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Novel Tamoxifen Citrate-Loaded Polymeric Nanoformulations for Improving Treatment and uses in Medical Applications: An *in Vitro* and *in Vivo* Study

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Abstract

Tamoxifen citrate (TAM) is one of the most regularly used therapy in hormone receptor-positive breast cancer. Although it is a successful treatment, there is a problem with its bioavailability, accordingly, this study was designed to improve TAM solubility and reduce its associated toxicity. TAM-Loaded poly (D, L-lactide – co- glycolide) nanostructure (TAM-loaded PLGA) has been synthesized and employed both *in vitro* and *in vivo* experiments. The blood hemolysis induced by TAM- loaded PLGA was 4.6 % at 200 µg mL^{-1}, indicating that this nano-construct led to increased red blood cell protection. DNA molecule integrity was assessed and results indicated that DNA strands were protected from destruction at 200 µg mL^{-1}. The antioxidant activity of TAM-loaded PLGA exceeded the pure TAM, it showed concentration-dependent scavenging action in the 100-200 µg mL^{-1} range, with a maximum activity of 79.2% at 200 µg mL^{-1}. Whereas pure TAM had a decrement of 60.57%. While the activity of positive control (Vit. C) was 92.12%. Finally, the safety of TAM-loaded PLGA was examined *in vivo*. The functions of the heart, liver, and kidney in male mice injected with TAM-loaded PLGA were detected. A histopathological investigation was also included. TAM-loaded PLGA offered better properties as a drug delivery system, nutritional supplements, and pharmaceutical products.

Keywords: Tamoxifen citrate (TAM), toxicity, antioxidant, animal model, biocompatibility, PLGA, P407.

تركيبات نانوية جديدة من سترات التاموكسفين محملة ضمن البوليمرات لتحسين العلاج والاستخدامات الطبية:دراسة في المختبر وفي داخل الجسم الحي

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الخلاصه

يعتبر عقار سترات التاموكسيفين (TAM) من الادوية الأكثر استخدامًا في العلاج الهرموني لسرطان

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الثدي. على الرغم من كونه شائعا في هذا المجال إلا أن هناك عائق في عملية اذابته بصورة كاملة ، لذلك تم تصميم هذه الدراسة لتحسين قابلية الذوبان وتقليل السمية المرتبطة في استخدامه. تم استخدام هذا النوع من البناء النانوي في مجموعة متنوعة من التطبيقات البيولوجية ، سواء في خارج أو داخل الجسم الحي. كان حجم اختبار انحلال الدم 4.6٪ عند 200 ميكروغرام مل$^{-1}$ ، مما يشير إلى أن هذا التركيب النانوي أدى إلى زيادة حماية لخلايا الدم الحمراء. قد يعني هذا أن بوليمر PLGA المحمّل بـ TAM يحمي خلايا الدم الحمراء بشكل فعال. لم يمنع TAM النقي ظهور انحلال الدم و ادى الى تحطيم واضح في خلايا الدم الحمراء ، وتم علاجه بواسطة بوليمر PLGA المحمل بـ TAM تم تقييم سلامة جزيء الحمض النووي من التدمير. كانت النتائج مذهلة بالنسبة للعقار الجديد TAM في كيفية حماية شريط الحمض النووي من التدمير عند 200 ميكروغرام مل $^{-1}$ ، كما تم دراسة في زيادة نشاط مضادات الأكسدة على TAM النقي. تم استخدام 1،1−2−diphenyl−picryl hydrazyl (DPPH)، ومقاييسات الكسح الجذري لبيروكسيد الهيدروجين في المختبر. أظهر بوليمر PLGAالمحمّل بـ TAM إجراء الكسح المعتمد على التركيز في نطاق 100−200 ميكروغرام مل $^{-1}$ ، مع نشاط أقصى قدره 79.2٪ عند 200 ميكروغرام مل. في حين أن TAM النقي انخفض بنسبة 60.57٪. بينما كان نشاط الضبط الإيجابي (فيتامين ج) 92.12٪. أخيرًا ، تم تقييم سلامة بوليمر PLGA المحملة بـ TAMفي داخل الجسم الحي. تم اختيار ذكور الفئران للكشف عن وظائف القلب والكبد والكلى. كما تم تضمين فحص الأنسجة المرضية. وفقًا للنتائج ، قد يوفر بوليمر PLGA المحمل بـ TAM خصائص أفضل كنظام توصيل الأدوية والمكملات الغذائية والمنتجات الصيدلانية.

Introduction:

Drug delivery systems have attracted considerable attention from cancer researchers for the improvement of drug features such as solubility, stability, and safety [1, 2]. While that Nanocarriers have got extensive concern as a promising carrier for chemotherapy in respect to their capability to carry drugs preferentially into tumor tissue [3]. New research techniques in the field of drug delivery have been enabled to thrive recently in nanoscale advances. Nanoparticles were developed as effective drug carriers with great interest. [4]. Pluronic (poloxamer) copolymers, widely used as nanocarriers, are surfactant moieties. These moieties consist of one lipophilic poly (propylene oxide) (PPO) and two lipophobic poly (ethylene oxide) (PEO) arranged in a PEO–PPO–PEO triblock [5]. Poloxamer triblock can be self-arranged into a spherical micelle temple constructed by propylene oxide (PO) as a lipophobic internal core and ethylene oxide (EO) as a lipophobic external coat [6]. This feature facilitates the encapsulation of the poorly water soluble drugs in the lipophilic core, which diminish the cytotoxicity of chemotherapeutics and sustained activity in patient due to longer circulation in the blood [7]. This new block exhibits several advantages such as solubility increments, metabolic stability, and sustained drug duration in the circulation. Pluronic copolymers are known as the inert carrier with low cytotoxicity [8]. Nanoparticle drug delivery systems have demonstrated the capacity to encapsulate a wide range of therapeutic agents, including small molecules (hydrophilic or hydrophobic), peptide protein-based medicines, and nucleic acids. By coating these molecules into a nanocarrier [9]. Polymeric nanoparticles provide substantial design freedom due to the utilization of various polymers derived from synthetic or natural sources. Some popular polymers used for nanoparticle production include polylactide-co-glycolide (PLGA), which is one of the most effective nanocarriers for targeted drug delivery [10].

PLGA (lactic-co-glycolic acid) is a linear natural Polymeric nanoparticle one of the best-studied biodegradable copolymers that breakdown into non-toxic compounds (H_2O and CO_2). PLGA is often made by catalyzing the ring-opening copolymerization of L-poly-lactic acid, and poly-glycolide is amorphous in the form [11]. *In vivo*, the ester bonds between the polymeric nanoparticle and its monomeric anions are hydrolyzed, causing it to disintegrate (lactate and glycolate). While D-lactate is not further metabolized before excretion, L-lactate

is transformed to CO_2 and then to pyruvate, which is then passed via the Krebs cycle. Glycolate, on the other hand, can be eliminated directly through the kidneys or oxidized to glyoxylate, which can then be converted to glycine, serine, and pyruvate. The latter can be converted into CO_2 and H_2O once more through the Krebs cycle [12]. High-affinity malignant tumors are targeted with PLGA nanoparticles coupled to targeting ligands. Large surface areas and functional groups are also beneficial for conjugating numerous diagnostic agents [13]. Nanoparticle carriers are more resistant to enzymatic metabolism than other colloidal carriers like liposomes or lipid vesicles in biological fluids [14].

Nanocapsules are nanoscale vesicular systems with a core-shell structure that confines a drug in a reservoir or cavity surrounded by a polymeric membrane or coating [15, 16]. TAM has been incorporated into drug-delivery systems, which has resulted in TAM molecules being given at a much lower concentration over a longer time, substantially lowering the risk of dose-dependent toxicity [17]. Only when a needed concentration of an active medication reaches its target location may desire therapeutic effectiveness be attained [18]. The body's defensive mechanism, or the presence of macrophages from the reticuloendothelial system and tumor-associated macrophages that destroy TAM or foreign molecules, may limit the optimal concentration at which TAM can be effective. As a result, in addition to being able to deliver TAM to tumors at a lower and safer dose, nano-enabled-formulations acting as carriers can protect hydrophobic TAM molecules from degradation by macrophages during their transportation through the bloodstream, extending their circulation time and ensuring that the required concentration of TAM reaches the tumor site [19].

Chemotherapeutic chemicals now available do not distinguish between normal and malignant cells, resulting in systemic toxicity and side effects. Some of these hormonal drugs are named estrogen blocker which works by stopping estrogen from helping breast cancer cells to grow such as tamoxifen, toremifene, and fulvestrant. This significantly reduces the drug's maximum permissible dosage [20, 21].

Tamoxifen citrate (TAM) is highly lipophilic non-steroidal triphenylethylene derivatives that has been the antiestrogen therapy for advanced or metastatic breast cancer for more than 20 years [22]. It has been used most commonly for the management of estrogen receptor breast cancer [23]. On oral administration, only a limited amount of TAM is absorbed and reaches the blood circulation due to the low water solubility and an extensive first-pass metabolism [24, 25]. However, most anticancer drugs that can successfully diminish the neoplastic cells exhibit remarkable side effects mainly because of the absence of precise targeting ability [26]. Despite the fact that TAM is widely utilized in the treatment of breast cancer, worries about TAM-induced endometrial and liver cancer have hindered its long-term therapeutic usage [27].

As mentioned previously, most TAM adverse effects are dosage and concentration dependent. As a result, it is plausible to assume that modest dosage is the key to balancing the advantages and disadvantages of TAM. Nanotechnology is well-known as one of the most effective techniques for improving therapeutic efficacy, safety profile, and precise medication delivery. Many nano-enabled devices have been created to transport TAM molecules and deliver them selectively to breast cancers, with great precision and low off target side effects, because of the beneficial characteristics of nanomaterials [18]. Thus, the current study was designed to evaluate the toxicity of pure TAM and the TAM-loaded PLGA formula and also their effect on the DNA molecule. In addition, the animal model was used to elucidate the toxicity issue against the heart, liver, and kidneys of mice.

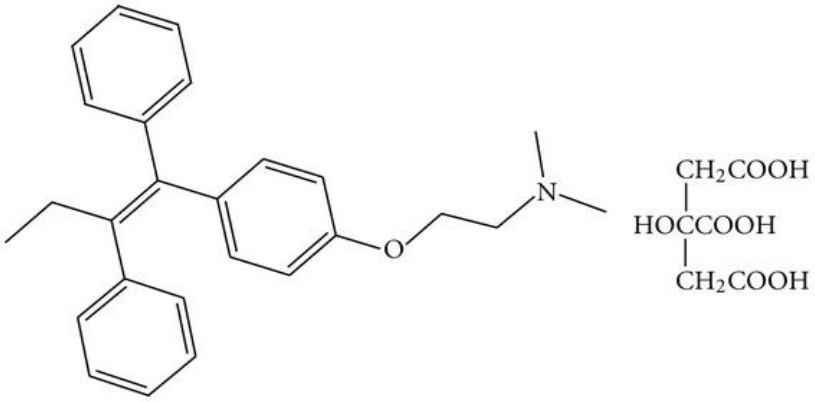

Figure 1- Chemical structure of Tamoxifen citrate (TAM) [28].

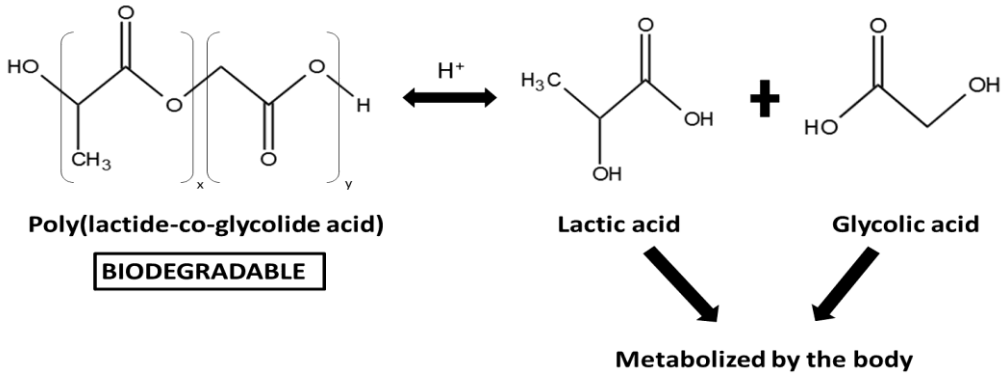

Figure 2-Chemical structure of PLGA [29].

MATERIAL AND METHODS

Chemicals and reagents

Ethanol and ethyl acetate (Chem-lab, Belgium), Tamoxifen citrate with (99% purity), Poloxamer 407(P407), PLGA poly(D, L-lactide –co- glycolide), ascorbic acid (Vitamin C) & 1-diphenyl-2-picrylhydrazyl (DPPH), All of these ingredient purchased from Sigma Chemical Co. USA, standard fish Deoxyribonucleic acid (DNA) (BDH, England).

Fabrication of TAM- loaded PLGA nanoparticles

TAM-Loaded PLGA was prepared by hydrotrope method as previously reported by Al-jubori, et al [30]. With minor modifications, briefly; TAM (50mg) was dissolved in 2 mL of ethanol, and (50 mg) of PLGA was dissolved in 2 mL of ethyl acetate and mixture together. The resulting solution was added drop wise by using a burette directly onto 10 mL of Poloxamer-407 solution (100 mg with 10 mL of deionized water) and also act as a surfactant and stabilizer. Then, the solution was kept immediately on sonication (50 Hz at 20 min), vortex, and mechanical stirrer at 1500 rpm for 30min at 25°C. temperature to obtaining a homogeneous solution. As a result, the mixture was stirred continuously at 500 rpm for 24 hours in order to evaporate the ethanol and ethyl acetate, as illustrated in Figure 3. The solution was then transferred to 20mL (deionized water) to promote the diffusion and maintained overnight at 25°C on a magnetic stirrer. To separate free unbound chemicals and stabilizer in the solution, the homogeneous solution was centrifuged at 13,000 rpm for 20 minutes. After that, the system was left at 25°C for 24 hours to reach equilibrium.

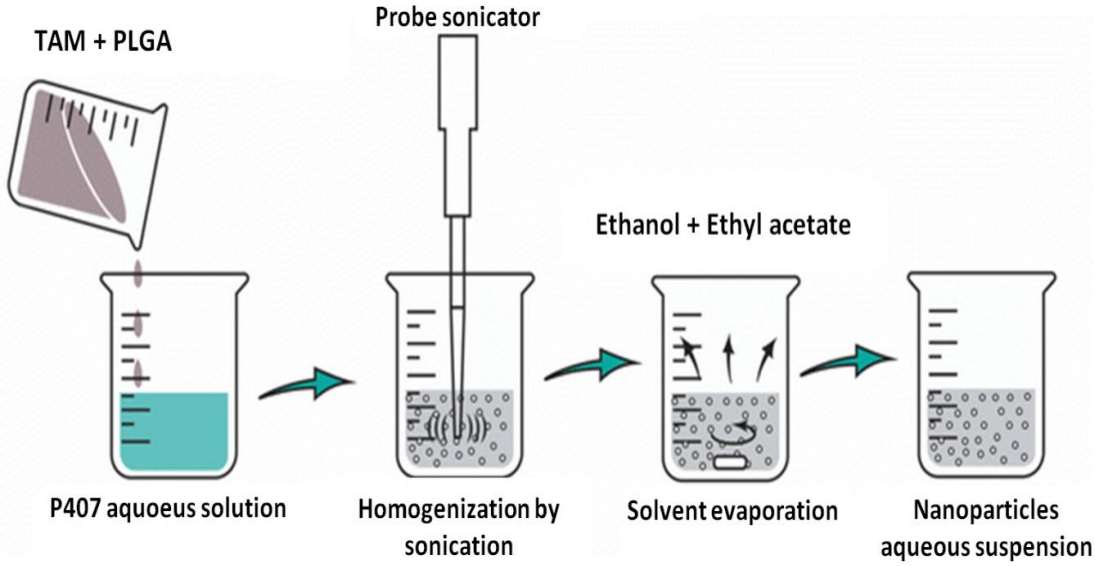

Figure 3- Preparation of TAM-loaded PLGA nanoparticles. P407:poloxamer407

Human blood collecting

Human blood samples were collected from five healthy volunteers (15 mL)and divided into tubes containing the anticoagulant agent (heparin "10 µL"), as directed by the National Institute of Health and the Food and Drug Administration, as well as the Declaration and Regulation of Helsinki as a statement of ethical principles.

Blood compatibility studies

The hemolytic toxicity of pure TAM and TAM-loaded PLGA was evaluated using the method described by Sulaiman et al. [31]. Briefly, 200 µL of the blood was mixed with 1600 µL normal saline. 200 µL of different concentrations of pure TAM and TAM-loaded PLGA (ranging from 100, 150, and 200µg mL^{-1} in three experiments) were added to the blood samples together with the controls. The distilled water was utilized as a positive control (100% lysis) and normal saline as negative control (0% lysis). All samples were incubated in a water bath for 1 h. at 37°C, one drop from each blood sample was taken to make a smear on the slide and was left to dry at room temperature. The samples were then centrifuged for 3 min at 700 rpm. The absorbance of each sample was measured using UV-Vis spectrophotometry at 541 nm and the percent of hemolysis was measured utilizing the following equation:

$$\textbf{Hemolysis } \% = \left(\frac{\textbf{Abs} - \textbf{Abc}\,(-\textbf{ve})}{\textbf{Abc}\,(+\textbf{ve}) - \textbf{Ab}\,(-\textbf{ve})}\right) \times \textbf{100} \qquad (\textbf{1})$$

When (Abs) is the sample absorbency, (Abc) is the absorbency of negative control and positive control, respectively. Blood smears were stained with Lishman stain for 15 min and fixed with 96% ethanol and then washed with D.W. The red blood cells were examined under a light microscope at a magnification of 40x (H.P.F.).

DNA Damage Assay

Standard fish DNA samples with an absorbance ratio of about A260/A280 were treated with different concentrations (100, 150, and 200 µg mL^{-1}), equal volumes of pure TAM and TAM-loaded PLGA, and standard DNA solution was mixed with a presence of 1×10^{-5} M hydrogen peroxide (H_2O_2) solution and then incubated for about 10 minutes at 37°C, after which the effect was determined by measuring the wavelength of the solution [32].

Antioxidant activity by (1, 1-diphenyl-2-picrylhydrazyl) DPPH radical scavenging assay

The DPPH test technique is based on a steady free radical decrease in DPPH. With a curious electron, the free radical DPPH ensures maximal absorption at 517 nm (purple color). In the presence of a hydrogen donor reduced to the DPPHH* and, as a result, the absorption's lowered result in the decline (yellow) is the next step, if antioxidants react with DPPH which is a stabilic free radical [34]. Absolute ethanol 0.5 mL was added to different test tubes and mixed with 0.5mL of different concentrations of pure TAM and TAM-loaded PLGA (100, 150, and 200 µg mL^{-1} in three experiments) and then 0.5 mL of DPPH was added to all test tubes and brooded about 30 min at room temperature. The DPPH with ethanol only was used as negative control and the positive control was 5 µg mL^{-1} of vitamin C. After that the absorbance of all samples was tested at 517 nm utilizing UV-VIS spectrophotometry. The percent of DPPH scavenging efficacy was measured utilizing the next formula:

$$\textbf{Anti} - \textbf{oxidant capacity}\ (\%) = \frac{\textbf{Ac} - \textbf{As}}{\textbf{Ac}} \times \textbf{100} \qquad (\textbf{2})$$

AC represents the peak intensity of DPPH and AS is the peak of pure TAM or TAM-loaded PLGA sample solvent [33].

Histopathology study

Experimental design and samples collection

Nine Swiss albino male mice weighing 24 - 28 g and aged 4 to 8 weeks were utilized in this study. Mice were kindly provided by Iraqi Center for Cancer and Medical Genetic Researches, Mustansiriyah University, Baghdad, Iraq. The mice were housed in a 12:12 hour light/dark cycle with a constant supply of water and food at a temperature of 24 ° C. and a humidity of 55%. All procedures were confirmed by the Animal Care and Ethics Committee at Biotechnology Division, Applied Sciences Department, University of Technology, Baghdad, Iraq.

These mice were assigned at random to one of three groups (three mice per group): Animals in Group I were not given any treatment and were instead given normal saline as a control. Animals in Group II were given a low dose (100 mg Kg) of TAM-loaded PLGA, whereas those in Group III were given a high dose (200 mg Kg) of TAM-loaded PLGA. The intraperitoneal injection was given to the treated groups for 14 days (single dose per day; 0.20-0.25 mL). The body weights of mice were recorded during the experiment [34]. The mice were given chloroform and sacrificed after 14 days. Additionally, for the histological examination of the selected organs in non-treated and treated mice, these organs (heart, liver, and kidney) were washed in PBS before being fixed in 10% formalin and embedded in paraffin. And a microtome was used to cut sections, which were then stained with hematoxylin and eosin (H&E). The sections were processed and stained in accordance with a standard method employed in histopathology laboratories [35].

Statistical data analysis

The obtained data were statistically analyzed by utilizing ANOVA (analysis of variance) by using SPSS software (SPSS/16.0; SPSS Inc., Chicago, IL, USA). Results are represented as the mean ± S.D. of the three independent experiments of each test [36].

RESULT AND DISCUSSION

Fabrication of TAM-loaded PLGA nanoparticles

The TAM-loaded PLGA is depicted in Figure 4. TAM-loaded PLGA were first formulated through dilution and then increased their water solubility using the hydrotrope technique, as shown in the figure. An organic PLGA polymer solution is emulsified in an aqueous solution with the help of a surfactant in this method. The organic solvent is removed by stirring, and nanoparticles are formed. As can be seen in Figure 5, the poloxamer is stable and homogeneous for a long time. When attempting to dissolve pure TAM in water, we noticed that it is extremely difficult to dissolve due to the foggy appearance of the solution and the presence of clear insoluble particles. But after applying the precipitation technique and converting pure TAM to nanotamoxifen by changing the color of the mixture to white, we found that the developed TAM became homogeneous and completely soluble. Modified TAM increased water solubility might be related to their greater surface area, which aids in dissolving. Drug solubility is frequently inversely proportional to particle size; as a particle gets smaller, the surface area to volume ratio rises. Because of the increased surface area, there is more contact with the solvent, resulting in an increase in solubility [37]. Previous research has discovered a hyperbolic association between particle size and surface specific dissolving rate when solubility is taken into account. Similar outcomes have been shown when the particle size of active substances is reduced to nanoparticle size, resulting in increased solubility and bioavailability [30, 38].

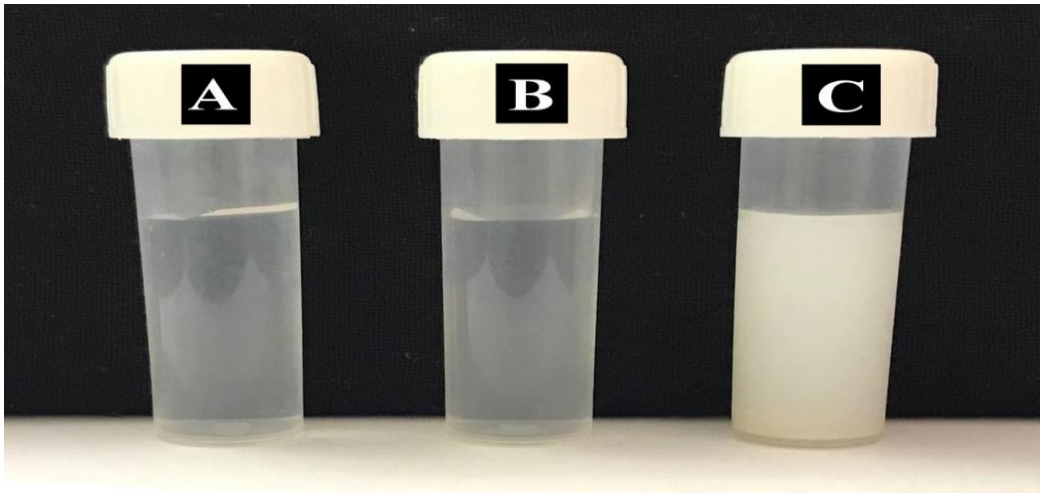

Figure 4- chemical reaction stages for PLGA loaded with TAM, then color change is detected with reaction **A**: TAM, **B**: PLGA, **C**: TAM-loaded PLGA.

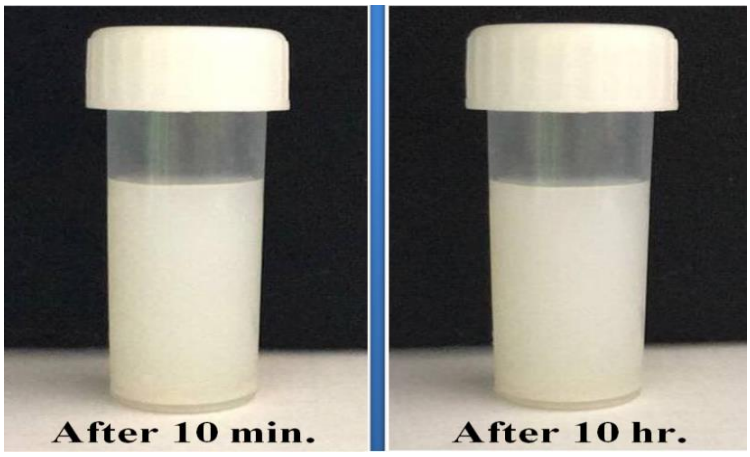

Figure 5- The high stability of the TAM-loaded PLGA after 10 hours of preparation.

Blood compatibility studies

Any parenteral delivery system must have low or negligible hemolytic activity. As a result, blood interaction studies were carried out on all formulations, including pure TAM and TAM-loaded PLGA. TAM-loaded PLGA was shown to have fewer effects on RBCs than pure TAM. The hemolysis activity of RBCs was measured after treatment with three concentrations (100, 150, and 200 µg mL^{-1}), as well as normal saline and distilled water as negative and positive controls, respectively. On RBCs, the effects of TAM-loaded PLGA were found to be minor. At concentrations of 100 to 200 µg mL^{-1}, the percent of hemolysis of pure TAM was above the permitted threshold (8.8, 12.3, and 19.9% respectively) but in TAM-loaded PLGA was below than that (2.8, 3.8, and 4.6 %, respectively) of less than 5% hemolysis. It was discovered that TAM-loaded PLGA had lesser impacts on RBCs than pure TAM. This finding might be explained by the surfactant characteristics of PLGA, which allowed for a higher quantity of TAM to be released in comparison to the hemolysis assay, which did not utilize PLGA owing to its intrinsic hemolytic function. As a result of the longer drug release from nanoparticles, TAM-loaded PLGA showed minimal hemolysis, resulting in a shorter duration of interaction between erythrocytes and the released drug. Blank nanoparticles did not induce cellular harm, implying that there are no interactions between nanoparticles and erythrocytes (the sizes of the synthesized nanoparticles were 200–205 nm as determined earlier by Al-jubori, et al) [30]. Nanostructured systems have the potential to lower the toxicity of hemolytic drugs. Studies have shown that using nanoparticles reduces the cytotoxicity of medicines having a high hemolytic potential, which supports the findings of this study. Because of the high polyunsaturated fatty acid composition of their membranes and their high cellular oxygen and hemoglobin concentrations, erythrocytes are particularly vulnerable to oxidative damage [39]. TAM is cytotoxic to Erythrocytes. It has a strong potential for inducing hemolytic anemia in human erythrocytes. TAM damages erythrocyte membrane structure via interacting with membrane proteins and/or changing the cytoskeleton's framework, causing structural rupture [40, 41].

Over the examined lower concentration range, notably at TAM-loaded PLGA Light microscopy picture of RBCs shown in Figure 6, the images of hemolysis assay indicated no detrimental action on the RBCs morphology. Native TAM, on the other hand, had more faulty effects on RBCs, causing cell membrane breakdown, and this effect was similar to that of the positive control. These findings, together with dosages ranging from 100 to 200 µg mL^{-1}, indicated that successful TAM-loaded PLGA was ready for systemic delivery with less than 5% hemolysis, as determined by the ASTM E2524-08 standard. (Test Method for Analysis of Hemolytic Properties of Nanoparticles) [42].

This is in agreement with previous research, pure TAM exhibited a high percentage of hemolysis, and both pure TAM concentrations showed hemolysis higher-level to 80% ($p > 0.05$). Conversely, TAM-NPs, exhibited negligible hemolytic activity, independent of TAM concentration ($p < 0.05$). Nanoparticles showed no erythrocyte lysis suggesting the polymer exhibits biocompatibility [43].

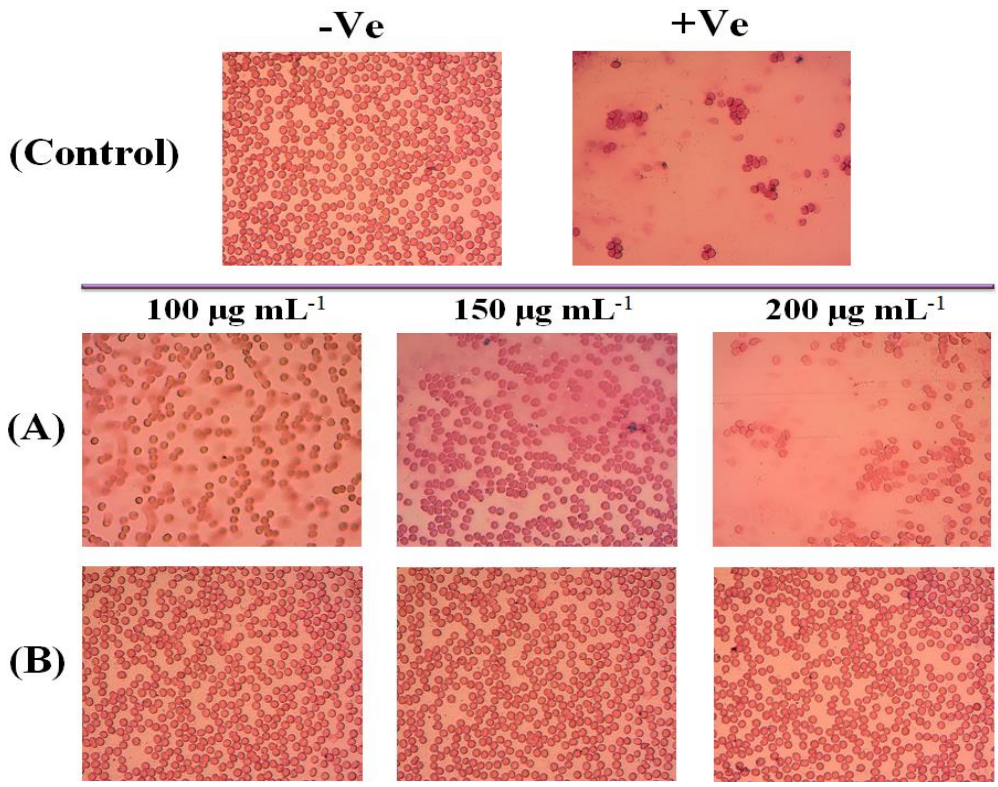

Figure 6- Light microscopic images showing the hemolytic toxicity **(A)** pure TAM **(B)** TAM-loaded PLGA at concentrations (100, 150, and 200 µg mL^{-1}) along with the positive and negative controls.

DNA Damage Assay

One of the relevant models for measuring the protective ability of pure TAM and TAM-loaded PLGA is DNA damage induced by hydrogen peroxide (H_2O_2). H_2O_2 is a non-radical yet highly reactive molecule that is generated in large quantities in living cells [44]. H_2O_2 is the result of O_2•- dismutation and other enzyme-mediated processes, and it participates in the oxidation reaction via a non-radical pathway [45]. As demonstrated in table 1, adding pure TAM and TAM-loaded PLGA in three concentrations (100, 150, and 200µgmL^{-1}) reduced H_2O_2-induced DNA damage. We noticed that when treating DNA with TAM-loaded PLGA, it had much less effective than pure TAM and there was a significant difference between the means ($P \leq 0.05$).

These findings suggest that DNA damage accumulation is a factor in pure TAM-induced cytotoxicity. Indeed, previous *in vitro* investigations in mouse and rat models revealed that TAM causes DNA single and double-strand breaks, as well as DNA adducts, through chromosomal breakage and the production of free radical species [46, 47].

Moreover, previous research has confirmed that long-term TAM treatment is genotoxic, and subsequent investigations have identified various genotoxic consequences of TAM *in vivo*. In female Wistar-Hannover rats, induction of micronuclei and reduction of polychromatic erythrocyte production was shown in bone marrow cells [48, 49]. TAM-loaded PLGA was more successful in this respect; as the concentration is increased, they show reduced absorbency, especially when treated with 200 µgmL^{-1} .as we demonstrated how DNA was prevented from breaking down.

Table 1- It demonstrates the effect of drugs pure TAM and TAM-loaded PLGA on the DNA molecule and its preservation from the effect of the oxidation reaction.

Compounds	Concentration (µg mL^{-1})	DNA	H_2O_2 + DNA (1×10^{-5}) M	H_2O_2+ DNA (1×10^{-5}) M +Compound
Pure TAM	0.0	0.244±0.023	0.329	0.329±0.01a*
	100			0.328±0.011a
	150			0.318±0.03a
	200			0.293±0.05b
TAM-loaded PLGA	0.0	0.244±0.023	0.329	0.329±0.01a
	100			0.296±0.06b
	150			0.277±0.04b
	200			0.269±0.03b

*Similar letters indicate non-significant differences and different latters indicate significant differences at $P \leq 0.05$

Antioxidant capacity of (DPPH radical scavenging assay)

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging process is based on electron transfer and produces a deep purple color in ethanol solution [50]. When they interact with an antioxidant molecule as a hydrogen donor, these free radicals, which are stable at 25°C, change colorless to pale yellow. The amounts of antioxidants have a direct relationship with the reduction in absorption. The antioxidant activity of pure TAM and TAM-loaded PLGA at three different concentrations is shown in Figure 7. Depending on the concentration inhibition observed, the results show that TAM-loaded PLGA has a higher scavenging capacity than pure TAM. The findings show that at 200µgmL^{-1}, pure TAM reduces the amount of the DPPH free radical in a concentration-dependent manner, which is better than the other concentrations in a concentration-dependent manner. Achieving 60.57% antioxidant activity while 92.12% inhibition of the positive control compared to TAM-loaded PLGA efficiency 79.2%, which has a higher activity of reduced DPPH radical scavenging compared to TAM-loaded PLGA efficiency, which has a higher activity of reduced DPPH radical scavenge the results showed that TAM-loaded PLGA inhibits DPPH more effectively than pure TAM, which might be explained by enhanced dissolvability.

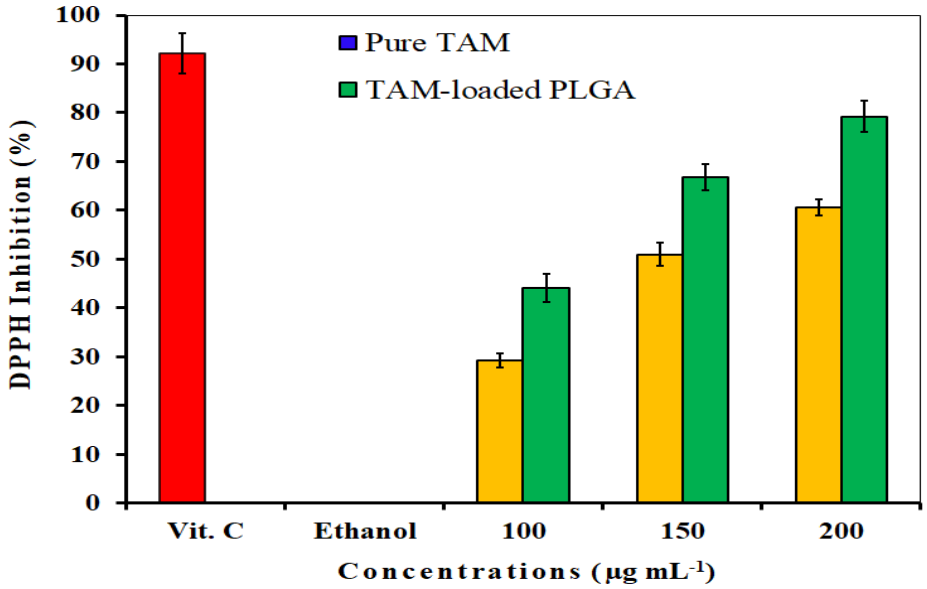

Figure 7-DPPH free radical scavenging activity of pure TAM (yellow) and TAM-loaded PLGA (green) at three different concentrations (100, 150, and 200µgmL^{-1}).

Histopathology study

Based on the *in vitro* evaluations, the *in vivo* experiments were further investigated to evaluate the toxicity of TAM-loaded PLGA nanoparticles using mice model (Figure 8). It can be seen that administration with TAM-loaded PLGA nanoparticles in the dose range of 100 and 200 mg Kg^{-1} for 14 days did not cause mortality, and no statistically significant differences ($P \geq 0.05$) in the body weight of mice were observed between the TAM-loaded PLGA nanoparticles -treated mice and control mice. However, the body weight of mice before treatment was 24.0±0.40 and 25.5±0.82, respectively, while, after treatment was 30.8±0.80 and 31.2±0.64, respectively. Furthermore, neither the control nor the treated groups showed any aberrant clinical symptoms or behavior. TAM-loaded PLGA nanoparticles therapy did not appear to cause any harm in mice, and they stayed healthy until the end of the trial. At the conclusion of the experiment, necropsy revealed no macroscopic organ alterations in the treated groups. According to a previous study, TAM nanoparticles did not display any death or odd behaviors, such as vocalizations, hard breathing, difficulties moving, or any abnormal interactions with cage mates. There were also no clinical signs such as redness, swelling, difficulty moving, or odd behaviors [30].

Furthermore, the heart, kidneys, and liver tissues of mice in the control and treatment groups were evaluated histopathologically to assess if two doses of TAM-loaded PLGA nanoparticles caused tissue damage, inflammation, or lesions, (Figure 8). The animals given TAM-loaded PLGA nanoparticles showed no visible harm or histological abnormalities. The hepatic cords, mild vacuolar lobules, and hepatic lobules of the animals in different treatment groups showed no significant changes. In the treated groups' kidney tissues, which were identical to those of the control groups, there was no atrophy of the glomerular and renal tubular epithelial cells. The histological study of the heart showed the cardiac muscle, the myocardium, consists of cross-striated muscle cells, and cardiomyocytes, with one centrally placed nucleus. Cardiac muscles showed intercalated discs which are specialized junctions between cardiac cells with no increased cytoplasmic vacuolization, myofibrillar loss. Therefore, the animals' hearts, livers, and kidneys did not show any histopathological abnormalities after being exposed to TAM-loaded PLGA, indicating that TAM-loaded PLGA has the potential for *in vivo* applications. According to a previous study, Jian et al. showed that TAM-PLGA (poly D, L-lactic-co-glycolic acid)-NPs significantly reduced the hepatotoxicity, while co-encapsulation of QT (quercetin) and TAM in PLGANPs fully eliminated TAM induced hepatotoxicity [51].

Moreover, when coupled with TAM and put into nanostructured lipid carriers, sulforaphane substantially decreased TAM toxicity. When combined with TAM and loaded into nanostructured lipid carriers, sulforaphane significantly reduced the toxicity associated with TAM. Showed that TAM-PLGA (poly D, L-lactic-co-glycolic acid)-NPs significantly reduced the hepatotoxicity, while co-encapsulation of QT (quercetin) and TAM in PLGANPs fully eliminated TAM induced hepatotoxicity [51]. Furthermore, when combined with TAM and loaded into nanostructured lipid carriers, sulforaphane significantly reduced the toxicity associated with TAM [52].

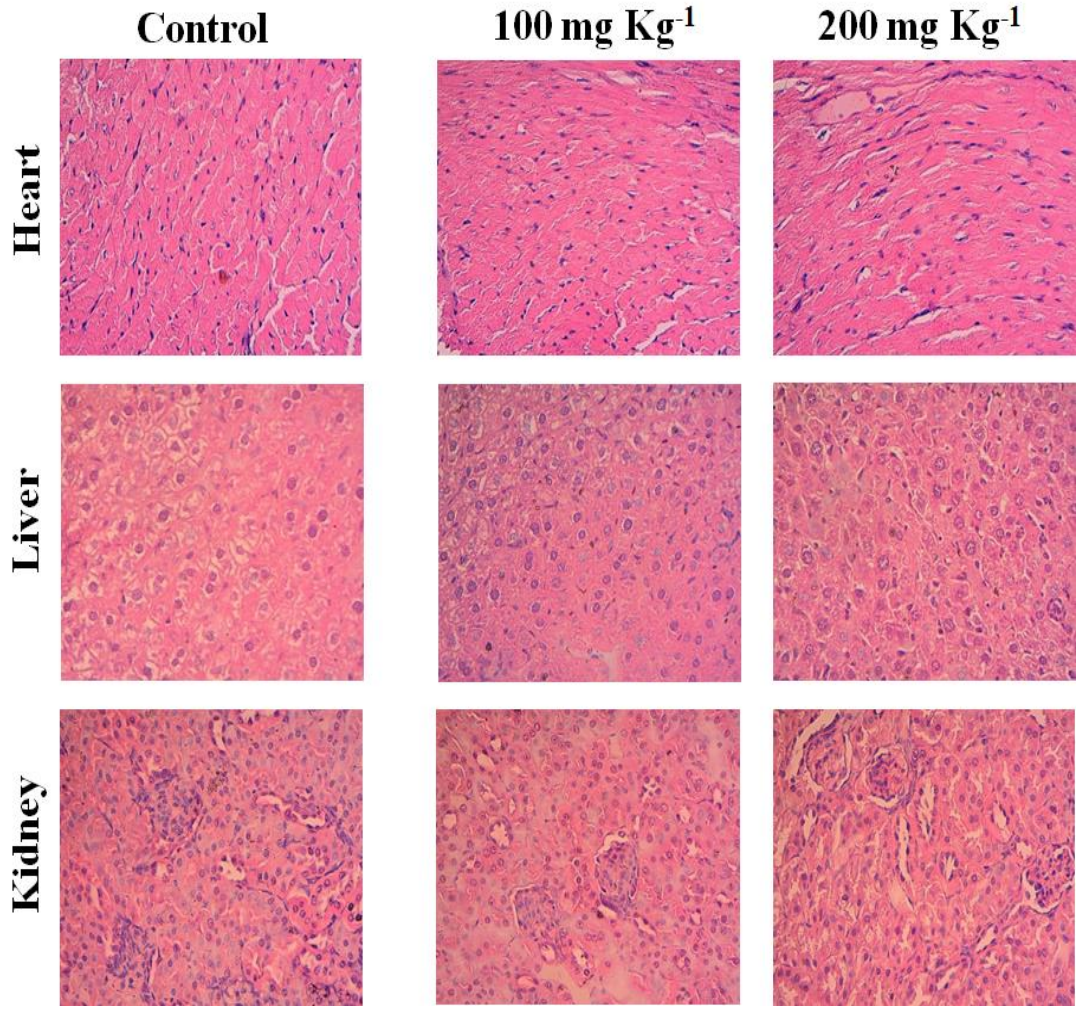

Figure 8- Histological images of the heart, liver, and kidney of mice treated with TAM-loaded PLGA by intraperitoneal injection at various dosages (100 mg Kg^{-1}, 200 mg Kg^{-1}).

Conclusions

According to the findings of this study, TAM-loaded PLGA was successfully synthesized using the hydrotrope method. Furthermore, certain trials were undertaken to demonstrate the bio-viability of the novel construct, and it was shown to be therapeutically effective, safe, and biocompatible for human red blood cells, with no toxicity and no harmful side effects or behavioral abnormalities in mice. It was also put to the test in terms of retaining the DNA molecule, with promising results. For this reason, it might be desirable to employ this formula in further studies using larger animal models in order to confirm it as a therapeutic agent for its durable biological activities, in particular for the prevention or treatment of various human disorders.

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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