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Effects of tamoxifen-loaded solid lipid nanoparticles on the estrogen receptor-α (ER-α) and vascular endothelial growth factor-A (VEGF-A) genes expression in the endometrial tissue of ovariectomized female Sprague-Dawley rats

Saman Javid, Nasrin Ziamajidi, Shadi Foroughi, Roghayeh Abbasalipourkabir*

Department of Clinical Biochemistry, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

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ABSTRACT

Background: The effect of tamoxifen on endometrial carcinogenesis stems from its estrogen agonist effect. An *in vivo* study was carried out to compare the effect of tamoxifen-loaded solid lipid nanoparticles and free drug on the ER-α and VEGF-A genes expression.
Material and methods: Twenty-four female Sprague-Dawley rats divided into 4 groups of six rats were used for this study. The first and second groups were ovariectomized and given tamoxifen and tamoxifen-loaded SLN respectively for six days continuously. Group 3 served as the untreated ovariectomized control group and group 4 was made up of untreated normal healthy rats. At the end of the study, the rats were sacrificed and study of the genes expression and serum oxidative stress were carried out.
Results: The results of this study showed that treatment with tamoxifen-loaded SLN significantly reduced the mRNA levels of ERα and VEGF-A gene and the total oxidant status compared to the ovariectomized control group.
Conclusions: The results of this study revealed that encapsulation of tamoxifen in solid lipid nanoparticles may have less adverse effects on the oxidative stress status and incidence of endometrial cancer.

1. Introduction

Breast cancer is one of the most important health concerns of contemporary society [16].

Although the mortality rates from breast cancers have decreased in most developed countries owing to more frequent mammographic screening and extensive use of tamoxifen (TMX), it remains the second highest cause of death in women [9].

In hormone-sensitive cancer, patients receive chemotherapy with cytotoxic drugs. The cytotoxic drugs treat cancers by causing cell death or growth arrest. Efficacious cancer chemotherapy has the capacity to shrink a tumor or to help destroy cancer cells [15].

ER-α gene and VEGF-A are involved in the pathogenesis of "tamoxifen dependent" endometrial cancer. In humans and adult mice, during the proliferative phase of the menstrual cycle, estradiol (E_2) is the major mitogen in the uterine cells that acts via the transcription factor receptor called ER-α [46]. Estrogen receptor stimulation in uterine cells increases the cell proliferation and risk of developing cancer [23]. In uterine cells, tamoxifen acts as an estrogen agonist [32]. Increased ER-α gene expression in the endometrial tissue during tamoxifen therapy has been reported. The risk of endometrial cancer increases due to estrogenic effect [13].

VEGF-A is a specific mitogen of endothelial cells with potential capability in angiogenesis. Physiological function of VEGF-A gene include reconstruction of the endometrium during monthly periods, but over expression can lead to an increase in cell proliferation and tumor formation in this tissue. It has been shown that the expression of VEGF-A in the uterus increases by two factors; estradiol (E2) and tamoxifen [17]. Considering the fact that the importance of this gene in the development of endometrial carcinoma has been substantiated, it was chosen as a representative of the growth factor genes.

Thus, alternative methods of drug administration like appropriate drug carrier system is required to surmount this problem. Depending on the route of administration, the size of drug carriers

* Corresponding author at: Department of Clinical Biochemistry, School of Medicine, Hamadan University of Medical Sciences, 65178 Hamadan, Iran.
E-mail addresses: rpourkabir@hotmail.com, abbasalipourkabir@umsha.ac.ir (R. Abbasalipourkabir).

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may range from a few nanometers (colloidal carriers) to micrometers (microparticles) and to several millimeters (implants). Among these carriers, nanoparticles have shown great promise for parenteral application of chemotherapeutic drugs [33].

In this study, the comparative effect of free tamoxifen (TMX) and tamoxifen-loaded solid lipid nanoparticles (TMX-SLN) on the estrogen receptor-α (ER-α) and vascular endothelial growth factor-A (VEGF-A) genes expression in the uterus of female ovariectomized Sprague-Dawley rats was assessed.

2. Materials and methods

2.1. Materials

Softisan® 154 (S154) or hydrogenated palm oil was given as a gift by CONDEA (Witten, Germany). Lipoid S100 (soy lecithin) was given as a gift by Lipoid KG (Ludwigshafen, Germany). Thimerosal, sorbitol and tamoxifen were purchased from Sigma.

2.2. Preparation and characterization of TMX-loaded SLN

SLN was prepared using the high-pressure homogenization (HPH) technique [41]. 70 g of palm oil (S154) and 30 g of soy lecithin (S100) were weighed within a short time, mixed and ground in a ceramic crucible and thereafter heated to 65–70 °C until a clear yellowish solution was obtained. A solution consisting of 1 ml of oleyl alcohol, 0.005 g of thimerosal, 4.75 g of sorbitol and 89.25 ml of bidistilled water was added to each of the lipid matrices. The mixtures were stirred on a magnetic stirrer using a teflon-coated magnet for 30 min at room temperature. The lipophilic drug model, TMX with a concentration of 10 mg was dissolved in 1 ml olive oil and mixed with 50 mg of SLN using an Ultra Turrax® (Ika, Staufen, Germany) at 13,000 rpm for 10 min. The mixture of TMX-SLN was then incubated at 50–60 °C while stirring overnight with a teflon-coated magnet at 500 rpm and then exposed to air until solidification was achieved. TMX-loaded SLN was then characterized by the particle size and polydispersity index (PDI), zeta potential (Zetasizer; Mal 1033452, Malvern Instruments, UK) and fourier transform infrared spectroscopy (FTIR; Perkin Elmer, Spectrum 65, England).

2.3. Experimental design and procedure

Twenty-four virgin female Sprague-Dawley rats aged 6–8 weeks, weighing 180–200 g were purchased from Pasteur Institute of Iran. The animals were housed on the basis of two rats per plastic cage and allowed to acclimatize under standard conditions (12 h light/dark cycles) for one week. The rats were given free access to distilled water and commercialized food throughout the experiment. The rats were anaesthetized with a mixture of ketamine/xylazin (100/5 mg/kg B.W.) by intraperitoneal injection and were bilaterally ovariectomized under standard method [22]. After operation, the animals were allowed to recover for 2 weeks before the commencement of the study. The rats were divided into 4 groups of 6 rats each. The first group (T) was given tamoxifen (2 mg/kg B.W.) dissolved in 1 ml olive oil, the second group (TS) was given 2 mg/kg B.W. tamoxifen-loaded SLN (a total of 2 mg/kg B.W. TMX loaded in 10 mg/kg B.W. SLN dispersed in 1 ml olive oil), the third group (C) was made up of untreated ovariectomized rats and served as the ovariectomized control group and group four (H) served as the healthy unovarietomized group. Treatments were given to the animals orally for 6 consecutive days using gastric intubations. At the end of the study, the rats were sacrificed using an overdose of ketamine and their uterus were harvested, washed with ice-cold normal saline and stored at −80° C until the commencement of the gene expression study. The rats' blood samples were also collected by cardiac puncture using 23 G needles and were allowed to clot at room temperature, and centrifuged at 1000*g* for 10 min. Serum were separated and analyzed for total antioxidant capacity (TAC), malondialdehyde (MDA), total oxidant status (TOS) and thiol levels. The experimental procedure was approved at the Hamadan University of Medical Sciences (UMSHA) and the research was conducted according to the guidelines for the care and use of laboratory animals of UMSHA.

Table 1
The temperatures and reactions times of Real-time PCR.

Reaction Phase	Temperature	Period
Initial activation	95 °C	10 min
50 cycles of:		
Denaturation	95 °C	15 s
Annealing	60 °C	60 s
Extension	60 °C	60 s

2.4. Quantitative real time polymerase chain reaction (qRT-PCR)

Both estrogen receptor-α (ER-α) and vascular endothelial growth factor-A (VEGF-A) mRNA expression were assessed by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using specific primers for amplification. In this study, by using trizol (Invitrogen), total RNA was extracted from frozen uterine tissue and t the RNA was later removed. The extracted total RNA purity ratio (A_{260}/A_{280}) was determined using a spectrophotometer (WPA Bio Wave II). For synthesis of the cDNA by reverse transcription of 1 μg RNA, we used Revert Aid™ First Strand cDNA Synthesis Kit (K1622, Fermentase, USA). Expression of both genes, estrogen receptor-α (ER-α) and vascular endothelial growth factor-A (VEGF-A) was determined by real time polymerase chain reaction (Applied Biosystem Step one plus) using Master Mix SYBR Green method and detection system (AB, Invitrogen). The temperatures and reactions times of real-time PCR are shown in Table 1. The agarose gel electrophoresis (1%) was used to confirm cDNA amplification. The characteristics of the forward and reverse primers of the target genes, ER-α (NM_012689.1), VEGF-A (NM_001110333.2) and house keeping gene, beta actin (NM_031144.3) are shown in Table 2. Cycle threshold (Ct) of both genes was determined and ΔCt and $2^{-\Delta\Delta ct}$ (fold change) of the genes were analyzed [30].

2.5. Determination of oxidative stress status

Total Antioxidant Capacity (TAC): TAC in serum samples was assessed using ferric reducing antioxidant power assay (FRAP) [5].

Malondialdehyde (MDA): MDA as a lipid peroxidation index was determined using fluorometric thiobarbituric acid method [6].

Total Oxidant Status (TOS): The oxidation of ferrous ion to ferric ion accompanied with a number of oxidant species in acidic pH was used for the measurement of TOS in serum. The ferric ion was determined using xylenol orange [14].

Protein Thiol Groups (SH): Protein SH (P-SH) is a marker of free radical damage to the cells and was determined using DTNB (Ellman's reagent) (2,2-dithiobisnitrobenzoic acid). The absorbance of yellowish complex was assayed at 412 nm [20].

2.6. Statistical analysis

The data were expressed as mean ± standard deviation. For statistical analysis, the experimental values were compared with their corresponding control values. One-way analysis of variance (ANOVA) incorporated in SPSS software (version 16.0) was used to show the significant difference between the experimental and