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## Ultrasonic waves improves the tumoricidal effect of 5-fluorouracil loaded on polymeric nanoparticles in chemically induced hepatocellular carcinoma in mice

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### ABSTRACT

A relatively novel strategy for drug delivery enhancement is application of polymeric nanoparticles in combination with relatively low-intensity ultrasound (US). Bovine serum albumin nanoparticles (BSA NPs) were prepared using intermittent desolvation method and investigated in combination with ultrasound irradiation as drug delivery system to improve the therapeutic efficacy of 5-fluorouracil (5-FU) against experimentally induced hepatocellular carcinoma in mice by 4-dimethylaminoazobenzene (DAB) and phenobarbital (PB). The prepared BSA NPs encapsulating 5-FU were prepared with mean particle size of 70 nm and spherical in shape. The in vitro drug release studies revealed an enhanced and controlled release of 5-FU from BSA NPs under the effect of ultrasound. Respecting results, mice bearing hepatocellular carcinoma (HCC) and injected with BSA NPs encapsulating 5-FU and irradiated with ultrasonic waves showed significant decreased levels of hepatic malondialdehyde (MDA) and hepatic alanine transaminase (H.ALT), serum aspartate transaminase (AST) and serum alanine transaminase (ALT). Histopathological examination showed increase in apoptosis, appearance of more or less normal cells and well defined liver cords. Regarding immunohistochemical results, alpha-fetoprotein (AFP) showed moderate positivity in hepatocyttoplasm. Our results nominate BSA NPs in combined with a local ultrasonic irradiation of the tumor to be a powerful new and non-invasive tool of drug targeting and treatment of cancerous tumors. © 2014 Trade Science Inc. - INDIA

### KEYWORDS

Bovine serum albumin nanoparticles;  
5-fluorouracil;  
Ultrasound;  
Hepatocellular carcinoma;  
Mice.

### INTRODUCTION

It is well known that feeding of carcinogenic azo dye produces liver damage followed by regeneration of parenchymal cells, proliferation of bile ducts and con-

nective tissue, and at later stages tumors develop from liver parenchyma that end up with neoplastic characteristics<sup>[1]</sup>.

4- Dimethylaminoazobenzene (DAB), an azo amine dye, is a well known liver carcinogen that induces hepa-

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tocellular carcinoma (HCC) in mice through multiple assays: cytogenetical, biochemical, histological and electron microscopical techniques<sup>[1]</sup>. Chronic feeding of phenobarbital (PB) has been reported to have various toxic effects and has also been shown to promote development and growth of liver cancer in rodents when administered subsequent to feeding of an initiating carcinogen like DAB<sup>[2,3]</sup>.

A glycoprotein, alpha-fetoprotein (AFP)<sup>[4]</sup>, is tumor-associated fetal (oncofetal) protein<sup>[5]</sup>. Increased AFP gene expression was considered to be a marker for liver cancer<sup>[6]</sup>. Thus, this tumor marker was considered fairly specific<sup>[7]</sup> and an indicator of tumor activity in liver cancer<sup>[8]</sup>; approximately 80% of liver cancers produce AFP at increased levels<sup>[9]</sup>.

The critical bottleneck of conventional cancer chemotherapeutics includes high toxicity of most anticancer drugs, due to indiscriminate distribution of drugs towards disease and healthy cells following systemic administration. In addition, anticancer drugs often suffer from poor solubility in water and thus need to use organic solvents or detergents for clinical applications, resulting in undesirable side effects such as venous irritation and respiratory distress<sup>[10]</sup>. Therefore, designing a distinct carrier system that encapsulates a large quantity of drugs and specifically targets tumor cells is indispensable for successful cancer therapy.

Bovine serum albumin (BSA) is a naturally-occurring polymer<sup>[11]</sup>. It has great potential as a nanocarrier as the NPs generated by it are easily adaptable to the human body<sup>[12]</sup>, since they can be metabolized with natural mechanisms into harmless endproducts<sup>[11]</sup>. (BSA) NPs can escape from the vasculature through the leaky endothelial tissue that surrounds the tumor and can accumulate into tumors via the enhanced permeability and retention (EPR) effect<sup>[13]</sup>. Prepared (BSA) NPs containing chemotherapeutic drug can bypass the efflux action of drug-resistant cancer cells, thus resulting in their enhanced cellular drug uptake, accumulations, and therapeutic effects<sup>[14]</sup>.

A relatively novel strategy for drug delivery enhancement is application of polymeric nanoparticles in combination with relatively low-intensity ultrasound (US). This method (referred to as "sonoporation") can induce cavitation of or near cellular membranes to enhance delivery of drugs. In general, low-intensity US

can induce beneficial and reversible cellular effects, which are more likely to induce cellular death. Sonoporation is an emerging and promising physical method for drug delivery enhancement *in vitro* and *in vivo*<sup>[10,15]</sup>.

The present work aimed to study the effect of 80 KHz pulsed ultrasonic waves in enhancing the delivery of 5-fluorouracil encapsulated into (BSA) NPs by evaluating the tumoricidal effect in hepatocellular carcinoma induced chemically in mice by (DAB).

## MATERIALS AND METHODS

### Chemicals and reagents

4-dimethylaminoazobenzene (DAB) (MWt 225.3 g/mol, Bio Basic Inc, Canada). Pheno barbital (PB) (MWt 232.24 KDa), Bovine serum albumin (MWt 66 KDa), 5-Fluorouracil (5-FU) (MWt 130.08 g/mol), Glutaraldehyde (MWt 100.12 KDa), Trypsin (MWt 23.8 KDa), were provided by Sigma Chemical Co. USA, and all other chemicals and solvents were of reagent grade.  $\alpha$ -fetoprotein (AFP) antibody (Mwt 70 KDa) from Lab Vision/NeoMarkers, USA). All other chemicals were of analytical grades.

### Preparation of 5-FU loaded BSA NPs

#### (A) Intermittent desolvation method

5-Fluorouracil-loaded BSA NPs were prepared by modified desolvation method<sup>[12]</sup> in which intermittent addition of the desolvating agent was applied to control and minimize the particle size of BSA NP. Briefly, 0.2 g BSA powder was added to 4 mg 5-FU in 2 ml distilled water, pH adjusted to 8.4 and incubated at room temperature for 30 min. Ethanol was added intermittently at the rate of 1 ml per 5 min, under magnetic stirring at room temperature. Subsequently, 176  $\mu$ l glutaraldehyde solution (8%) was added to induce particle cross-linking. The cross-linking process was performed under stirring of the suspension over night. Microparticles were precipitated by centrifugation at 14,000 rpm for 4 min and then discarded. 5-FU BSA NPs were separated by two cycles of centrifugation at 14,000 rpm for 30 min. The pellet was redispersed in 10 mM NaCl at pH 9 using ultrasonication for 5 min, refrigerated at -80 °C for one day, then frozen by vacuum freeze-drying machine (Lyophilizer) (Model/CRYODOS-50, Spain) at

230 V, 50 Hz, at a pressure of 26.5 Pa and 5 % saccharose was used as cytoprotectant. The lyophilized particles were then characterized.

### Characterization of BSA nanoparticles

- I The particle size distribution of the nanoparticles was determined by laser light scattering on a Beckman Coulter Particle Size Analyzer (N5 sub-micron particle size analyzer, Japan) equipped with a He-Ne Laser beam at a fixed scattering angle of 11.1°.
- II The physical morphology and shape of the prepared nanoparticles were determined by transmission electron microscope and scanning electron microscope (JEOL-JSM-6360LA, Japan).
- III The percentage of DLE and EE of the entrapped 5-FU in BSA NPs was calculated using the equations (1) and (2) respectively<sup>[16,17]</sup>:

$$\text{DLE (\%)} = \left( \frac{\text{Amount of drug in nanoparticles}}{\text{weight of nanoparticles}} \right) \times 100 \quad (1)$$

$$\text{EE (\%)} = \left( \frac{\% \text{ Drug Content}}{\% \text{ Theoretical Drug Content}} \right) \times 100 \quad (2)$$

Theoretical drug content was determined assuming that the entire drug present in the BSA solution used gets entrapped in nanoparticles and no loss occurs.

The actual drug content in the 5-FU loaded BSA nanoparticles was quantitatively determined by the following procedure:

First standard curve from known concentration of 5-FU was constructed. Freeze-dried nanoparticles were weighted and resuspended in phosphate buffered saline (PBS) at pH 7.4 using ultrasonication for 5 min. For complete liberation of entrapped drug, 50 µl of trypsin solution (1 mg/ml) was added and the resulted solution was incubated at 37°C for 8 h. Later, total drug concentration was determined using Nano UV/VIS spectrophotometer at 266 nm.

### (B) Studying the effect of ultrasonic waves on drug release

#### Ultrasonic apparatus

Ultrasonic therapy instrument (Ultrasonic Therapy Model CSL, Shanghai, No.822 factory, China) were used. This instrument uses calcium zirconate titanate circular transducer which operates at frequency of 0.8 MHz at pulsed mode with adjustable power of 0.5-3 W (pulse- repetition rate 1000 Hz, duty ratio 1:3 and

average intensity from 0.15 to 1 W/cm). Transducers were mounted on a brass holder cooled with water. The ultrasonic emission beam diameter (BeD) was calculated from the equation:

$$\text{BeD} = 0.2568 (D) * S_f \quad (3)$$

Where D transducer diameter and  $S_f$  normalized focal length of the transducer surface = 1 for flat surface. For the 1-cm diameter flat surface transducer used in the present work,  $\text{BeD} \approx 0.26$  cm.

#### Procedure<sup>[18]</sup>

5 mg of 5-FU loaded BSA NPs were redispersed in 10mL of pH 7.4 PBS in a glass flask and sonicated at frequency of 0.8 MHz with ultrasound intensity at 3 Watt/cm<sup>2</sup> for pulsed waves. 1.5 ml of drug-releasing medium was withdrawn at various time intervals. The samples were centrifuged at 18,000 rpm for 15 min. A quantity of 1 ml of the supernatant was taken for determination of concentration using Nano UV/VIS spectrophotometer at 266 nm. 1 ml of fresh PBS buffer was added to the remaining 0.5 ml sample and then 1.5 ml of the suspension was agitated vigorously by vortexing and replaced in the flasks. The cumulative percentage of 5-FU released from the nanoparticles at a certain time point was obtained using Eq. (3).

**Cumulative 5- FU released%=**

$$100 - (4 - 5\text{-FU remaining \%}) \quad (4)$$

The same procedures were repeated in the absence of ultrasonication.

### (C) Animal care

*In vivo* studies were done on a total of 60 male Swiss albino mice 6–8 weeks old, weighing 16–25 g, purchased from animal house at National Cancer Institute, Cairo University. Ten animals each were housed in plastic cages a well-ventilated room (25 ± 2 °C) with a relative humidity of (43 ± 3 %) and maintained on a standard diet at the animal House Unit at Medical Technology Center-University of Alexandria. Animals were handled according to the rules and regulations of Medical research Institute Animal Ethics Committee.

### (D) Experimental design

60 male mice were fed on 0.06% DAB<sup>[19]</sup> dissolved in paraffin oil at a daily dose of 165 mg/kg body weight per mouse through a specially made fine pipette and water was replaced with 0.05% aqueous solution

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of PB till appearance of liver tumor (two months)<sup>[20]</sup>.

### After confirming HCC development, animals were divided into three main groups

Group A: 20 mice were served as control group and injected intraperitoneally (i.p) with saline.

Group B: 20 mice were injected i.p with 5-FU as free drug with the dose of 5 mg/kg every two days for 15 days.

Group C: 20 mice were injected i.p with 40 mg/kg body weight per mouse of 5-FU loaded BSA NPs which equivalent to the dose of 5 mg/kg of free 5-FU every two days for 15 days.

Each group were divided into two subgroups each of 10 mice, where one subgroup was exposed to ultrasonic waves for 15 minutes<sup>[21]</sup> after 30 minutes of the i.p injection every two days, while the other one was not exposed to ultrasonic waves. After 15 days, animals were sacrificed. Blood and liver tissues were collected for biochemical analysis, histopathological, and immunohistochemical examinations.

### Biochemical analysis

I Liver tissue was homogenized in 5% w/v cold PBS solution at pH 7.4. The sample was centrifugated at 4000 rpm for 15 min at 4°C and then the supernatant was removed for assay. Tissue MDA content was determined using spectrophotometric methods (using Shimadzu, Double beam Spectrophotometer UV1700, Japan) adopted for assay of MDA. Briefly, thiobarbituric acid (TBA) reacts with MDA in acidic medium at 95°C for 30 minutes to form thiobarbituric acid reactive product the absorbance of the resultant pink product can be measured at 534 nm<sup>[22,23]</sup>. The sample concentrations of MDA was calculated from the following equation (5)

$$\text{MDA tissue} = \frac{A_{(\text{Sample})}}{A_{(\text{standard})}} \times \frac{10}{\text{gram (g.) tissue used}} \text{ nmol/g.tissue} \quad (5)$$

II For the analysis of hepatic ALT, 0.1 ml of tissue homogenate was made to react with 0.5 ml of the substrate solution (L-alanine) and incubated for 60 min at 37 °C. This was followed by addition of 0.5 ml of dinitrophenolhydrazine (DNPH) and then by 5.0 ml 0.4 N NaOH. The absorbance was measured at 510 nm.

III Sera were obtained from blood harvested by centrifugation at 3500 rpm at 4 °C and stored in aliquots at -80 °C until assayed. Serum alanine and aspartate aminotransferases (ALT and AST, respectively) were determined spectrophotometrically using ready-for-use colorimetric kits.

### Histopathological studies

#### H & E for histopathological studies

Part of the liver was preserved in neutral buffered formalin and was processed for paraffin section. Deparaffinization and hydration of sections in descending series of ethyl alcohol till distilled water, then stained with H&E, dehydrated, cleared with xylene and mounted using Canada balsam for histopathological investigations<sup>[24]</sup>.

#### Immunohistochemical study of AFP

Peroxidase antiperoxidase (PAP) technique was used<sup>[25]</sup>.

#### Image analysis

Image analysis is a tool for extraction of quantitative information contained in an image. Slides were randomly analyzed and quantitated for the optical density of the signals using a PC computer<sup>[26]</sup>. The video image was digitized for image analysis at 255 grey levels, in which the light intensities are represented as numbers from 0 to 255<sup>[27]</sup>. Imported data were analyzed quantitatively by Leica Q500 MC Software-Qwin<sup>[26]</sup>.

#### Statistical analysis

Statistical analysis was performed using the PASW 18 software package. Non-parametric Kruskal - Wallis test was used for comparison among multiple groups. P < 0.05 was considered significant. While ANOVA was used for comparisons of AFP and p < 0.05 was considered significant.

## RESULTS

### Characterization of BSA NPs

#### Particle size analysis

The particle size distribution of 5-FU loaded BSA NPs was 60-80 nm with mean of 70 nm, base line error at 0.01% at diffraction angle of 11.1o and polydis-

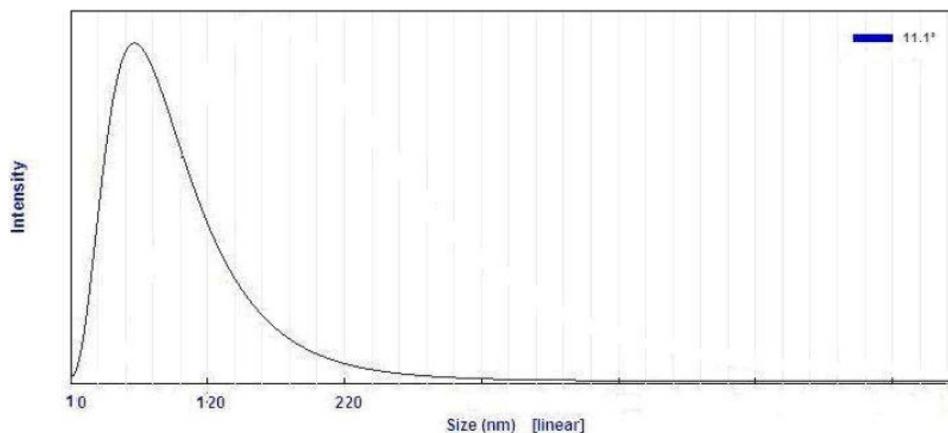


Figure 1 : Particle size distribution curve of 5-FU loaded BSA NPs

persity index (PI) of 0.784 (Figure 1):

### Electron microscope examination

Morphology of prepared particles was studied using scanning microscope examination (SEM) that indicates the formation of completely spherical particle with smooth surface and low level of agglomeration (Figure 2, 3). Transmission microscope examination (TEM) revealed the preparation of blank BSA NPs in the shape of completely spherical NPs with no agglomeration (Figure 4).

### Drug loading efficiency (DLE) and encapsulation efficacy (EE)

Amount of entrapped 5-FU in BSA NPs was determined by encapsulation efficiency (EE) which was 62.5% and drug loading efficacy (DLE) which was 19.23%.

### In vitro release profile

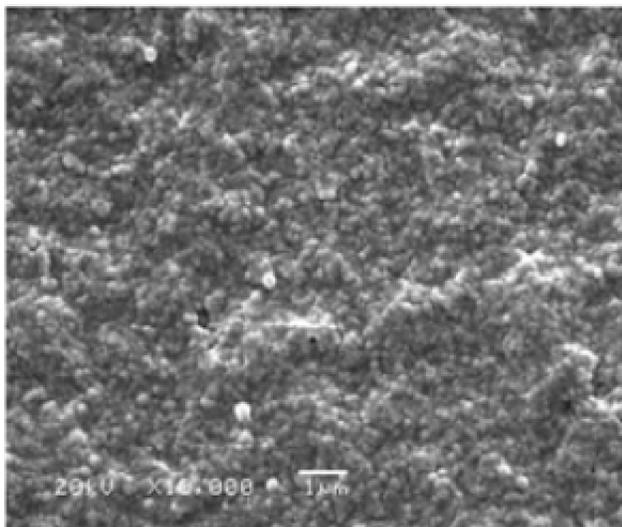


Figure 2 : SEM of prepared particles (Mag. x10.000, 20 kV)

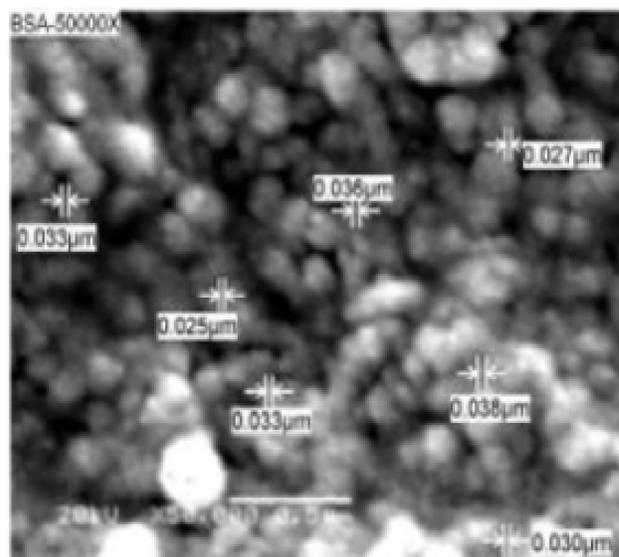


Figure 3 : SEM showing particle size range (Mag. x50.000, 20 kV)

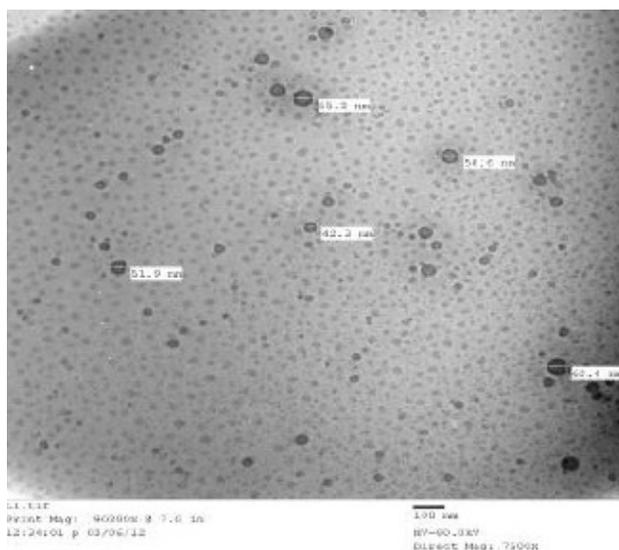
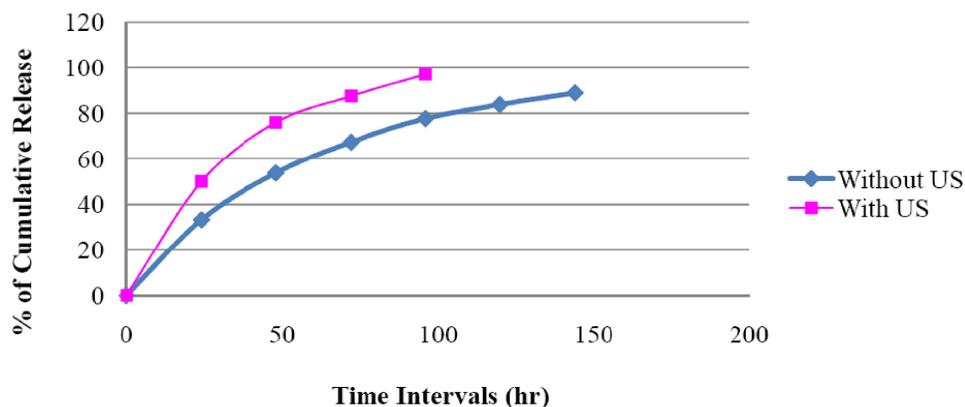


Figure 4 : TEM showing 5-FU loaded BSA NPs (Mag. x7.500, 80 kV)

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**Figure 5 : In vitro cumulative release curve of 5-FU from BSA NPs with and without ultrasound irradiation**

The *in vitro* drug release of 5-FU under the effect of ultrasound (US) irradiation reached 50% in the first 24 hrs compared with 33.3% in the absence of ultrasound irradiation. After 48 hrs the effect reached 76% compared with 53.8%, and then after 72 hrs the effect reached 87.5% compared with 67.4%. The comparison showed a higher release percentage with the use of ultrasound irradiation (Figure 5). A maximum release of 5-FU under the effect of ultrasound irradiation reached 97% after 96 hrs, while maximum release of 88.9% reached after 144 hrs from NPs in the absence of ultrasound irradiation.

### Biochemical parameters

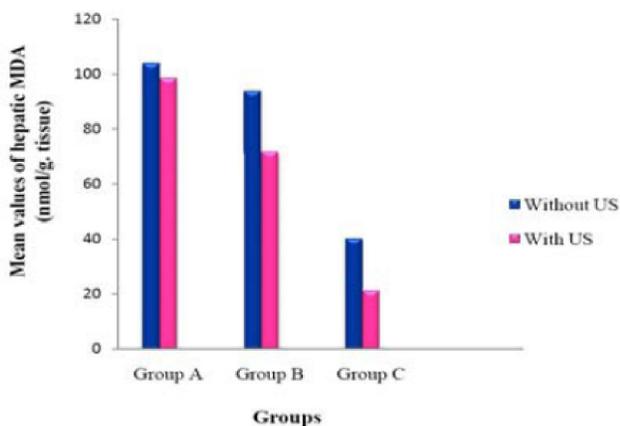
#### Hepatic MDA level

As shown in Figure 6, for subgroups that did not exposed to ultrasound irradiation, mice treated with free 5-FU (group B) showed no significant decrease when compared to mice bearing the liver nodules in group A ( $p1 = 0.217$ ), while treating mice with 5-FU loaded BSA NPs (group C) caused a highly significant decrease

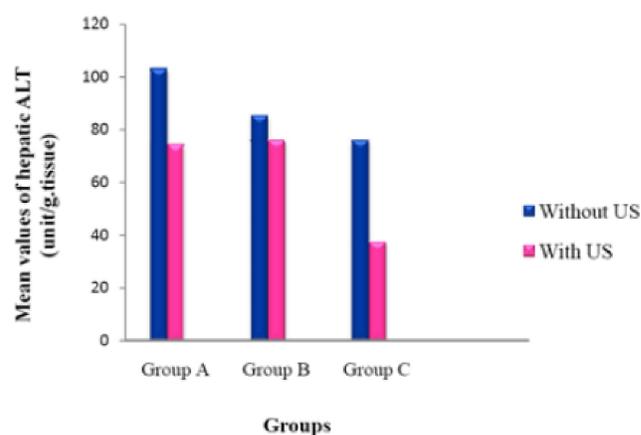
in MDA level when compared to group A ( $p1 = 0.001^*$ ). A significant decrease in MDA level was obtained when comparing group C with group B ( $p1 < 0.001^*$ ). On the other hand, exposing mice to ultrasound irradiation caused a significant decrease in MDA level when comparing both groups B and C to group A being significantly decreased in group C ( $p2 = 0.010^*$ ,  $0.001^*$ ), ( $p2 < 0.001^*$ ) respectively. Within the same group, ultrasound irradiation triggered a significant decline in MDA level in groups B and C ( $p = 0.049^*$ ,  $0.009^*$ ; respectively).

#### Hepatic ALT activity

Mice treated with free 5-FU (group B) showed a significant decrease when compared to mice bearing the liver nodules in group A ( $p1 = 0.035^*$ ), while treating mice with 5-FU loaded BSA NPs (group C) caused a highly significant decrease in HALT activity when compared to group A ( $p1 = 0.003^*$ ). A non-significant decrease in HALT activity was obtained when comparing group C with group B ( $p1 = 0.246$ ). On the other hand,



**Figure 6 : Bar graph illustrated the mean values of hepatic MDA (nmol/g. tissue) in different treated groups**



**Figure 7 : Bar graph illustrated the mean values of hepatic ALT (unit/g. tissue) in different treated groups**

exposing mice to US caused a significant decrease in ALT activity when comparing group C to group A being more significantly decreased in group C ( $p_2 = 0.002^*$ ,  $< 0.001^*$ ) respectively. While in group B, HALT activity showed a no significant decrease compared to group A ( $p_2 = 1.000$ ). Within the same group, US triggered a significant decline in HALT activity in all groups ( $p = 0.047^*$ ,  $0.022^*$ ,  $< 0.001^*$ ) respectively (Figure 7).

### Serum ALT activity

Mice treated with free 5-FU (group B) showed no significant difference when compared to mice bearing the liver nodules in group A ( $p_1 = 0.217$ ), while treating mice with 5-FU loaded BSA NPs (group C) caused a highly significant decrease in ALT activity when compared to group A ( $p_1 = 0.043^*$ ). A non-significant decrease in ALT activity was obtained when comparing group C with group B ( $p_1 = 0.149$ ).

On the other hand, exposing mice to US caused a significant decrease in ALT activity when comparing both groups B and C to group A being more significantly decreased in group C ( $p_2 = 0.014^*$ ,  $0.001^*$ ), ( $p_2 = < 0.001^*$ ) respectively. Within the same group, US triggered a significant decline in ALT activity only in group C ( $p = 0.001^*$ ) (Figure 8).

### Histopathological findings

#### H&E

#### Control group

Control liver sections stained with H&E showed hexagonal unit structure called "hepatic lobule" which was centered on "hepatic venule" (centrilobular venule).

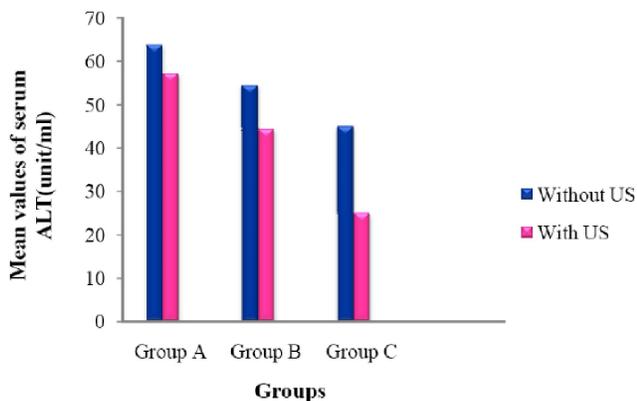


Figure 8 : Bar graph illustrated the mean values of serum ALT (unit/ml) in different treated groups

Each lobule showed anastomosing plates of hepatocytes radiating from the centrilobular venule toward the periphery of the hexagon. Hepatocytes had pink stain

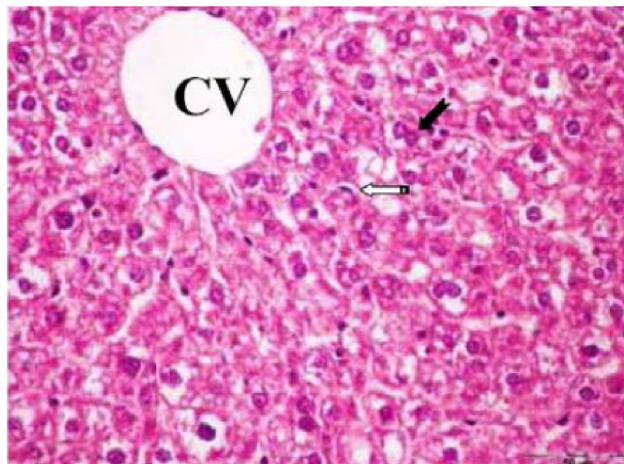


Figure 9 : Paraffin section of control mouse liver showing normal structure, binucleated (black arrow), Kupffer cells (white arrow) and central vein (CV). H&E (bar = 50  $\mu$ m)

with round nuclei containing prominent nucleoli. Most of the hepatic cells contained single nucleus, but some binucleated cells were found (Figure 9).

#### Treated groups

By 60 days, the sections showed well differentiated HCC, acinic cells and mitotic cells (Figure 10). Examination of liver sections of group treated with free 5-FU revealed less necrosis and disappearance of inflammation (Figure 11), while group treated with free 5-FU and exposed to US showed well circumscribed cells and defined liver cords (Figure 12). The group treated with

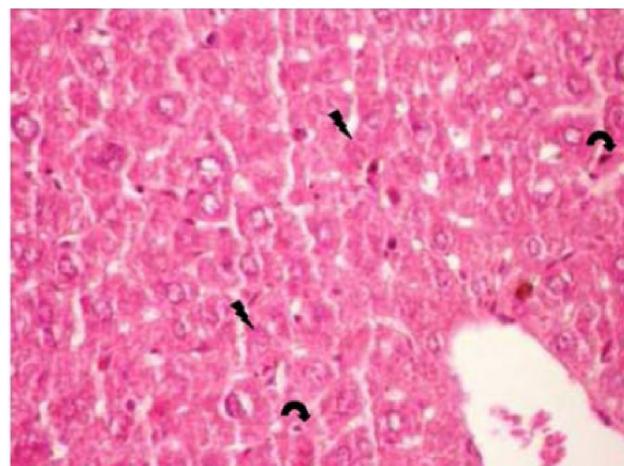
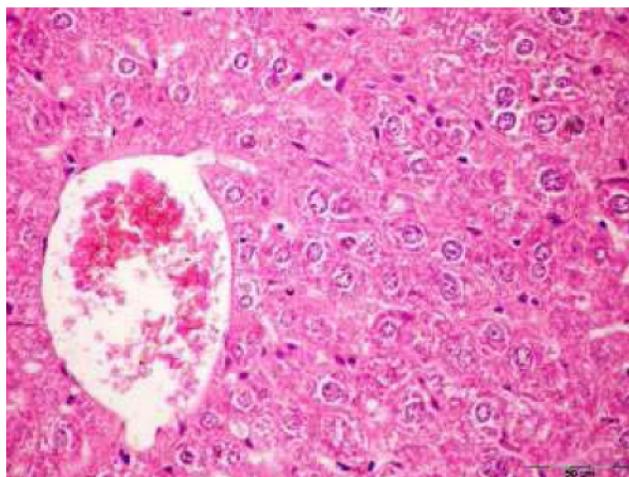
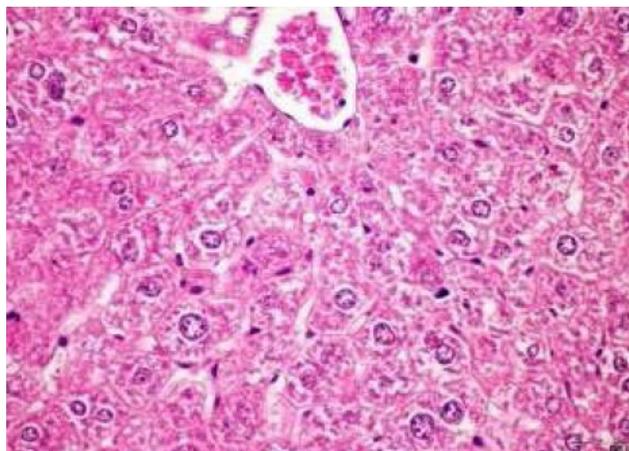


Figure 10 : Paraffin section of mouse liver 60 days post DAB + PB receiving showing acinic cells (black arrow) and mitotic cells (black arrowhead). H&E (bar = 50  $\mu$ m)

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**Figure 11 :** Paraffin section of mouse liver treated with free 5-FU post DAB + PB receiving showing less necrosis and disappearance of inflammation. H&E (bar = 50 μm)



**Figure 12 :** Paraffin section of mouse liver treated with free 5-FU drug and exposed to ultrasonic waves post DAB + PB receiving showing well circumscribed cells and defined liver cords. H&E (bar = 50 μm)

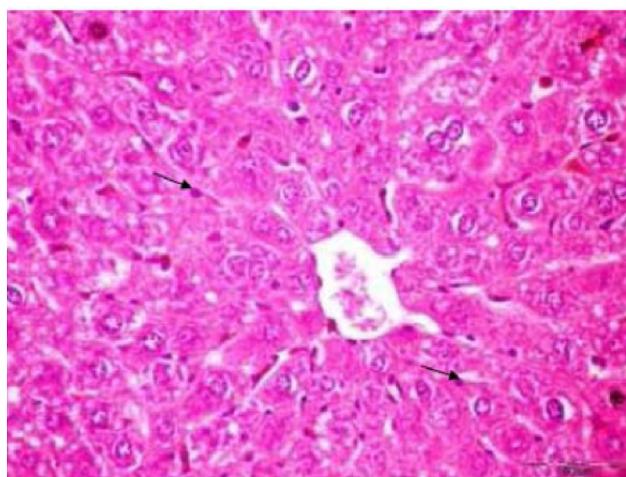
5-FU loaded BSA NPs showed the presence of apoptosis, the appearance of cords and disappearance of inflammation (Figure 13). Liver sections treated with 5-FU loaded BSA NPs and exposed to US showed increased apoptosis, appearance of more or less normal cells and well defined liver cords (Figure 14).

### AFP

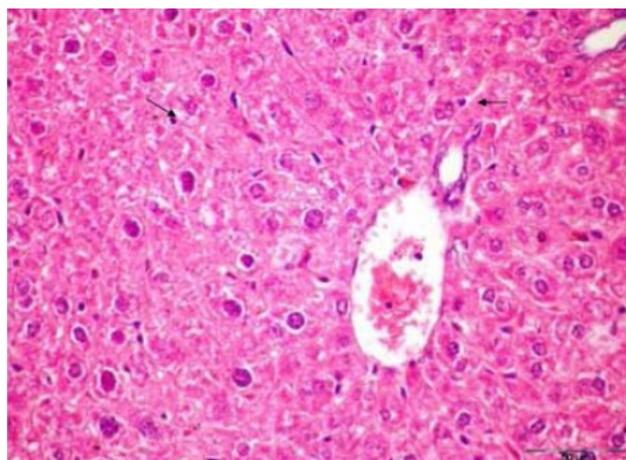
The expression of AFP is well demonstrated as brown granules in the cytoplasm and arranged around cell membranes either of hepatocytes or sinusoids.

### Control group

Immunohistochemical staining of AFP in fetal mouse liver revealed positive AFP reaction in hepatocyte cytoplasm (Figure 15).



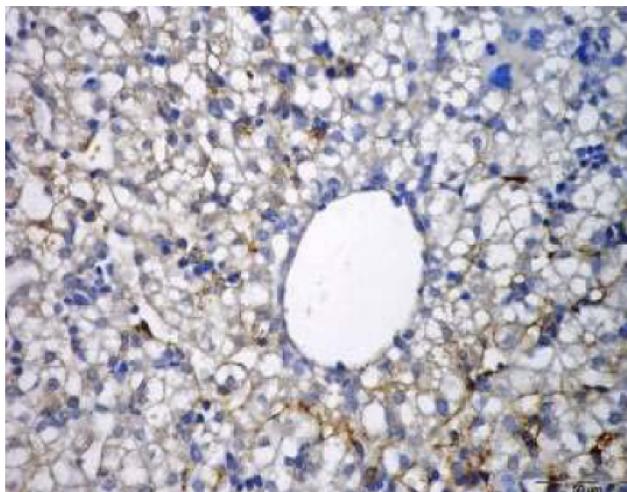
**Figure 13 :** Paraffin section of mouse liver treated with 5-FU loaded BSA NPs post DAB + PB receiving showing apoptosis (→), the appearance of cords and disappearance of inflammation. H&E (bar = 50 μm)



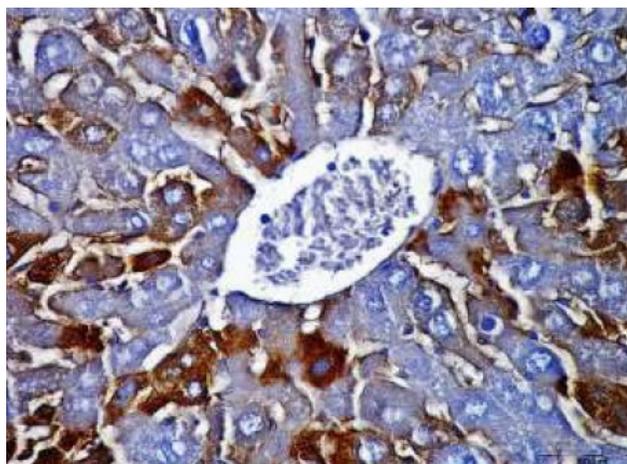
**Figure 14 :** Paraffin section of mouse liver treated with 5-FU loaded BSA NPs and exposed to ultrasonic waves post DAB + PB receiving showing increased apoptotic bodies (→) and defined liver cords. H&E (bar = 50 μm)

### Treated groups

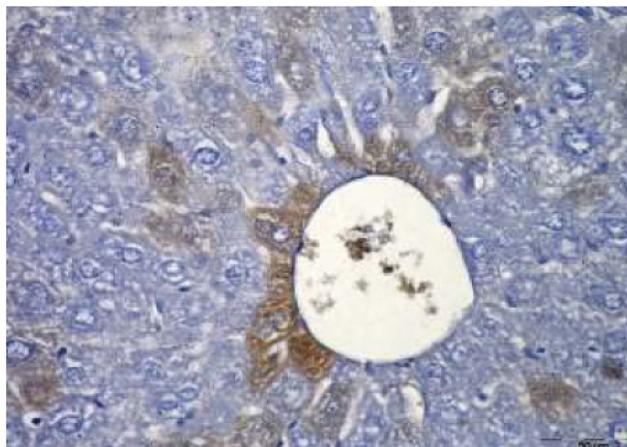
After 60 days, this stage showed intense positivity in hepatocyte cytoplasm compared to the normal group (Figure 16). Groups treated with free 5-FU showed marked positivity in hepatocyte cytoplasm compared to 60 days group (Figure 17). Groups treated with free 5-FU and exposed to US showed moderate positivity in hepatocyte cytoplasm (Figure 18). Groups treated with 5-FU loaded BSA NPs showed strong positivity in hepatocyte cytoplasm (Figure 19). Groups treated with 5-FU loaded BSA NPs and exposed to US showed moderate positivity in hepatocyte cytoplasm compared to 60 days group (Figure 20).



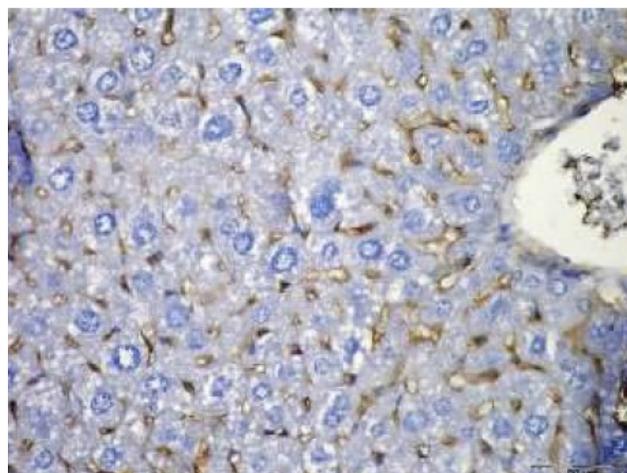
**Figure 15 :** Immunohistochemical staining of AFP in fetal mouse liver showing positive AFP positivity in hepatocyte cytoplasm. PAP method (bar = 50 μm)



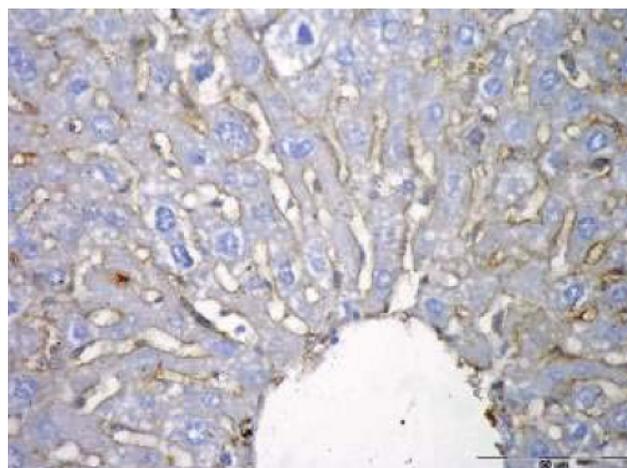
**Figure 16 :** Immunohistochemical staining of AFP in mouse liver 60 days post DAB + PB receiving showing intense positivity in hepatocyte cytoplasm. PAP method (bar = 50 μm)



**Figure 17 :** Immunohistochemical staining of AFP in mouse liver treated with free 5-FU post DAB + PB receiving showing marked reaction in hepatocyte cytoplasm. PAP method (bar = 50 μm)



**Figure 18 :** Immunohistochemical staining of AFP in mouse liver treated with free 5-FU and exposed to ultrasonic waves post DAB + PB receiving showing moderate positivity in hepatocyte cytoplasm. PAP method (bar = 50 μm)

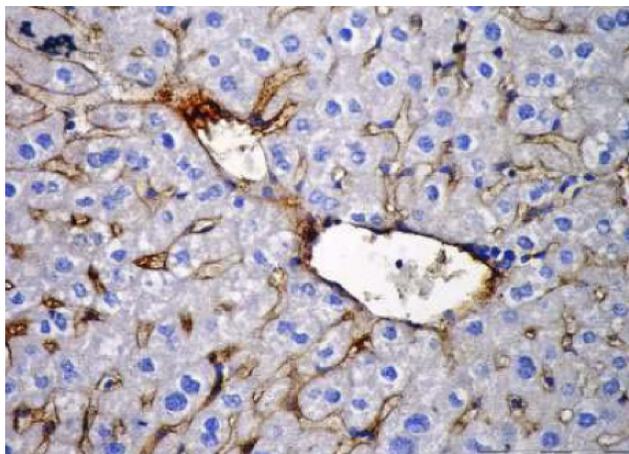


**Figure 19 :** Immunohistochemical staining of AFP in mouse liver treated with 5-FU loaded BSA NPs post DAB + PB receiving showing strong positivity in hepatocyte cytoplasm. PAP method (bar = 50 μm).

### Image analysis

Chronic feeding of DAB + PB caused a progressive significant increase in AFP expression when compared 60 days after induction to the control animals ( $p < 0.001^*$ ). Mice treated with free 5-FU (group B) showed a highly significant decrease when compared 60 days after induction in group A ( $p_2 = < 0.001^*$ ), also treating mice with 5-FU loaded BSA NPs (group C) caused a highly significant decrease in AFP expression when compared to group A ( $p_2 = < 0.001^*$ ). A significant decrease in AFP expression was obtained when comparing group C with group B ( $p_3 = 0.001^*$ ). On the other hand, exposing mice to US caused no

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**Figure 20 :** Immunohistochemical staining of AFP in mouse liver treated with 5-FU loaded BSA NPs and exposed to ultrasonic waves post DAB + PB receiving showing moderate positivity in hepatocyte cytoplasm. PAP method (bar = 50  $\mu$ m)

significant decrease in AFP expression when comparing group B with group C ( $p_4 = 0.318$ ). Within the

same group, US triggered a significant decline in AFP expression in group B ( $p_1 = 0.022^*$ ) and no significant decrease in AFP expression in group C ( $p_1 = 0.946$ ) (TABLE 1, Figure 21).

## DISCUSSION

The chronic feeding of both DAB (initiator) and PB (promoter) has been successfully used to develop liver tumors and cancer<sup>[28]</sup>, and an elevated toxicity level of biomarkers. Biswas et al. (2008)<sup>[1]</sup> induced hepatocellular carcinoma in mice using DAB; their results showed that liver nodules appeared after chronic feeding of DAB + PB for 60 days or more.

From this point of view, in this study, we choose to chemically induce HCC in Swiss albino mice chemically using DAB and PB, by feeding mice on 0.06% DAB at a daily dose of 165 mg/kg body weight per

**TABLE 1 :** Image analysis of AFP expression in liver tissue in different treated groups

Groups	Control	60 Days [Group A]	Free 5-FU [Group B]		5-FU loaded BSA NPs [Group C]	
	(5)	(5)	(5)	(5)	(5)	(5)
Ultrasound			Without US	With US	Without US	With US
Min.-Max.	270.0-310.0	470.0-590.0	400.0-460.0	340.0-370.0	300.0-350.0	290.0-330.0
Mean $\pm$ SD	294.0 $\pm$ 16.73	542.0 $\pm$ 50.20	420.0 $\pm$ 25.50	352.0 $\pm$ 13.04	328.0 $\pm$ 19.24	310.0 $\pm$ 15.81
Median	290.0	570.0	410.0	350.0	330.0	310.0
P	↓					
P <sub>1</sub>			↓		↓	
P <sub>2</sub>		↓			↓	
P <sub>3</sub>					↓	
P <sub>4</sub>					↓	

p: p value for Post Hoc test (Scheffe) for comparing between control and 60 days groups

p<sub>1</sub>: p value for Post Hoc test for comparing between with and without US in group B and C

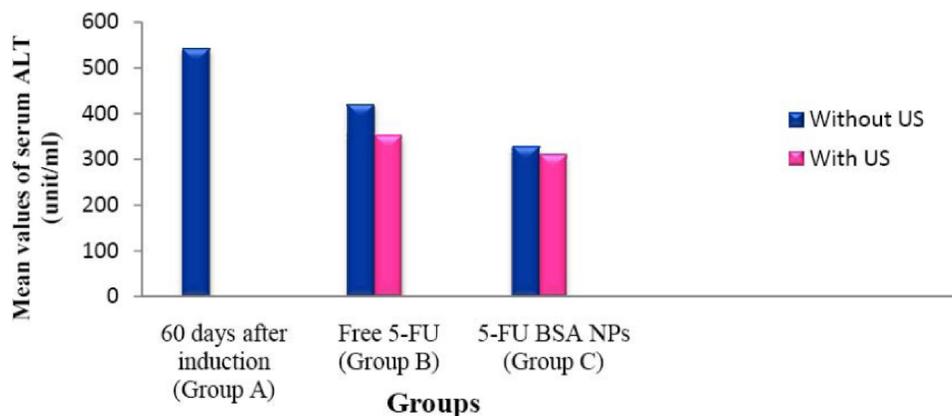
p<sub>2</sub>: p value for Post Hoc test for comparing between 60 days and without US in group B and C

p<sub>3</sub>: p value for Post Hoc test for comparing between without US in group B and C

p<sub>4</sub>: p value for Post Hoc test for comparing between with US in group B and C

\*: Statistically significant at  $p \leq 0.05$

n: number of animals



**Figure 21 :** Bar graph illustrated the mean values of image analysis of tissue AFP expression (unit/g.tissue) in different treated groups

mouse and 0.05% aqueous solution of PB for 60 days<sup>[20]</sup>. After 2 months, all the mice fed on DAB and PB established well differentiated HCC that was confirmed by the histopathological findings. Our present results showed degenerated changes ranged from the presence of few inflammatory cells near the central vein and few apoptosis till reaching the appearance of well differentiated HCC during 60 days. This was in accordance with Hassan (2012)<sup>[28]</sup> investigation that showed HCC appearance with loss of hepatic architecture and having proliferating streaks and cords of malignant hepatocytes.

It is well documented that AFP estimation remains a useful test in the management of patients with hepatic malignancies (HCC)<sup>[29]</sup>. The stained sections showed a significant increase in positivity after 60 days compared with control mice. This was in accordance with Hassan (2012)<sup>[28]</sup> who found that AFP was reexpressed in HCC induced by 2-nitropropane (2-NP) and diethylnitrosamine (DEN).

In the present study, 5-FU was selected to prepare a delivery system that can be used for targeting anti-cancer drugs. 5-FU is a cytotoxic drug, which interferes with nucleic acid synthesis, inhibits DNA synthesis, and eventually halts cell growth. It is extensively used to treat solid tumors such as liver, breast, colorectum and brain cancer<sup>[30]</sup>.

5-FU has a narrow therapeutic index<sup>[30]</sup> and a very short biological half-life (10-20 minutes) that necessitates frequent administration of the drug which may lead to severe side effects such as anorexia, diarrhea, shock<sup>[31]</sup>, severe gastrointestinal toxicity, hematologic disturbance, and severe bone marrow deficiency<sup>[30]</sup>.

Therefore, attempts have been made to control these complications by encapsulation of 5-FU using biodegradable polymers<sup>[31]</sup>.

Albumin-based NP carrier systems represent an attractive strategy, since a significant amount of drug can be incorporated into the particle matrix because of the different drug binding sites present in the albumin molecule<sup>[32]</sup>. Due to the high protein binding of various drugs, the matrix of albumin NPs can be used for effective incorporation of these compounds<sup>[33]</sup>.

In our study, BSA NPs were loaded with 5-FU to be the therapeutic modality for treatment of HCC. 5-FU loaded BSA NPs were prepared using intermittent desolvation method. According to these methods we obtained BSA NPs which are completely spherical particles in shape, as shown by SEM and TEM, with the mean size of 70 nm for the 5-FU loaded BSA NPs by using PSA. This was in accordance with Cheng M et al (2012)<sup>[30]</sup>.

The primary objective of our research is to achieve drug release profiles with desired characteristics in terms of time period and rate of drug release. This was realized by first interpreting the effect of ultrasound on drug release in terms of the pertinent scientific principle and then employing these effects to tailor the release profiles.

From our preparation, the obtained NPs had DLE of 19.23 and EE of 62.5%. The result was coincided with Arnedo et al (2002)<sup>[34]</sup>. While the result of drug release under effect of pulsed US with power intensity in the range of 3 W/cm<sup>2</sup> showed an increase in 5-FU release from BSA NPs and revealed from 0 h to 96 h a cumulative release percentage of 97%. This result was

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in accordance with Santhi et al (1999)<sup>[35]</sup> and Das et al (2005)<sup>[36]</sup>.

Comparing between release profiles of free drug and NP suspension confirms that this colloidal drug carrier is capable of releasing drug in a controlled manner with the ability of revealing burst release.

Using US power as a drug release mechanism is advantageous for being noninvasive. The main advantage that renders US so useful is that no insertion or surgery is needed; acoustic transducers are placed in contact with a water-soluble gel that is spread on the skin. In addition, ultrasonic waves can penetrate deep into the interior of the body<sup>[37]</sup>.

Gao et al (2004)<sup>[38]</sup> proposed the use of US enhancing the nanocarrier uptake towards the tumor site. By using drug free polymeric micelles, the authors found a highly increased deposition of their carriers inside the tumor after i.v or i.p administration suggesting such carriers as a promising approach.

After confirming development of HCC, we divided mice into group treated with i.p injection of 40 mg/kg body weight per mouse of 5-FU as free drug and the other treated with i.p injection of 40 mg/kg body weight per mouse<sup>[39]</sup> of 5-FU loaded BSA NPs with the dose of 5 mg/kg every two days for 15 days<sup>[40]</sup>; and then from each group, half of the mice were exposed to US for 15 minutes<sup>[21]</sup> after 30 minutes of the i.p injection<sup>[10]</sup> every two days for 15 days.

After 15 days of treatment<sup>[40]</sup>, treated mice showed improvement mostly after the use of 5-FU loaded BSA NPs and exposed to US. By measuring the biochemical parameters, hepatic MDA compared with mice bearing the liver nodules showed decreased levels by 9% and 58% after being treated with free 5-FU, and treated with 5-FU loaded BSA NPs respectively. While compared with mice bearing the liver nodules and exposed to US showed decrease levels by 27% and 75% after being treated with free 5-FU and exposed to US, and being treated with 5-FU loaded BSA NPs and exposed to US respectively.

Serum ALT compared with mice bearing the liver nodules showed decreased levels by 10% and 36% after being treated with free 5-FU, and treated with 5-FU loaded BSA NPs respectively. While comparing with mice bearing the liver nodules and exposed to US, it showed decreased levels by 25% and 50% after be-

ing treated with free 5-FU and exposed to US, and being treated with 5-FU loaded BSA NPs and exposed to US respectively.

Finally, hepatic ALT compared with mice bearing the liver nodules showed decrease levels by 19% and 30% after being treated with free 5-FU, and treated with 5-FU loaded BSA NPs respectively. While compared with mice bearing the liver nodules and exposed to US showed decrease levels by 0% and 53% after being treated with free 5-FU and exposed to US, and being treated with 5-FU loaded BSA NPs and exposed to US respectively.

This result was confirmed by the histopathological findings that revealed less necrosis and disappearance of inflammation after being treated with free 5-FU, while the treatment with free 5-FU and exposure to US showed well circumscribed cells and defined liver cords. By using 5-FU loaded BSA NPs, apoptosis was present; while treatment with 5-FU loaded BSA NPs and exposure to US, we noticed increase in apoptosis, appearance of more or less normal cells and well defined liver cords.

Our results were in accordance with Dai et al (2009)<sup>[41]</sup>, Varshosaz et al (2012)<sup>[42]</sup> and Cochran et al (2011)<sup>[43]</sup>. Their results suggested that in situ generation of NPs provides a superior treatment over injection of free drug and also de novo synthesized NPs.

$\alpha$ -Fetoprotein sections showed a significant decrease in positivity compared with mice bearing liver nodules. This was in accordance with Dai et al (2009)<sup>[41]</sup>.

The rationale behind the approach of nanoparticles for drug delivery is that drug encapsulation decreases the systemic concentration of free drug, inhibits intracellular drug uptake, and provides a passive drug targeting to the tumor interstitium via the EPR effect. The unwanted drug interactions with healthy tissues are therefore inhibited<sup>[38]</sup>. The US induced drug release and enhanced the intracellular uptake of both released and encapsulated drug; the latter was presumably caused by the perturbation of cell membranes<sup>[44]</sup>. This effect is expected to increase the selectivity of the drug accumulation in the tumor<sup>[38]</sup>.

In conclusion, the investigation of BSA NPs containing 5-FU, through studies on the carrier capacity, in vitro release and anti-tumor efficacy reveals its suitability as a delivery system through their enhanced efficacy

against cancer cells. Also the drug delivery in combined with a local ultrasonic irradiation of the tumor may be developed into a powerful new tool of drug targeting and treatment of cancerous tumors.

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