug concentration and cell viability for each drug concentration[18].

LD50 estimation based on in vitro IC50 value

Using the following formula, the in vivo LD50 of acute oral toxicity was calculated from the in vitro IC50: log LD50 = 0.372 log IC50 (g/mL) + 2.024[19].

In vivo study

Experimental Animals

Adult female Swiss albino mice (25-30g) were procured from the National Research Centre's Animal House in Giza, Egypt. They were maintained in stainless steel cages in a controlled environment (temperature 20 ± 2 ºC) with regular laboratory diet and ad libitum water at the National Research Centre's Animal House in Giza, Egypt. . The National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, updated 1985) was followed for animal procedures, and the experiment followed the recommendations and criteria of the National Research Centre's (NRC) ethical committee.

Transplantation of a tumor

Dr. Gklien kindly provided an EAC cell line, which was maintained in experimental female Swiss albino mice via intraperitoneal injection of 2.5×10^6 cells per mouse. Fluid tumor was detected after 5-7 days of EAC cell inoculation[20, 21].

Experimental design

Thirty-two Swiss albino mice were weighted and their weight volumes were recorded to compare with their weight after the experimental period; they were then randomly assigned to four experimental groups (8 mice per group) as appeared in chart 1 and categorised as follows:

Group 1: Healthy mice.

Group II : EAC cell line was administered intraperitoneally into healthy mice.

Group lll : mice with EAC were given selenium (as Na_2SeO_3 dissolved in water) orally for ten days[22, 23] at a dose one-tenth of the LD50.

Group IV : mice with EAC were given DOX (1/20 of the LD50) .

Following the 10-day study period, rats were fasted overnight and blood was collected from the orbital vein, centrifuged at 4000 rpm for 20 minutes, and the separated plasma was stored at -80 ºC for subsequent examination. The comet assay was performed on blood, and other biochemical parameters such as plasma caspase 3, Alpha fetoprotein (AFP), tumor necrosis factor-alpha (TNF-alpha), and Bcl2 were determined using an ELISA technique with commercial kits from R&D Systems GmbH (Wiesbaden, Germany), according to the manufacturer's instructions . In addition blood transaminases (ALT and AST) were estimated colorimetrically using spectroUV-VIS Double Beam UVD-3500 .EAC fluid volume was sucked and measured its volume by ml using a syringe.

Comet test

The comet test was created to detect cellular DNA damage24. Lymphocytes were separated and washed in pH 7.4 phosphate buffered saline (PBS). Ten microliters of cells were suspended in 75 l of 0.5% low melting agarose to pipette on microscope slides with a layer of 1% agarose, spread with a cover slip, and solidified for 5 minutes on an ice-cold flat tray. After removing the cover slip, the slides were immersed in cold lysis solution for 1 hour, followed by 40 minutes of electrophoresis at 25 V, 300 mA, before being carefully removed from the tank and washed three times with 0.4 M Trizma base at pH 7.5 for 10 minutes. Each slide received 20 microliters of ethidium bromide (10 g/ml). The slides were examined at 40 magnification with a fluorescence microscope (Leica Microsystems, CMS GM b H, Wetzlar, Germany. Model DM 2500) and power Max. 160 W equipped with a 549 nm excitation filter and a 590 nm barrier filter. The "comet appearance" of damaged cells was seen, with a brilliantly fluorescent head and a tail to one side generated by DNA strand breaks that will draw away during electrophoresis. The percentage of damage was calculated by counting the damaged cell out of 100 cells each slide.

Statistical analysis

The statistical package for the social sciences (SPSS) application, version 16, and Microsoft Excel 2007 were used to conduct data analysis. The data were displayed as means standard error (SE). The significance of the difference in results was calculated using one-way ANOVA and the Student's t-test. A statistically significant difference was defined as P 0.05.

RESULTS

The in vitro study indicated that , IC 50 for selenium was 11.585 ìg/ml as appeared in table, and 0.816 ìg/ml for doxorubicin (table1,2&fig1,2).

Table (3): showed significant increase in body weight gain with a percentage increase of 55.96 % in ehrlich bearing mice related to control .While medication of mice with EAC treated with

Se and DOX showed an insignificant change in body weight with percentages reached to -6.12 and- 8.92% respectively from EAC group . The percent of change in the volume of EAC ascites fluid in Se and DOX treated mice were -13.39 and -15.68 respectively (Table 4). Significant elevation in the mortality rate of EAC mice with percentage increase amounted 40% relative to control level . While ,marked reduction in EAC treated mice with Se (30%) was recorded. However ,the mortality rate in DOX –treated EAC mice showed insignificant difference compared to untreated EAC bearing mice (Table 4) . Additionally, Table (6): declared noticeable elevation in ALT, AST in EAC bearing mice relative to control group . Treated EAC mice with Se and DOX revealed an improvement of these values . TNF-á ,caspase 3, AFP and Bcl2 in plasma of EAC bearing mice were significantly increased in EAC compared to
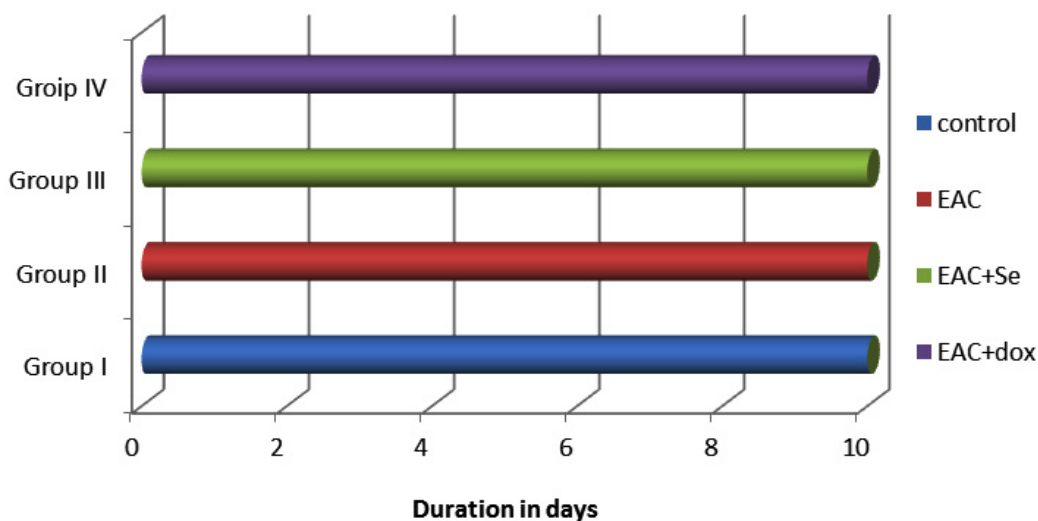

Chart 1. Experimental study appeared groups and duration of the experiment

Table 1. Cell viability and cytotoxicity of selenium' different concentrations against HepG2

Selenium concentration (µg/ml)	Cell viability %	Cytotoxicity %	IC50 (µg/ml)	LD50 (mg/kg b.w.)
50	7	93	11.585	262.887
25	37	63		
12.5	48	52		
6.25	54	46		
3.125	60	40		
1.562	62	38		

control . However, the treatment with either Se or DOX showed marked amelioration in their levels (Table 7). Significant increase in percentage of DNA damage as recorded by the test of comet in EAC bearing mice compared to control mice . Howevere ,treated mice with Se showed marked significant reduction in DNA damage % which is significantly lower compared to the recorded for DOX treated group (Table 8, Fig.3).

DISCUSSION

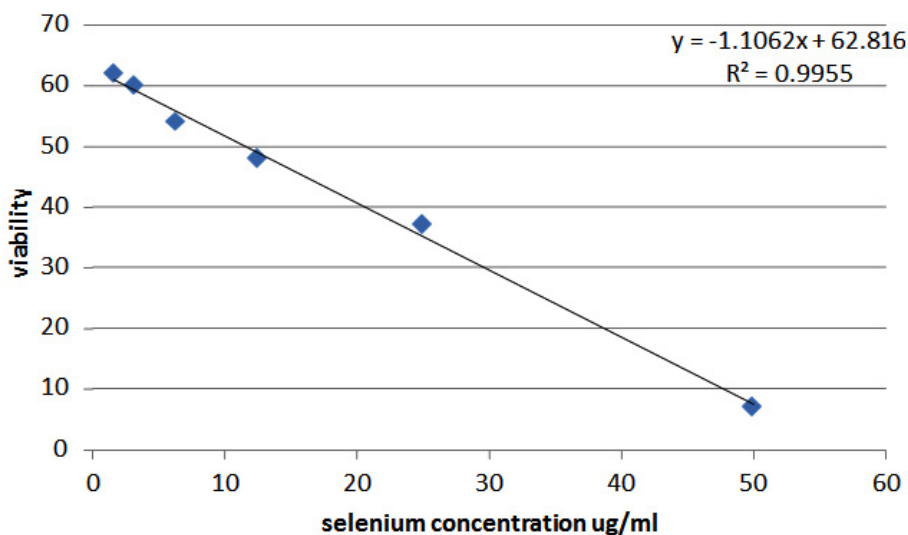We seek to describe the novel molecular mechanism of HCC related with liver disease using

$$y = -1.1062x + 62.816$$
$$R^2 = 0.9955$$

Fig. 1. IC50 for different concentration of selenium against HepG2

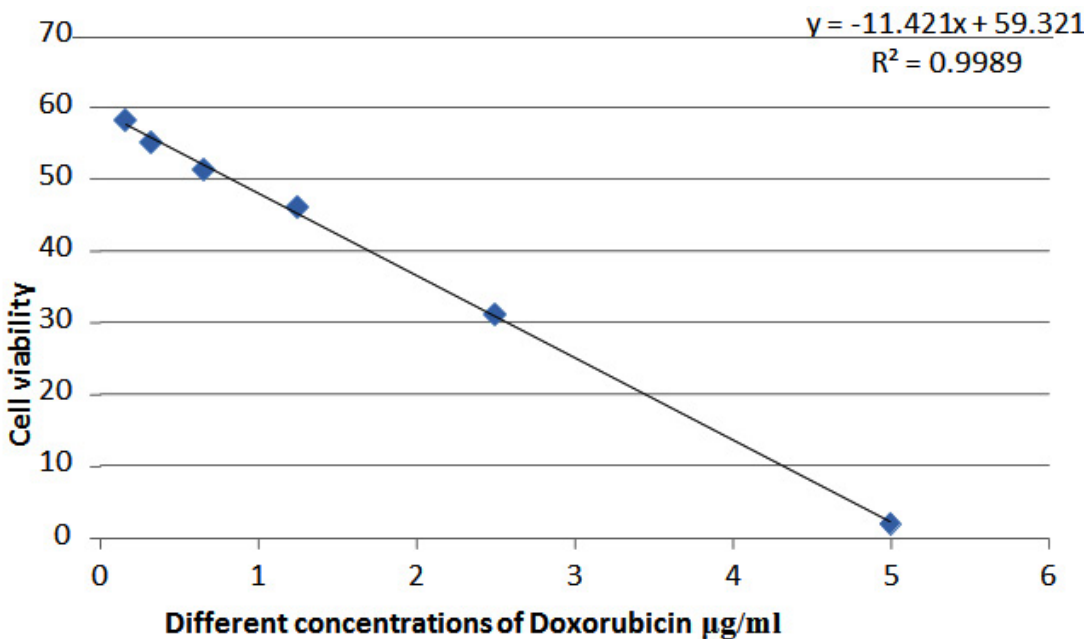$$y = -11.421x + 59.321$$
$$R^2 = 0.9989$$

Fig. 2. IC50 for different concentration of DOX against HepG2

the EAC mouse model and the therapeutic role of selenium based on this experimental observation. As a result, this model can be utilized to investigate new signaling systems, as well as offer additional insight on how cancer causes hepatitis and related liver illness. Few additional research have revealed that EAC tumor metastasizes to multiple organs, which may be related to the activation of angiogenic pathways, which can cause liver inflammation and fibrosis by various angiogenic growth factors as well as various cytokines[2]. Tumor angiogenesis and its detrimental consequences in comorbidities are known to be the primary causes of cancer-related liver dysfunction[25]. Our findings were comparable, and we believe that inflammation-induced hepatitis may be a cause of HCC-related mortality, even after therapeutic recovery[26]. Cancer cells, as previously stated, activate angiogenesis by raising the expression of AFP, Caspase 3, Bcl2, and the

release of angiogenic growth factors such as VEGF, TNFá, and TGF á, as well as different cytokines[27], as evidenced by these findings. Angiogenic factors and previously generated cytokines enter the portal circulatory system and portal vein before reaching the liver, where they bind to particular receptors and activate numerous signal transduction pathways. This pathogenic event then activates hepatic stellate cells, Kupffer cells, and mast cells, which are the mediators of hepatic inflammation, fibrosis, and, in other words, liver damage[14]. Activation of these hepatic stellate cells, on the other hand, produces chemokines that promote angiogenesis, inflammation, and fibrosis. EGF and EGFR implicated in various biological activities; including mitogenic and angiogenic purposes; EGF-mediated signaling pathway was shown to play a key role in motivating proliferation of microvascular endothelial cells and also lymphatic

Table 2. Cell viability and cytotoxicity of doxorubicin' different concentrations against HepG2

Doxorubicin concentration (µg/ml)	Cell viability %	Cytotoxicity %	IC50(µg/ml)	LD50(mg/kg b.w.)
5	2	93	0.816	97.982
2.5	31	63		
1.25	46	52		
0.625	51	46		
0.3125	55	40		
0.156	58	38		

Table 3. The mean value of body weight (g) of mice in different groups

	Control	Ehrlich	Treated- selenium	Treated -DOX
Mean ±SE	26.16±1.10	40.80±3.11[a]	38.30±2.87[a]	37.16±3.18[a]
% change from control group	-	+55.96	+46.41	+42.10
%of change from ehrlichgroup	-	-	-6.12	-8.92

Data are expressed as mean ±SE, where a: indicated significant at $P \leq 0.05$ from the control, and b: indicated significant at $P \leq 0.05$ from ehrlich group

Table 4. The mean Volume (ml) of EAC ascites fluid of mice in different groups

	Control group	Ehrlich	Se-treated mice	DOX -treated mice
Mean ±SD	0.00	15.30 ±0.56 [a]	13.25±0.89[a,b]	12.90±0.65[a,b]
%of change from EAC group	—	—	-13.39	-15.68

Data are expressed as mean ±SE, where a: indicated significant at $P \leq 0.05$ from the control, and b: indicated significant at $P \leq 0.05$ from ehrlich group

endothelial cells . EGF can act through both paracrine and autocrine mechanisms to facilitate the expression of key proteases on endothelial cells to remodel surrounding extracellular matrix permitting endothelial cell migration, regulate the expression of VEGF and other growth factors, and induce angiogenesis via PI3k, MAPK, and eNOS pathways in a VEGF-independent [28]. The increase in TNFá levels in our results may be related to its participation in the neovascularization process. TNFá is a significant inflammatory mediator that causes a variety of alterations in endothelial cells (EC) , including the activation of adhesion molecules, integrines, and matrix metalloproteinases[29]. Different types of cancer have altered levels of pro-inflammatory and pro-angiogenic proteins, and TNF-expression has been associated to tumor differentiation, invasiveness, and angiogenesis[30]. According to the results presented, an increase in TNFá is associated by an increase in tumor volume. The tumor's ability to develop, as well as its invasiveness and metastatic

Table 5. Mortality rate(%)post 10 days of treatment

	Control group	Ehrlich group	Seleniumtreated group	DOX treated group
day 1	10	10	10	10
day 10	9	6	7	6
dead animals	1	4	3	4
% of death	10%	40%	30%	40%

Table 6. Liver functions in different groups

Groups	Control	Ehrlich group	Se treated group	DOX treated group
ALT (U/L)	11.17±1.00	33.50±2.00[a]	17.83±1.21[ab,c]	21.33± 1.10[ab]
AST (U/L)% change	38.17±2.50	73.00±4.22[a]	40.5±3.11[ab,c]	48.50±3.07[ab]

Data are expressed as mean ±SE, where a: indicated significant at P ≤0.05 from the control group;b: indicated significant at P ≤0.05 from the ehrlich group;c: indicated significant at P ≤0.05 from the DOX treated group.

**Table 7.** Serum levels of TNF-α, caspase 3,AFP, and Bcl 2 in different groups

Groups / Markers	Control group	Ehrlich group	Se- treated group	DOX t-reated group
TNF-α (ng/L)	5.30± 0.70	14.72± 1.00 [a]	11.22± 0.50 [a,b]	12.90± 0.77 [a,b]
Caspase 3(ng/ml)	0.49 ± 0.02	1.30 ± 0.04 [a]	0.72± 0.05 [a,b]	0.79 ± 0.05 [a,b]
AFP(IU/ml)	0.41± 0.01	1.50± 0.07 [a]	0.72 ± 0.02 [a,b]	0.81 ± 0.03 [a,b]
Bcl 2 (ng/ml)	4.47 ± 0.04	9.32 ± 0.05 [a]	5.70 ± 0.03 [a,b]	6.30± 0.05 [a,b]

Data are expressed as mean ±SE, where a: indicated significant at P ≤ 0.05 from the control group;b: indicated significant at P ≤ 0.05 from the ehrlich group;c: indicated significant at P ≤ 0.05 from the DOX treated group.

Table 8. Percentage of DNA damage in different studied groups

groups	Control group	Ehrlich group	Se- treated group	DOX- treated group
DNA %	2.20±0.33	32.00±3.60[a]	16.00±1.03[a,b,c]	22.00±2.30[a,b]

Data are expressed as mean ±SE, where a: indicated significant at P ≤ 0.05 from the control group;b: indicated significant at P≤0.05 from the ehrlich group;c: indicated significant at P≤0.05 from the DOX treated group
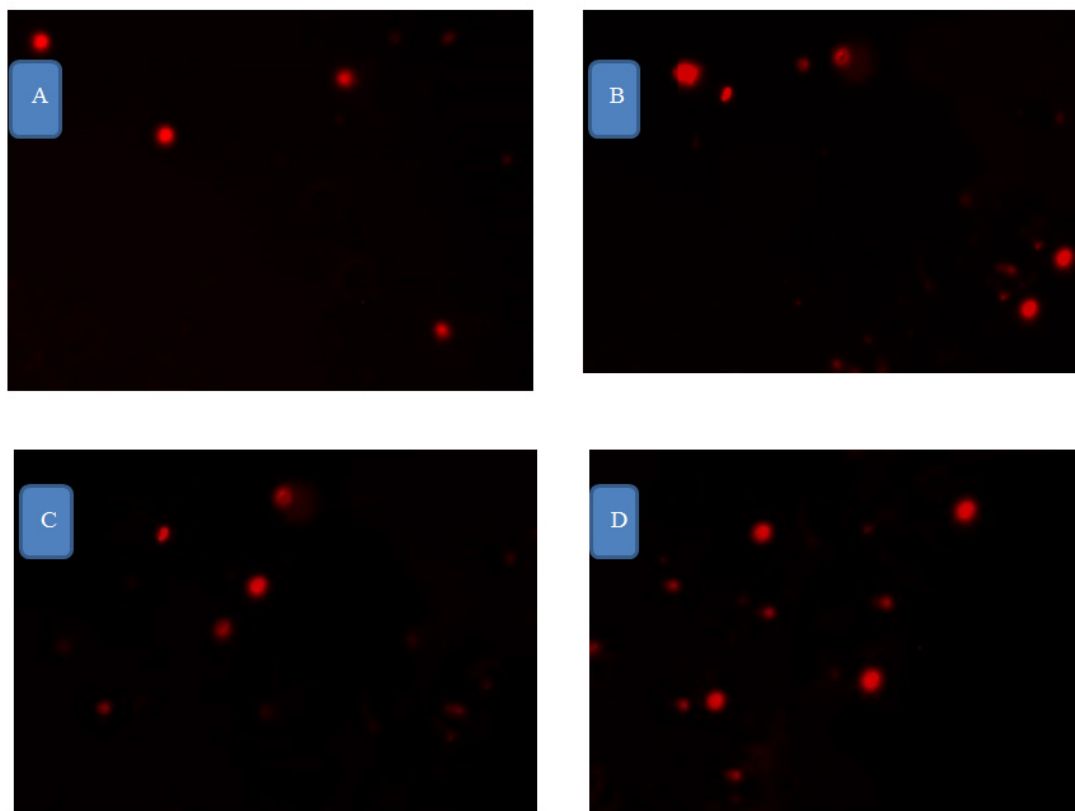
Fig. 3. DNA damage in different studied groups appeared the percent of damage in A) control group, B) EAC group, C) se treated group and D) DOX treated group.

ability, is enhanced by neovasculaturization[15]. Furthermore, a rise in tumor volume in mice with EAC is related with an increase in BcL2, AFP, Caspase 3, and DNA damage (Table 7,8).

As we discovered in our in vivo investigation, body weight increases with cancer growth (as seen by the current findings, which reveal a considerable increase in EAC ascitic fluid volumes due to inflammation (infiltration of immune cells) and concomitant fibrosis (collagen fiber accumulation). These pathological alterations damage hepatocytes' and the liver's overall physiological function. In support of these findings in liver damage, our biochemical assays demonstrated elevated blood levels of AST and ALT in EAC tumor-bearing mice compared to control mice. Another published study31 backed up our hypothesis. The increased level of liver enzyme activity in serum in EAC tumor-bearing mice definitely suggested liver injury. These new findings further suggest that the EAC tumor

promotes peritoneal angiogenesis and is involved in the circulation of proangiogenic substances, which may contribute to the advancement of liver inflammation and fibrosis[32]. The considerable rise in AFP in the current study suggests that AFP reduces tumor immunity and promotes tumor development, decreasing cancer immunity and increasing tumor [33].Based on our findings, there is a considerable rise in TNFá, BcL2, and Caspse

3 in the serum of EAC-bearing mice compared to the control. These biomarkers are considered key regulators of inflammation and fibrosis. These indicators are thought to be important regulators of inflammation and fibrosis. Overall, elevated expression of these markers in EAC mouse serum can cause various clinical outcomes, such as hepatitis-like symptoms in breast cancer patients. This is the most likely source of liver inflammation and fibrosis in mice with EAC tumors [2]. According to the findings, it was discovered that higher tumor volume in

EAC- bearing mice is connected with a significant decrease in antioxidant biomarkers SOD, CAT and GSH[15] . Selenium treatment showed a significant improvement in different biomarkers examined in EAC-bearing mice related to control group. These findings can be explained by the fact that Se can inhibit hepatocarcinoma angiogenesis in rats by down-regulating the expression of TNF- and VEGF34, and it also inhibits TNF- in human umbilical vein endothelial cells [HUVEC], which leads to suppression of MMP-2 and MMP-9 activities15. Sodium selenite and other different forms of selenium might be able to inhibit cancer metastasis and primary tumor growth in several cancers in animals [15]. Many researchers have observed that inhibiting antioxidant mechanisms in rat blood and tissues after irradiation35 and in irradiated carrier mice by EAC[15] is associated with an increase in lipid peroxide products. Numerous investigations, on the other hand, have revealed that tumor growth can generate antioxidant disruptions in some tumor host tissues[15]. In fact, tumor growth may be to blame for the depletion of antioxidants in the liver as well as an increase in the quantity of lipid peroxidation products[36]. Past lipid peroxidation appears to be begun by the extraction of hydrogen from lipid molecules by lipid radiolysis products, resulting in permeability disruptions due to variations in membrane proteins and polysaccharides. It has also been shown that the rate of LPx increase following irradiation is proportional to the radiation dose and time[37].When oxidative damage caused by tumor development is severe, ROS scavenging enzymes (SOD & CAT) and GSH are degraded[15]. In turn, free radicals and oxidative stress enhance the expression of TNF- and AFP, which are involved in the angiogenesis process and contribute to tumor formation. Furthermore, the anticancer efficacy of several Se compounds in the EAC mouse model has previously been described[16]. Se as adjuvant therapy with cyclophosphamide shown significant anticancer and antioxidant benefits in EAC-bearing mice[16]. Thus, Se played an important role in the reduction of Bcl2 in EAC cells, which may lead to the induction of mitochondria-mediated apoptosis by altering mitochondrial permeability and releasing specific apoptotic proteins such as cytochrome c [38]; this, in turn, increases the cascade of caspases in EAC cells, particularly caspase-3, known as the death protein. [39], [40]. These pathways have been approved in tumor cell execution in vitro in human colon cancer cells and human oral squamous cell carcinoma, respectively. Furthermore, our findings support the findings of Jin et al[41], who discovered a strong apoptotic effect on colorectal cancer cells in vitro and in vivo related with Bcl-2/Bax/Caspase-3 signaling. These findings were supported by a recent experiment[42] that validated these apoptotic processes in human prostate cancer cells in vitro. The most recent data also revealed higher DNA damage in EAC mice, as well as the modulatory action of Se. This could be because Se can impact the level of p53 in EAC cells; also, the P53 gene is recognized as the tumor suppressor gene and is in charge of DNA repair or damage in cells[38]. In cancer cells, the p53 gene is mutated, and the p53 proteins are changed into mutant p53 proteins that supply energy and dietary antioxidants to cancer cells, making them more resistant to chemotherapy medicines. and growing their numbers[38]. Finally, the data reported here show the establishment of a novel evidence-based mechanism for analyzing Ehlish-induced tumor angiogenesis in mice. In this mouse EAC model, we also demonstrated how tumor angiogenesis contributes to the activation of liver enzymes, the elevation of TNF-á, AFP, Bcl2, caspase 3, as well as an increase in DNA damage, and ultimately leads to the development of hepatitis due to inflammatory cell infiltration and collagen deposition. As a result, our findings may give more evidence that inhibiting tumor angiogenesis may be a promising therapeutic strategy in the treatment of liver impairment associated with advanced hepatitis. Furthermore, Se was able to lower inflammatory markers as well as Bcl2 activity, which supported an increase in caspases, particularly caspase-3, which caused cancer cells to be executed.

CONCLUSION

From the *in vitro* study (using HepG2 cell line) we can calculate LD50 that was used in determination of using selenium dose. This work appeared that Ehrlich Ascites Carcinoma (EAC) elevated liver functions and the inflammatory markers as appeared by measuring TNF-á, AFP in addition to increasing DNA damage, BcL2 and AFP; whereas treatment with selenium could

attenuate these disturbances . We can consider selenium as a promising agent that can regulat angiogenesis and the development of tumors in liver inflammation; and we recommended more studies on different types of cancer.

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