International Immunopharmacology 62 (2018) 326–333

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Mechanisms underlying the protective effect of montelukast in prevention of endometrial hyperplasia in female rats

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ARTICLE INFO

Keywords:
Endometrial hyperplasia
Estradiol valerate
Female rats
Montelukast

ABSTRACT

Objective: To study the possible protective role of montelukast in endometrial hyperplesia (EH) rat model, induced by estradiol valerate (EV).
Methods/materials: Thirty six female albino Wistar rats were classified into 7 groups: normal control, EV (2 mg/kg/day, p.o.), montelukast (10 mg/kg/day, p.o.), montelukast (1 mg/kg/day, p.o.) + EV (2 mg/kg/day, p.o.), montelukast (10 mg/kg/day, p.o.) + EV (2 mg/kg/day, p.o.), montelukast (20 mg/kg/day, p.o.) + EV (2 mg/kg/day, p.o.) groups. Uterine malondialdehyde (MDA), superoxide dismutase (SOD), total nitrites (NO) and serum total antioxidant capacity (TAC) were determined. Uterine, serum total cholesterol, high density lipoprotein (HDL) and tumor necrosis factor (TNF)-α were measured. Histopathological examination of the uterine tissue was also done. In addition, immunohistochemistry was done using Phosphatase and tensin homolog (PTEN) and inducible nitric oxide synthase (iNOS) antibodies.
Results: Our results showed that montelukast in dose dependant manner improves oxidative stress, lipids profile and TNF α which were affected by EV. Moreover, immunohistochemical examination revealed that montelukast markedly reduced iNOS expression, while expression of PTEN was markedly enhanced, as compared to EV group. The protective effects of montelukast were also verified histopathologically.
Conclusions: Montelukast in dose dependant manner provided biochemical and histo-pathological improvement in EV induced EH, through its anti-inflammatory, antioxidant activity and inhibition of iNOS expression with induction of PTEN expression.

1. Introduction

Endometrium, the functioning lining of the uterus, is susceptible to the cyclic effect of estrogen and progesterone hormones [1]. The equilibrium between both hormones is necessary to maintain the physiological and morphological structure of the endometrium. Abnormal excessive estrogen stimulation unchecked by progesterone effect results in endometrial hyperplasia (EH) [2]. EH is classified into two groups according to 2014 WHO revised classification [3]. EH without atypia and atypical EH. In hyperplasia, the proliferated endometrial glands show irregular sizes and shapes, along with increasing glands to stroma ratio. Epithelial cells may show atypia in atypical hyperplasia with a significantly high risk to develop endometrial carcinoma (EC). Thus, their early detection and treatment is mandatory for cancer prevention [3].

The mechanisms of development of EC remain unclear [4]. However, several mechanisms have been suggested. First, the most important predisposing factor is the hyperestrogenemia which stimulates glandular epithelial cell division [5]. Second, endometrial inflammation is another important factor that regulates the cell proliferation of the endometrium [6]. The relationship between inflammation and carcinogenesis has been proved in many organs; for example in liver [7] and colon [8]. The possible role of inflammatory cytokines in initiating carcinogenesis had been reported [4,9].

Mechanistically, inflammatory cytokines stimulate mitosis and inhibit apoptosis; increasing the possibility for replication errors, ineffective DNA repair, mutations and carcinogenesis [10]. Inflammation can also increase estrogen production directly which raises the risk of EH development [6].

Leukotrienes are fatty molecules of the immune system derived from the5-lipoxygenase pathway of arachidonic acid metabolism which are related to inflammation [11]. CysLTs interact with CysLTR1 and

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https://doi.org/10.1016/j.intimp.2018.07.008
Received 29 December 2017; Received in revised form 9 May 2018; Accepted 10 July 2018
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CysLTR2 receptors which are expressed on the outer membrane of immune/inflammatory cells [12]. Leukotrienes have a role in cancer progression [13]. The expression of LTB_4 receptors is increased in human pancreatic and prostatic cancer [14,15], respectively. Also treatment with bestatin (LTA_4 hydrolase inhibitor) decreased the burden of oesophageal adenocarcinoma in rats [16].

Montelukast is a Cys-LT1-receptor antagonist that blocks the pro-inflammatory action of LTD4 [17]. Montelukast is well established for prophylaxis of seasonal allergies and bronchial asthma. It balances oxidant-antioxidant status, regulates the generation of inflammatory mediators and inhibits neutrophil infiltration [18].

Our current study aimed at investigating the effect of monelukast in treatment of EH in rats. The mechanisms involved in its effect were also explored. Thus will help to develop more effective EH and EC chemo-preventive and/or therapeutic agents.

2. Materials and methods

2.1. Animals

Thirty six female albino Wistar rats (180–200 g) were purchased from the National Center of Research, El-Giza, Egypt. After two weeks of proper acclimatization to the animal house conditions (12 h lighting cycle, 25 ± 2 °C temperature and 45 ± 5% humidity) with free access to standard rodent chow (El-Nile Company, Egypt) and water, rats were used. Procedures involving animals and their care were conducted in conformity with the protocols of the Research Advisory Ethical Committee of Faculty of Medicine, Minia University, Egypt.

2.2. Chemicals and antibodies

Montelukast was provided by Egyptphar Co., Egypt; estradiol valerate was provided from Bayer pharma AG, Germany; total antioxidant capacity (TAC), superoxide dismutase (SOD) and high density lipoprotein (HDL) kits (Biodiagnostics, Egypt); cholesterol kit (human, Germany); tumor necrosis factor (TNF)-α ELISA kit (IDlabsT-Minc. Biotechnology, Canada); PTEN and iNOS antibodies were provided from Lab Vision (Fremont, CA, USA). Other chemicals were of analytical grade and were obtained from commercial sources.

2.3. Experimental protocol

Rats were divided into 6groups (n = 6) and treated for one week.

Group 1: normal control group received carboxy methyl cellulose as a vehicle (p.o); group 2: received estradiol valerate (2 mg/kg/day, p.o.) [19,20]; group 3: received montelukast (10 mg/kg/day, p.o.) [21, 22]; group 4: received montelukast (1 mg/kg/day, p.o.) [23] + estradiol valerate (2 mg/kg/day, p.o.); group 5: received montelukast (10 mg/kg/day, p.o.) + estradiol valerate (2 mg/kg/day, p.o.); group 6: received montelukast (20 mg/kg/day, p.o.) [21, 22] + estradiol valerate (2 mg/kg/day, p.o.). The drugs were suspended in carboxy methyl cellulose.

2.4. Biochemical analysis

At the end of experimental period, rats were anaesthetized with ether, and blood samples were collected, sera were separated by centrifugation at 4000*g* for 10 min and stored at −80 °C for assessment of TAC, HDL, total cholesterol and TNFα by enzymatic colorimetric methods using their commercial kits.

2.5. Preparation of tissue homogenates

Uterine tissue was homogenized in potassium phosphate buffer 10 mM pH (7.4). The ratio of tissue weight to homogenization buffer was 1: 5. The homogenates were centrifuged at 4000*g* for 10 min at 4 °C. The resulting supernatant was used for the determination of MDA, SOD, total nitrite/nitrate levels, histological and immunohistochemical studies. Determination of cholesterol in uterine tissue was achieved following lipid extraction [24].

Malondialdehyde (MDA) level was measured by the method of Mihara and Uchiyama [25]. NO content was measured as total nitrite/nitrate (NOx), the stable degradation products of nitric oxide, by reduction of nitrate into nitrite (NOx) using copperized cadmium, followed by colour development with Griess reagent in acidic medium [26]. SOD was measured by enzymatic colorimetric method using commercial kit (Biodiagnostics, Egypt).

2.6. Histopathological examination

The uteri of the studied animals were examined grossly and microscopically by a pathologist who was blind to the random groups but aware of the study design. The uteri were examined grossly, measured and weighed on the Mettler-Toledo scale. The middle 3rd of the uteri were dissected and fixed for 24 h in 10% formaldehyde, processed and paraffin embedded. Serial sections were cut in 5 μm to be stained by H& E. Light microscopy examination using Olympus microscope CX41 (Olympus, Tokyo, Japan) to examine the endometrium for assessing the luminal dilation, normal distribution of glands and gland-to-stroma ratio. Inflammatory cell infiltrate quantification/10hpf randomly selected per slide. Photographs for selected sections were taken using an Olympus camera (C5050Z, Olympus, Tokyo, Japan). Histopathological grading of endometrial hyperplasia was done according to WHO classification [3].

2.7. Immunohistochemical analysis

Multiple 5 μm sections were cut from all uteri studied then; they are all subjected to de-paraffinization by xylene, rehydration using descending alcohol grades, blocking endogenous peroxidases by 3% H_2O_2 and finally washing in phosphate buffer solution (PBS). Antigen retrieval was performed for all slides by citrate buffer pH 6.0 in microwave for 30 min. After cooling down to room temperature the slides were rinsed in PBS. The slides then incubated with primary antibodies in a humidity chamber at 4 °C overnight. Biotinylated 2ry antibody was applied for 30 min after rinsing with PBS (Lab Vision Laboratories). Slides were washed in PBS followed by incubation with streptavidinbiotin (Lab Vision Laboratories) for 30 min. Treatment of the slides with DAB (Lab Vision Laboratories) for 5 min to get a brown staining of the positive cases, then washing in water and counterstaining with Mayer's hematoxylin. The sides then dehydrated, cleared, mounted and covered slips. Positive and negative controls were performed win each run for both antibodies. The following antibodies were used: antibody for PTEN (Zymed Laboratories, dilution 1:100), and iNOS (Santa Cruz Biotechnology, dilution 1:200).

2.8. Immunohistochemical scoring

Sections were examined under light microscope to assess the positivity for PTEN and iNOS. Sections stained for PTEN were examined and both the intensity and percentage of the stained cells in the nucleus or cytoplasm was calculated as follows: According to the percentage, they were scored as if < 10% = negative, 10–50% = +1 and > 50% = +2. The intensity of the brown stain was scored as negative if absent, +1 if light brown and +2 if dark brown [27]. Sections stained for iNOS were examined and the intensity of staining qualitatively evaluated using the following score: 0 (no staining), 1 (mild), 2 (moderate), and 3 (strong) [27].

2.9. Statistical analysis

Results were expressed as means ± standard error of mean (SEM).

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