Therapeutic efficacy and toxicity of tamoxifen loaded PLA nanoparticles for breast cancer

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This study was carried out to assess the therapeutic efficacy and toxicity of tamoxifen (Tmx) loaded poly(ε-l-lactic acid) (PLA) nanoparticles (Tmx-NPs) for breast cancer. An in vivo study was conducted to determine the effect of Tmx-NPs on DMBA induced mammary tumor in female Wistar rat. The experimental results showed that the mean diameter of Tmx-NPs was 224 ± 2 nm with 68 ± 2% (w/w) of entrapment efficiency. In in vivo study, the tumor size in rat was significantly reduced (P < 0.001) by treating Tmx-NPs as compared to pure Tmx and untreated group (control DMBA). Tmx-NPs showed the marked reduction in hepatotoxicity and renal toxicity when compared to pure Tmx as evidenced by histopathological examination of liver and kidney tissues as well as estimation of AST, ALT levels, and creatinine, urea, blood urea nitrogen levels. Oxidative stress and lipid peroxidation was estimated in spleen, liver and kidney and was found significantly high in pure Tmx treated group as compared to Tmx-NPs and control group. Immunological parameters like blastogenic response of splenocytes, TLC, DLC were studied and found significantly high in pure Tmx treated group but the variations were nonsignificant in Tmx-NPs group as compared to control. Thus, Tmx-NPs have significant therapeutic efficacy with reduced side effects.

1. Introduction

Breast cancer is a major health problem worldwide and is second leading cause of cancer death especially for women. Chemotherapy is a very complicated and high risk procedure due to its toxicity. Patients tolerate severe side effects even after successful chemotherapy [1,2]. One of the major problems of cancer chemotherapy is to administer the required therapeutic concentration of drug at the tumor site for the desired period of time without causing undesirable effects on other organs [3,4]. Tamoxifen is an estrogen receptor modulator [5] that exhibits good bioavailability upon oral administration [6]. Following long term therapy of tamoxifen causes major side effects. To overcome the undesirable side effects of tamoxifen and increase the concentration at the tumor site, tamoxifen has been entrapped in various polymeric nanoparticles to improve better delivery by increasing local concentration of the drug at the receptor site [7,8]. Various anticancer drugs have been entrapped in various polymeric nanoparticles to enhance their activity by reducing side effects [9–11]. Various biodegradable polymeric nanoparticles have been extensively used for controlled delivery of active molecules and drugs [12–16]. The polymeric nanoparticles have the potential to act as a carrier of drugs at target sites by enhancing the biological activity and reducing the adverse side effects [17,18]. Various biodegradable polymers such as poly(lactic-co-glycolic acid) (PLGA), poly(ε-caprolactone) (PCL), chitosan, gelatin and poly(alkyl cyanoacrylates) have been extensively used as polymeric nanoparticles for targeted delivery of drugs related to cancer, diabetes, malaria and other harmful diseases [19–23]. Among them, PLA has been also approved by Food and Drug Administration (FDA) for the clinical uses as a carrier of drug delivery processes [24]. During this process, when polymeric nanoparticles are administered orally, the M-cells (specialized cells staying over mucosa-associated lymphoid tissue) in payer’s patches uptake the nanoparticles and transport them from the gut lumen to intraepithelial lymphoid cells and then through the lymphatic system into the blood stream [25–27]. Polymeric nanoparticles follow this particular way and thus improve the bioavailability of encapsulated drug by avoiding the enzymatic degradation in enterocytes. In the present study, tamoxifen loaded PLA nanoparticles (Tmx-NPs) have been developed, optimized and characterized. The dried Tmx-NPs have been explored for in vitro release characteristics and in vivo
antitumor efficacy. Hepatotoxicity, renal toxicity, oxidative stress and lipid peroxidation have been evaluated after administration of pure Tmx and Tmx-NPs. General immunological parameters have also been evaluated after administration of Tmx and Tmx-NPs.

2. Materials and methods

2.1. Materials

Poly(N-isopropyl acrylamide) (PNI) (Mn ~ 120,000) was obtained from Biomer, Germany. Poly (vinyl alcohol) (PVA) (Mw ~ 30,000), tamoxifen and 7,12-dimethylbenz(a)anthracene (DMBA) were purchased from Sigma, USA. Dichloromethane (DCM) was obtained from Qualigens Fine Chemical, India. All other reagents were used of analytical grade for in vitro and in vivo studies.

2.2. Animals and management

Female Wistar rats of 100 ± 10 g and 90 days old were obtained from the central animal facility of Department of Zoology, Banaras Hindu University, Varanasi, India. The animals were acclimatized at temperature of 25 ± 2 °C and relative humidity of 50–60% under natural light/dark conditions for one week before experiments. The animals were fed upon commercially available mice feed pellet and water ad libitum. In vivo studies were conducted in accordance with Institutional practice and within the framework of experimental animals (Scientific Procedure) Act 2007, of the Committee for the Purpose of Supervision and Control on Experiments on Animals (CPSCEA), Government of India, on animal welfare.

2.3. Nanoparticles preparation

Tamoxifen loaded PLA nanoparticles (Tmx-NPs) were prepared using the modified spontaneous nanoprecipitation method [28–30]. Briefly, different concentrations of Tmx and PLA were prepared by dissolving in dichloromethane (DCM). The resulting organic solution was then added drop wise into the aqueous phase containing PVA (emulsifier) under high speed stirring using vortex. The resulting suspension was allowed for mechanical stirring at room temperature (~25 °C) to evaporate the organic solvent. Nanoparticles were collected by ultracentrifugation (15,000 × g, 30 min, 10 °C) of the suspension and were washed with distilled water at least three times. DCM was removed from nanoparticles suspension using rotor evaporation technique under reduced pressure at 37 °C to get dried powder of nanoparticles. The final dried nanoparticles were stored at 4 °C until further use.

2.4. Optimization of tamoxifen loaded PLA nanoparticles (Tmx-NPs)

Investigation was carried out to optimize the different conditions to get high entrapment efficiency and preferred size of nanoparticles intended as delivery of Tmx for breast cancer. Tmx-NPs were prepared by emulsified nanoprecipitation method to encapsulate Tmx a lipophilic antiestrogenic drug into the PLA matrix. Tmx solution was prepared by mixing into an organic phase composed of DCM as solvent and dissolved PLA. The solution of Tmx and PLA was mixed and solubilized properly using vortex. For emulsification, the prepared organic solution was dispersed in an aqueous phase containing PVA as emulsifier and stabilizer. Briefly, different concentration of Tmx (5.0–15%, w/w) was prepared with DCM containing 10–30 mg/ml dissolved PLA. The concentration of PVA was changed in the range of 1.0–3.0% (w/v) aqueous solution. The resulting PLA solution including Tmx was added into PVA emulsifier for oil-in-water (o/w) emulsion. This emulsion was broken down into nanodroplets by applying external energy (sonicator) and these nanodroplets form nanoparticles upon evaporation of the highly volatile organic solvent (DCM) under reduced pressure at 37 °C. The surrounding temperature of emulsification nanoprecipitation process was varied from 20 to 35 °C for development of desired Tmx-NPs with high entrapment efficiency.

2.5. Nanoparticles characterization

Particle size and its distribution were measured by using Delsa, Beckman Coulter particle size analyzer. Nanosuspensions were diluted with ultrapure water for the analysis. Surface morphology of nanoparticles was determined using scanning electron microscope (SEM), Zeiss, EVO-LS-10. Samples of nanosuspension were prepared by placing one drop on a glass plate and dried under reduced pressure. Nanoparticles were gold coated using sputtering apparatus before observation in SEM. Transmission electron microscopy (TEM: Tecnai 12 G², FEI, The Netherlands) was performed to visualize the nature and the size distribution of Tmx-NPs. Thermal properties have been measured using Mettler 832 DSC at the scan rate of 10 °C per minute to compare the polymer and drug loaded particles (Tmx-NPs). 2.64 mg of Tmx-NPs as sample size was used for DSC. Fourier transform infrared (FTIR) spectroscopy (Nicolet-6700, USA) measurements were performed to understand the nature of interaction in drug loaded samples.

2.6. In vivo study

Animals were distributed into four groups containing six animals in each group. Pure Tmx suspension and Tmx-NPs suspension were administered orally to first and second group of animals respectively at a dose of 10 mg/kg body weight. Third group of animals act as control group (healthy group) by orally receiving PBS (pH 7.4) and fourth group was not treated by any formulation after DMBA induction that was represented as control DMBA group.

Female Wistar rats of 100 ± 10 g and 90 days old were used as chemical induced breast cancer animal model. A solution of 7,12-dimethylbenz(a)anthracene (DMBA) in soya bean oil was prepared and administered orally to rats at 60 mg/kg dose at weekly interval for four successive weeks. Tumor size was measured in rats and tumor bearing rats were separated randomly into different treatment groups. Drug treatment was started after two months of the last dose of DMBA. Animals were treated once in three days repeatedly of pure Tmx suspension (group A) and Tmx-NPs suspension (group B) both in a dose of 10 mg/kg body weight. The control group C (healthy group) was received PBS (pH 7.4) orally and the control DMBA group D was untreated group. The tumor size and percentage weight loss of the animals was observed during treatment period.

2.7. Toxicity evaluation

After treatment, the animals were sacrificed and blood was collected by cardiac puncture for all biochemical estimation. Hepatotoxicity and renal toxicity were evaluated by measuring toxicity markers like aspartate aminotransferase (AST), alanine aminotransferase (ALT) levels, and creatinine (CRE), urea, blood urea nitrogen (BUN) levels by commercially available diagnostic kits (Span Diagnostics Ltd., India). Oxidative stress was measured by estimating anti-oxidant enzymes like superoxide dismutase (SOD) and catalase (CAT) level in spleen, liver and kidney tissues. Lipid peroxidation was detected by estimating malondialdehyde (MDA) level in spleen, liver and kidney. Immunological parameters like blastogenic response (% stimulation ratio) of splenocytes, and TLC, DLC were studied for evaluation of inflammatory/immunogenic side effects.
2.8. Histological evaluation

Histological analysis of liver and kidney of each experimental group was carried out by the method of Kharwar and Haldar [31]. The tissues that were perfused in saline and formalin were fixed for 7 days in 10% formaldehyde after which dehydration was carried out in ascending grade of alcohol. The tissues were then cleared by xylene to remove the alcohol for overnight. Infiltration/impregnation was prepared in molten soft paraffin wax at 62 °C for 1 h each. Embedding and casting in paraffin wax with wooden block was carried out and sectioning of 5 μm thick using a microtome (Leica RM2245, Germany). The sectioned tissues of liver and kidney were spread on slides using a thin film of gelatin smeared on each slide. The sections were de-paraffinized in xylene, dehydrated by passing through grades of alcohol, stained with hematoxylin and eosin, mounted in neutral DPX medium and finally slides were evaluated for pathological changes under microscope.

2.9. Biochemical estimation

2.9.1. Assessment of total leukocyte count (TLC) and differential leukocyte count (DLC)

Total leukocyte count (TLC) and differential leukocyte count (DLC) were assessed following the staining method of Singh and Haldar [32]. In brief, blood was collected in a sterile tube and later into a WBC pipette of Haemometer (Spencer, USA) and diluted 20 times in Turk’s fluid (2.0 ml glacial acetic acid, 0.1 g mercuric chloride, one drop aniline, and 0.2 g Gentian Violet). The number of white blood cells was counted (cells × 10³ mm⁻³) in a Neubauer counting chamber (Spencer, USA), under oil immersion lens of Nikon Microscope (Nikon, Japan). Thin film of blood was prepared and stained with Leishman’s stain and differential leukocyte number was counted under oil immersion lens of Nikon microscope (Nikon, Japan).

2.9.2. Estimation of serum urea, blood urea nitrogen (BUN) and creatinine

Urea and BUN were estimated by commercially available urea estimation kit (Span Diagnostics Ltd., Surat, India) following manufacturer’s protocol. The assay followed the principle of Berthelot method (End Point Assay). In brief, urea is hydrolyzed in presence of water and urease to produce ammonia and carbon dioxide. Under alkaline condition ammonia is formed which reacts with hypochlorite and phenolic chromogen to form colored indophenols which was measured at 578 nm in an ELISA reader (BioTek, USA).

The serum creatinine level was estimated by commercially available creatinine estimation kit (Span Diagnostics Ltd., Surat, India). In brief, serum creatinine when reacts with alkaline picrate solution produces a yellowish red complex which was measured by at 490 nm using ELISA reader (BioTek, USA).

2.9.3. Estimation of superoxide dismutase (SOD)

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed following the method of Das et al. [33]. Just after sacrifice, the desired tissues were taken out, cleaned in chilled PBS and 10% homogenates of all tissues were prepared in 150 mM phosphate buffered saline (pH 7.4) and centrifuged for 30 min at 12,000 × g at 4 °C and then processed for enzyme activity based on a modified spectrophotometric method using nitrite formation by superoxide radicals. 0.5 ml of homogenate was added to 1.4 ml of reaction mixture comprised of 50 mM phosphate buffer (pH 7.4), 20 mM L-methionine, 1% (v/v) Triton X–100, 10 mM hydroxylamine hydrochloride, 50 mM ethylene diamine tetraacetic acid (EDTA) followed by a brief pre-incubation at 37 °C for 5 min. Next, 0.8 ml of riboflavin was added to all samples along with a control containing buffer instead of sample and then exposed to two 20 W fluorescent lamps fitted parallel to each other in an aluminum foil coated wooden box. After 10 min of exposure, 1 ml of Greiss reagent was added and absorbance of the color formed was measured at 543 nm using ELISA reader (BioTek, USA). One unit of enzyme activity is defined as the amount of SOD inhibiting 50% of nitrite formation under assay conditions.

2.9.4. Estimation of catalase (CAT)

Catalase (EC 1.11.1.6) activity was measured following the procedure of Sinha [34]. This method is based on the principle that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H₂O₂, with the formation of perchormic acid as an unstable intermediate. The chromic acetate thus produced is measured colorimetrically. The Catalase activity is allowed to split H₂O₂ for different periods of time. The reaction is stopped at a definite time by the addition of dichromate/acetic acid mixture and the remaining H₂O₂ is determined by measuring chromic acetate colorimetrically after heating the reaction mixture. There is production of green color at the end of the process. Immediately after sacrifice, 20% homogenate of tissue was prepared in PBS (10 mM; pH 7.0) and then centrifuged at 12,000 × g for 20 min at 4 °C. Supernatant was taken for enzyme estimation. 5 ml of PBS was added to 4 ml of H₂O₂ (200 μM) and then 1 ml of enzyme extract was added. After 1 min, 1 ml of this solution was taken in a tube and 2 ml of K₂Cr₂O₇ (5%) solution was added. Then it was boiled for 10 min and absorbance was measured at 570 nm. The activity of CAT was expressed as amount of H₂O₂ depleted per minute.

2.9.5. Lipid peroxidation (LPO) assay by thiobarbituric acid reactive substances (TBARS) level

After sacrifice of rat, the tissue was dissected out on a sterile watch glass placed in ice box, cleaned from adherent tissues and processed immediately for estimation of lipid peroxidation. 10% homogenates of spleen tissue in 20 mM Tris hydrochloride (HCl) buffer (pH 7.4) was centrifuged for 15 min at 5000 × g at 4 °C, and supernatant was subjected to thiobarbituric acid (TBA) assay by mixing it with 8.1% sodium dodecyl sulfate (SDS), 20% acetic acid, 0.8% thiobarbituric acid (TBA) and boiled for 1 h at 95 °C. The reaction mixture was cooled and shaken with n-butanol and pyridine reagent (15:1) and centrifuged [35]. The absorbance of reaction mixture was measured at 534 nm. Lipid peroxidation was expressed as thiobarbituric acid reactive substances (TBARS) in nmol/g tissue weight having 1,1,3,3-tetraethoxy propane (TEP) as standard. The standard curve was calibrated using 10 nM concentration of TEP.

2.9.6. Blastogenic response (% stimulation ratio) of splenocytes

Cell-mediated immune function was assessed by measuring splenocyte proliferation in response to the T-cell specific mitogen, Concanavalin A (Con A), using a colorimetric assay based on the reduction of tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [36]. Splenins of rats were removed in sterile condition and a single-cell suspension was prepared by mincing and grinding them between sterile frosted glass slides. Erythrocytes were lysed by hypotonic shock using equal volume of cold ammonium chloride tris buffer (tris hydroxymethylene amino methane, SRL, Mumbai, India); 0.5% tris buffer and 0.84% NH₄Cl mixed in 1:10 ratio; pH 7.2). This single cell suspension along with ice cold culture medium (RPMI-1640 supplemented with 1% penicillin (5000 U/ml) streptomycin (100 g/ml), 1% l-glutamine (2 mM/ml), 0.1% 2-mercaptoethanol (5 × 10⁻² M/ml), and 10% heat-inactivated fetal calf serum). The cell suspension was washed thrice. The cells were counted using a hemacytometer and viability was determined by trypan blue exclusion method. Viable cells (which exceeded 95%) were adjusted to 1 × 10⁷ cells/ml in culture
were 312 meric in when small result which oration Excel lization and controlled to ∼10% which the phosphate h volume used medium as (w/w) that is used to control concentration of higher polymer concentration and found 20 mg/ml formulation as appropriate based on high entrapment efficiency and controlled release of Tmx. Fig. 2(C) and (D) shows the TEM and AFM micrographs of 20 mg/ml of Tmx-NPs formulation also show almost similar size distribution with less than 200 nm of particles.

3.2. Thermal properties

The melting temperatures and heats of fusion of Tmx-NPs were measured using Mettler T82 DSC instrument. The small enthalpic peak at 65 °C is the glass transition temperature (T_g) of pure PLA which remains unchanged in PLA nanoparticles. The melting temperature of the Tmx-NPs remains almost unchanged as compared to pure PLA nanoparticles, while the heat of fusion (ΔH) is considerably reduced in Tmx-NPs indicating considerable interaction between the polymer and drug (Tmx) (Fig. 3A). ΔH is a parameter to measure the interactions between the two components. The lower value of heat of fusion of Tmx-NPs (ΔH = 30 J g⁻¹) shows good interaction between the components as compared to ΔH of 34 J g⁻¹ of pure PLA nanoparticles.

3.2.1. Analysis of Tmx-PLA interaction

The Tmx-PLA interaction was evaluated by using FTIR. The FTIR spectra of PLA nanoparticles, pure Tmx and Tmx loaded PLA NPs were recorded in the range of 800–2500 cm⁻¹ and at 1 cm⁻¹ resolution (Fig. 3B). The peak at 1752 cm⁻¹ due to C=O stretching frequency in Tmx-NPs which gets shifted from 1747 cm⁻¹ in pure PLA NPs showing considerable interaction in drug loaded system. The peak at 1365 cm⁻¹ in Tmx-NPs due to C–H bending against 1374 cm⁻¹ in pure PLA NPs also exhibit better interactions. On the other hand, the shifting of peaks at 1188, 1086 and 1039 cm⁻¹ in Tmx-NPs against peaks at 1182, 1080, 1004 cm⁻¹, respectively, in PLA nanoparticles due to C–O stretching frequency confirm the good interactions between drug and PLA.

3.3. In vitro drug release

In vitro release characteristics of Tmx from Tmx-NPs were carried out in phosphate buffer saline (PBS) at pH 7.4 and 37 °C. The released concentration of Tmx was determined at different time intervals using calibration curve drawn at 275 nm using UV–vis spectrophotometer. The release profiles of Tmx were studied from Tmx-NPs prepared from different concentrations such as 10, 20 and 30% of PLA. Fig. 4 indicates that Tmx-NPs prepared from high concentration of PLA are appropriate to control the release of Tmx as compared to low concentration of PLA. At 24 h of immersion, 78, 68 and 62% of Tmx were released from Tmx-NPs prepared from 10, 20 and 30 mg/ml of PLA, respectively. Particles prepared using 30 mg/ml of PLA showed ~65% of the entrapped drug released in the first 24 h and ~80% in 4 days. On the other hand, ~78% of the entrapped drug was released in the first 24 h and ~90% was released in 4 days from Tmx-NPs prepared using 10 mg/ml of PLA. The fast release of Tmx from low concentration of polymer was due to lower particle dimension resulting higher surface area and thereby facilitating drug diffusion as compared to relatively larger size of particle from higher concentrated solution where drug diffusion is slowed down because of lower surface area of larger particle. Hence, the release of drug can be controlled by regulating the site of drug embedded nanoparticles prepared simply by monitoring the concentration of polymer in solution.
3.4. In vivo antitumor efficacy

In vivo studies of tumor progression are shown in Fig. 5(A) and (B) after repetitive oral administration of pure Tmx and Tmx-NPs formulations. Administration of Tmx-NPs resulted in significant change \( (P<0.01) \) in tumor growth inhibition as compared to pure Tmx. Tmx-NPs significantly suppressed the tumor growth as compared to pure Tmx \( (P<0.01) \). After 30 days, the residual tumor burdens were 74 and 82% in the case of Tmx-NPs and Tmx, respectively, whereas the untreated animal group (control DMBA) showed an increase in tumor volume up to 130% \( (\text{inset \ Fig. 5A}) \). Body weights of the rats were measured throughout the study. Fig. 5B shows percentage weight loss of tumor-bearing rat by treating with Tmx and Tmx-NPs for 60 days of the study. Administration of Tmx and Tmx-NPs resulted 23 ± 4% and 17 ± 3% of weight loss as compared to starting weight of treatment. On the other hand, the weight of control DMBA group was reduced up to 44 ± 6% and the weight of control group (healthy group) was increased up to 142 ± 6%.

3.5. Histological examination of liver and kidney

Histological analysis of liver and kidney of each experimental group was carried out by the method of Kharwar and Haldar [31]. After one month of oral administration of Tmx and Tmx-NPs, conventional histological examinations were carried out to determine the possibility of Tmx and Tmx-NPs induced histopathological changes in hepato- and renal microanatomy in rats. Histological studies revealed that Tmx administration resulted with severe micro-anatomical changes and tissue injury in liver and kidney. On the other hand, Tmx-NPs treatment showed no considerable tissue injury in liver and kidney. Microscopic observations of control liver show normal and regular structure of hepatic lobules, central vein, normal sinuscles and parenchymatous polyhedral cell, while in Tmx treated group the liver shows portal inflammation, sinusoidal dilatation and dilatation of central vein \( (\text{Fig. 6}) \). Tmx-NPs supplementation to DMBA treated rat showed lesser dilatation of central vein \( (\text{Fig. 6}) \). Control kidney shows prominent regular structure of Bowman’s capsule and glomerulus \( (\text{Fig. 7}) \) while in the Tmx treated group the kidney was extensively degenerated with severe vasocongestion and edema in the renal parenchyma and also wide dilation in the Bowman’s capsule. In most areas the Bowman’s capsule lost its normal morphology \( (\text{Fig. 7}) \). Supplementation of Tmx-NPs caused no significant loss in the structural morphology of Bowman’s capsule \( (\text{Fig. 7}) \).

3.6. Biochemical estimation of hepatotoxicity and renal toxicity

Markers of hepatotoxicity \( (\text{i.e. ALT, AST}) \) were measured in the plasma. AST, ALT levels were significantly low \( (P<0.001) \) in Tmx-NPs treated group as compared to control and Tmx treated groups. AST and particularly ALT presented significant increase in Tmx treated rats \( (\text{Table 1}) \). Level of urea was found to be significantly high \( (P<0.05) \) in the Tmx-NPs treated group as compared with control. On the other hand level of creatinine and BUN did not show any significant variations as compared to control and Tmx treated groups \( (\text{Table 2}) \).
Fig. 2. SEM image of Tmx-NPs (A) 10 mg/ml, (B) 20 mg/ml; and (C) TEM image of 20 mg/ml, (D) AFM image of 20 mg/ml.

Fig. 3. Analysis of Tmx-PLA interaction by using DSC thermogram (A) and FTIR spectra (B) of Tmx, PLA and Tmx-NPs.
3.7. Estimation of total leukocyte count (TLC) and differential leukocyte count (DLC)

Total leukocyte count (TLC) and differential leukocyte count (DLC) were assessed following the staining method of Singh and Haldar [32]. TLC and DLC were significantly decreased in peripheral circulation due to treatment of Tmx-NPs as compared to control and Tmx treated group (Table 3). Overall, the TLC level was significantly low (P<0.05) in Tmx-NPs treated groups in comparison to Tmx treatment only. The results indicate that the controlled delivery of Tmx from Tmx-NPs is more suitable than direct administration of Tmx.

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>TLC (× 10³ mm⁻¹)</th>
<th>Neutrophils</th>
<th>Eosinophils</th>
<th>Basophil</th>
<th>Lymphocyte</th>
<th>Monocyte</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>12.5 ± 2.2</td>
<td>17.7 ± 2.1</td>
<td>2.2 ± 0.08</td>
<td>0.0 ± 0.00</td>
<td>79.1 ± 5.2</td>
<td>1.0 ± 0.03</td>
</tr>
<tr>
<td>Tmx</td>
<td>22.8 ± 4.6</td>
<td>32.0 ± 2.7</td>
<td>6.8 ± 0.36</td>
<td>1.2 ± 0.09</td>
<td>58.7 ± 4.3</td>
<td>1.2 ± 0.05</td>
</tr>
<tr>
<td>Tmx-NPs</td>
<td>18.7 ± 2.1</td>
<td>28.7 ± 3.2</td>
<td>5.7 ± 0.34</td>
<td>1.5 ± 0.06</td>
<td>61.7 ± 5.3</td>
<td>2.2 ± 0.06</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD (n = 6); Significance of difference; *P<0.05, **P<0.01, ***P<0.001, control vs. Tmx and Tmx-NPs groups; *P<0.05, **P<0.01, ***P<0.001, Tmx vs. Tmx-NPs.

3.8. Estimation of antioxidant enzymes [superoxide dismutase (SOD) and catalase (CAT)]

The levels of SOD and CAT activity were measured in liver, kidney and spleen. SOD activity was significantly high (16.42 ± 1.06, P<0.05) in kidney tissue of Tmx group but it was not significant in spleen and liver of the same group when compared to control. The Tmx-NPs showed non-significant differences in SOD activities in all of the tissues when compared to control, however, SOD activity of kidney was significantly low (12.08 ± 0.733, P<0.05) as compared to Tmx treated rat (Fig. 8). On the other hand, CAT activity was significantly low in spleen (13.07 ± 0.623, P<0.05)
Fig. 6. Histological examination of liver after treatment with formulations: control (healthy group); Tmx treated and Tmx-NPs treated.

Fig. 7. Histological examination of kidney after treatment with formulations: control (healthy group); Tmx treated and Tmx-NPs treated.

and high in kidney (17.06 ± 0.75, P < 0.001) and liver (18.36 ± 0.85, P < 0.01) respectively in Tmx treated groups. Only the kidney tissue of Tmx-NPs showed significant increase in Catalase activity (17.22 ± 1.01, P < 0.001) when compared to control but liver and spleen did not show any significant increase. However the Tmx-NPs treatment showed significant increase in Catalase activity in spleen (20.19 ± 1.02, P < 0.01) but the Catalase activity was significantly low in liver (7.23 ± 0.71, P < 0.001) in the same group when compared with Tmx treated rats. Tmx-NPs treatment did not show any significant variation in Catalase activity in kidney tissue when compared to Tmx treatment (Fig. 9).

3.9. Lipid per oxidation assays by thiobarbituric acid reactive substances (TBARS) level

Tmx treatment showed significant increase in MDA level in liver (207.18 ± 5.552, P < 0.01), spleen (186.86 ± 5.32, P < 0.001) and

Fig. 8. Superoxide dismutase (SOD) activity in spleen, liver, kidney, for control, Tmx and Tmx-NPs treated groups of female Wistar rat. Values are expressed as means ± SD, n = 6. Significance of difference; *P < 0.05, **P < 0.01, ***P < 0.001, Control vs. Tmx and Tmx-NPs groups; *P < 0.05, **P < 0.01, ***P < 0.001, Tmx vs. Tmx-NPs.

Fig. 9. Catalase activity in spleen, liver and kidney for control, Tmx and Tmx-NPs treated female Wistar rat. Values are expressed as means ± SD, n = 6. Significance of difference; *P < 0.05, **P < 0.01, ***P < 0.001, control vs. Tmx and Tmx-NPs groups; *P < 0.05, **P < 0.01, ***P < 0.001, Tmx vs. Tmx-NPs.
kidney (147.5 ± 6.85, P < 0.001) as compared to control group. In Tmx-NPs group MDA level was significantly high in liver as compared to control (189.40 ± 7.01, P < 0.05), however the level was not significant in cases of spleen and kidney. The level of MDA was significantly low in Tmx-NPs treatment in spleen (125.53 ± 4.72, P < 0.001) and kidney (136.53 ± 5.01, P < 0.01) (Fig. 10).

3.10. Assessment of cell-mediated immune function by measuring blastogenic response

The blastogenic response (% stimulation ratio) of spleenocytes showed significant increase in both the Tmx (126.91 ± 4.32, P < 0.001) and Tmx-NPs (118.6 ± 5.72, P < 0.001) groups as compared to control. But, the level was significantly low in Tmx-NPs treated rats (P < 0.01) when compared to Tmx treatment. In present study the peripheral immune parameters were found to be significantly low in Tmx-NPs group than Tmx and control groups (Fig. 11). Further, the blastogenic response of spleen lymphocytes were found to be significantly high in Tmx-NPs group than control but was low when compared to Tmx treated group. Thus, we may suggest that Tmx-NPs treatment is non-vulnerable in terms of inflammation and inflammatory stress.

4. Discussion

Different parameters such as PLA, PVA, Tmx and surrounding temperature were optimized on the basis of particle size and entrapment efficiency. The concentration of polymer plays an important role for controlling the size of particles and entrapment efficiency using general emulsification method by applying PLGA/PLA systems [38–40]. Increasing the polymer concentration leads to an increase in the viscous forces which resist the breakdown of droplets by homogenization/sonication. High viscous force opposes the proper emulsification and stabilization of nanodroplets, which causes the destabilization and coalescence of nanoparticles. Thus high polymer concentration resulted to increase the size of Tmx-NPs as well as entrapment efficiency [41].

PVA plays an important role to stabilize uniform and smaller size of nanoparticles. During removal of organic solvent, the presence of PVA hinders the coalescence of droplets and then promotes the formation of small nanoparticles. After removal of organic solvent, more PVA molecules can be physically incorporated onto the nanoparticles surface, and then a large number of hydroxyl groups extend into the continuous phase could be hydrated, hence forming a hydrated layer at the surface to hinder nanoparticles aggregation that causes uniform and stabilized nanoparticles [40,42,43]. On the other hand, high concentration of PVA forms a thick layer around the nanoparticles, which also cause to hinder the entrapment of drugs.

The solubility of drug and polymer plays an important role for the entrapment efficiency and the particle size [41]. PLA and Tmx both have lipophilic properties, therefore effect of Tmx concentration on the particles size and entrapment efficiency was studied. Results indicate that due to the saturation of the polymer with Tmx the entrapment efficiency was not increased upon increase in Tmx concentration. Considering the results, 10% (w/w) of Tmx is optimum to get 234 ± 2 nm of Tmx-NPs with 67 ± 2% of entrapment efficiency.

Temperature plays an important role to improve the thermal stability and mechanical properties of PLA. Therefore an optimum temperature is required to stabilize thermal and mechanical properties of PLA during preparation of Tmx-NPs [44]. High and also low temperature can cause coalescence of nanodroplets. An optimal temperature of ~30° C is suitable to control the evaporation of organic solvent, which causes high yield and uniform particle size by reducing the coalescence and resolubilization of nanoparticles.

DSC studies were carried out to confirm the possible interaction between the drug and the polymer within the matrix [45] and also to assess the physical state of drug in nanoparticles whether it exists in amorphous or crystalline state [46]. The present finding confirms that the lower value of heat of fusion of Tmx-NPs (ΔH = 30 J g⁻¹) suggests enhanced interaction among Tmx and PLA as compared with pure PLA nanoparticles where ΔH = 34 J g⁻¹. This result also indicates that the polymer may have inhibited the crystallization in nanoparticles presumably due to interaction. The obtained change in heat of fusion values for pure PLA nanoparticles and Tmx-NPs are in agreement with the earlier reports, which have been given for drug loaded PLA as well as PLGA nanoparticles [47,48]. Hence, it could be concluded that Tmx formulated in the nanoparticles was in an amorphous or disordered state.

In vitro release characteristics of Tmx from Tmx-NPs were carried out in phosphate buffer saline (PBS) at pH 7.4 and 37 °C. The fast release of Tmx from low concentration of polymer was probably due to lower encapsulation as compared to high concentration. On the other hand, an increase in polymer concentration increases the length of diffusional pathways into the aqueous phase, thereby reducing the drug loss through diffusion and increasing the drug content. The controlled release profile of drugs from PLA nanoparticles have been reported by several authors [30,47,49].
In vivo antitumor efficacy of pure Tmx and Tmx-NPs were evaluated on DMBA induced breast cancer rat model. The in vivo antitumor efficacy of Tmx-NPs is significantly more effective as compared to pure Tmx. Oral administration of Tmx-NPs significantly reduced the tumor burden as compared to the Tmx. On the other hand, continue increase in the tumor volume was observed in DMBA control group. The percentage weight loss of DMBA induced rat model was significantly lower in Tmx-NPs treated group as compared to pure Tmx and DMBA control group. The observed results confirm the enhanced efficacy and bioavailability of released Tmx from Tmx-NPs.

To study the effect of Tmx-NPs on different organs the histological examination of liver and kidney was carried out. In comparison to control the pure Tmx treatment showed significant disruption in micro-anatomy of liver and kidney but the Tmx-NPs did not show considerable micro-anatomical changes. Thus, it can be suggested that liver and kidney which are the most important organs of the body to maintain body homeostasis remain unaffected upon Tmx-NPs treatment. This can be predicted as an important result that neither Tmx nor the NPs can produce any significant biological changes in terms of body homeostasis by affecting other organs. This result was further supported by the estimations of marker enzymes for hepatotoxicity (i.e. ALT and AST) and renal function test (urea, BUN and creatinine). Considering some reports, it has been confirmed that pure Tmx increases hepato- and renal toxicity markers levels like ALT, AST, MDA, urea, creatine and BUN [50,51]. Thus Tmx-NPs may be used to reduce the hepato- and renal toxicity. The ALT and AST levels were found to be significantly high in Tmx treated rats and significantly low in Tmx-NPs rats. Cumulatively from these results it can be inferred that the liver function was normal in Tmx-NPs groups but liver function was significantly affected by both the processes, i.e. induction of tumor and its further treatment by pure Tmx. Further the urea level was found to be maximum in Tmx-NPs group but creatine and BUN levels were non-significant. It can also be inferred that the livers of Tmx-NPs treated rats are having some different supply of nitrogenous substrates which could be due to the supply of some amino acids from kidney to liver. Elevated level of urea in body can even be due to the degradation of poly(3,4-lactic acid) in body which may affect some other biological/metabolic processes and thus may be responsible for elevated level of urea. But simultaneously, the urea was not converted into BUN and creatinine and thus can be nullified as a side effect of Tmx-NPs treatment. Another important aspect of cancer induction and treatment is their effects on immunity. The immunological aspect can be divided into peripheral immune parameters and organs of immune-progenitor cells. In present study the peripheral immune parameters were found to be significantly low in Tmx-NPs treated groups as compared to pure Tmx and control groups. Further, the blastogenic response of splenocytes were found to be significantly high in Tmx-NPs group than control but was low when compared to pure Tmx treated group. Thus, we may suggest that Tmx-NPs treatment is non-vulnerable both in terms of body homeostasis, inflammation and inflammatory stress. The latter was further supported by the results of radical marker enzymes (i.e. SOD, CAT and level of MDA). Tmx induces oxidative stress during therapy which causes the formation of radicals [52]. SOD level was found to be almost static in all the tissues (spleen, liver and kidney) in pure Tmx and Tmx-NPs treated groups as compared to control. The Catalase activity was found to be significantly high in the kidney of Tmx-NPs treated groups but the level was significantly high in spleen and low in liver when compared to Tmx treated groups. The results are quite interesting in the sense that only Tmx-NPs treatment may somehow generated peroxide ions in spleen and kidney which is the main cause behind elevated level of catalase. This can be explained that the Tmx-NPs may have increased the local metabolism process of kidney and hence have generated more peroxide ions which support our previous results of elevated level of serum urea which is a main product of kidney. In spleen, the catalase level was elevated mainly due to the fact that the Tmx-NPs may be initially provoked some inflammatory response in body, which had affected the activity of spleen and elevated spleenic Catalase activity. Thus, the blastogenic response of splenic lymphocyte was high in Tmx-NPs treated groups. Interestingly, the liver catalase and SOD levels were low and non-significant in Tmx-NPs treated groups than Tmx treated groups which suggest that Tmx-NPs treatment have provided negative impact on metabolic process of liver. This was further supported by elevated level of lipid peroxidation in liver of Tmx and Tmx-NPs treated groups than control. The present findings confirm the reduced hepato- and renal toxicity of Tmx after the encapsulation in Tmx-NPs. Therefore Tmx-NPs have significant therapeutic efficacy for the treatment of breast cancer with reduced side effects as compared to chronic Tmx therapy.

5. Conclusions

In summary, Tmx-NPs were synthesized using emulsified nanoprecipitation technique with high entrapment efficiency with 2% (w/v) PVA, 10% (w/w) Tmx and 20 mg/ml of PLA at 30 °C. Tmx-NPs were characterized for evaluation of particle size by using SEM, TEM, AFM and particle size analyzer and results showed that the mean diameter of Tmx-NPs was 224 ± 3 nm with 68 ± 2% (w/w) of entrapment efficiency. The lower value of heat of fusion of Tmx-PLA nanoparticles (∆H = 30 J g⁻¹) shows enhanced interaction between Tmx and PLA as compared with pure PLA nanoparticles (∆H = 34 J g⁻¹). In in vivo study, the tumor size significantly reduced by treating Tmx-NPs as compared to pure Tmx and untreated group. Tmx-NPs showed the marked reduction in hepatotoxicity and renal toxicity, when compared with pure Tmx as evidenced by histopathological as well as biochemical examinations. Oxidative stress and lipid peroxidation was estimated in spleen, liver and kidney and was significantly high in Tmx treated group as compared to Tmx-NPs and control group. Immunological parameters like blastogenic response of splenocytes, and TLC, DLC was studied and found significantly high in Tmx treated groups but the variations were nonsignificant in Tmx-NPs group as compared with control group. In conclusion our study may suggest that Tmx-NPs have significant therapeutic efficacy for the chronic breast cancer with reduced hepatotoxicity, renal toxicity and also reduced inflammatory/immunogenic side effects.

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