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Tubal cytokine changes accompany the epithelial atypia of letrozole-stimulated ovaries

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ABSTRACT

Letrozole (LTZ), one of ovulation induction medications, is increasingly prescribed in many gynecological conditions. Although its hazardous effect on the ovarian surface epithelium (OSE) as well as tubal epithelium cells (TEC) has been previously studied, the associated changes occurring in the inflammatory cytokines have not been elucidated. Therefore, the objective of our study is to investigate these changes that may accompany LTZ-induced tubo-ovarian epithelial abnormalities.

A total of 45 Sprague-Dawley rats were used in this study, divided equally into; control, LTZ6 and LTZ12 groups (received saline, 6 and 12 cycles LTZ i.p. respectively). Samples from ovaries (OVs) as well as fallopian tubes (FTs) were histologically studied for the associated changes.

An increased proliferative activity, Ki67 immunoexpression and abnormal invaginations were observed in the OSE of LTZ6 group accompanied with occasional pseudostratification and loss of cilia of TEC. These changes became more pronounced in the LTZ12 where micropapillae, hyperchromasia, frequent deep invaginations, cysts of OSE as well as papillae and multilayering of TEC were noticed. The tubal level of IL-1β, IL-6, TNF-α and serum MCP-1 progressively increased in LTZ6 and LTZ12 groups compared with the control group. The significant positive correlation observed between these cytokines in the LTZ6 group became stronger in the LTZ12 one. However, no significant changes in the tubal IL-10 and TGF-β were detected. Therefore, further studies are required to consider these cytokines as objective markers to precisely assess severity of the associated epithelial changes particularly in long periods of stimulation.

1. Introduction

Ovulation dysfunction represents about 30–40% of infertile ladies (Nahid and Sirous, 2012). Therefore, the use of ovulation inducing drugs has been increasing (Yun et al., 2015). A possible relationship between fertility drugs, particularly clomiphene citrate (CC) and gonadotrophins, and various malignancies, including those of female reproductive system, has been proposed (Impicciatore and Tiboni, 2011).

Among all gynecological malignancies, epithelial ovarian malignancy is associated with the highest rate of mortality (Erickson et al., 2013; Dietl, 2014). Serous tumors form about 70% of all epithelial ovarian tumors and are responsible for the majority of deaths (Kurman, 2013).

It was thought that ovarian high-grade serous carcinoma (HGSC) arises from the ovarian surface epithelium (OSE) (Vang et al., 2013), however there is an increasing evidence that it originates from the distal FT, by implantation of TEC on the denuded ovarian surface to form cortical inclusion cysts (CICs) (Crum et al., 2012; Reade et al., 2014; Gilks et al., 2015). Therefore, prophylactic post-reproductive salpingectomy is nowadays seriously considered as a procedure which can provide protection against a frequently fatal disease (Chene et al., 2013; Dietl, 2014).

Letrozole (LTZ), a third generation nonsteroidal aromatase inhibitor, is used for ovulation induction and is effective as the first-line treatment, CC (Usluogullari et al., 2015). It has a higher pregnancy rate and minimal complications relative to CC (Franik et al., 2014), particularly when minimal stimulation using two alternate-day gonadotropin with LTZ is prescribed (Yun et al., 2015).

Although the possible link between fertility drugs and ovarian cancer remains controversial (Gadducci et al., 2013; Rizzuto et al., 2013; Diergaarde and Kurta, 2014), the relationship between ovarian epithelial dysplasia (OED) and the use of these drugs has been demonstrated (Dauplat et al., 2009; Chene et al., 2012a).

In addition, studies have demonstrated a close relationship between prolonged presence of the inflammatory environment and

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the increased risk for developing cancer (Keibel et al., 2009). Most of the inflammatory cytokines such as IL-6, TNF-α and TGF-β are produced by many cells including macrophages and are associated with poor prognosis of ovarian tumors (Thibault et al., 2014).

To our knowledge no experimental study has investigated changes occurring in the inflammatory cytokines that may accompany the expected LTZ-induced tubo-ovarian epithelial abnormalities. Therefore, our concern in this study was to explore these changes at different periods of LTZ stimulation.

2. Materials and methods

2.1. Experimental animals

Adult female Sprague-Dawley rats (220 ± 30 g and having regular menstrual cycles) were obtained from Mansoura Faculty of Pharmacy animal house, where the experiment was performed. Animals (n = 45) were divided equally into 3 groups (n = 15 each) and were maintained on 12/12 h light/dark cycle at 24 ± 2 °C. All animals received standard laboratory animal's chow and water *ad libitum* during the period of the experiment. The experimental techniques were approved by the Institutional Laboratory Animal Care and Use Committee of Mansoura Faculty of Medicine and were performed in accordance with their guidelines.

2.2. Experimental design

The animals were divided equally into 3 groups. The control group received normal saline (2.5 ml/kg/day, i.p. injection). In LTZ6 and LTZ12 groups, ovulation was induced by LTZ (0.5 mg/kg/day, i.p. injection, Femara; Novartis Pharma) for 6 and 12 menstrual cycles respectively.

The menstrual cycle lasts for four days in rodents (Caligioni, 2009). LTZ was diluted in normal saline before its administration on the first and second days of the menstrual cycle. Then human chorionic gonadotropin (HCG) (Pregnil; Schering–Plough, 100 UI/kg) was administrated on the third day of the cycle, while the fourth day remained free of any medications. This regimen was repeated for 6 and 12 cycles in the LTZ6 and LTZ12 groups respectively. At the end of the experiment, salpingo-oophorectomy was carried out by a professional obstetrician (Y.M.) in each animal under strict sterile conditions and ketamine anesthesia.

2.3. Histological procedure

The resected OVs and FTs were immediately fixated in 10% formol solution, from which paraffin blocks were prepared. The latter were cut at 4–5 μm thickness for the routine hematoxylin and eosin (H & E) as well as for immunohistochemical staining.

All histological evaluations were performed by a light microscope (Olympus CX31) mounted to digital camera connected to a computer.

2.4. Immunohistochemical study

Paraffin sections from specimens of the OVs were used for detection of Ki67 expression immunohistochemically by the monoclonal anti-Rat Ki-67 antibody, according to instructions provided by the manufacturer (1/20 dilution, Code No. M7248, Clone MIB-5, Dako, Glostrup, Denmark).

2.5. Biochemical assays

2.5.1. Tubal cytokines assessment

Tissue homogenates were prepared from parts of the freshly resected FTs for detection of their levels of interleukin (IL)-1β (Cat. no BMS630), IL-6 (Cat. no BMS625), IL-10 (Cat. no BMS629), tumor necrosis factor-α (TNF-α, Cat. no BMS622) and transforming growth factor-β1 (TGF-β1, Cat. no BMS623/2) by ELISA kits according to the manufacture instructions (eBioscience; Vienna, Austria).

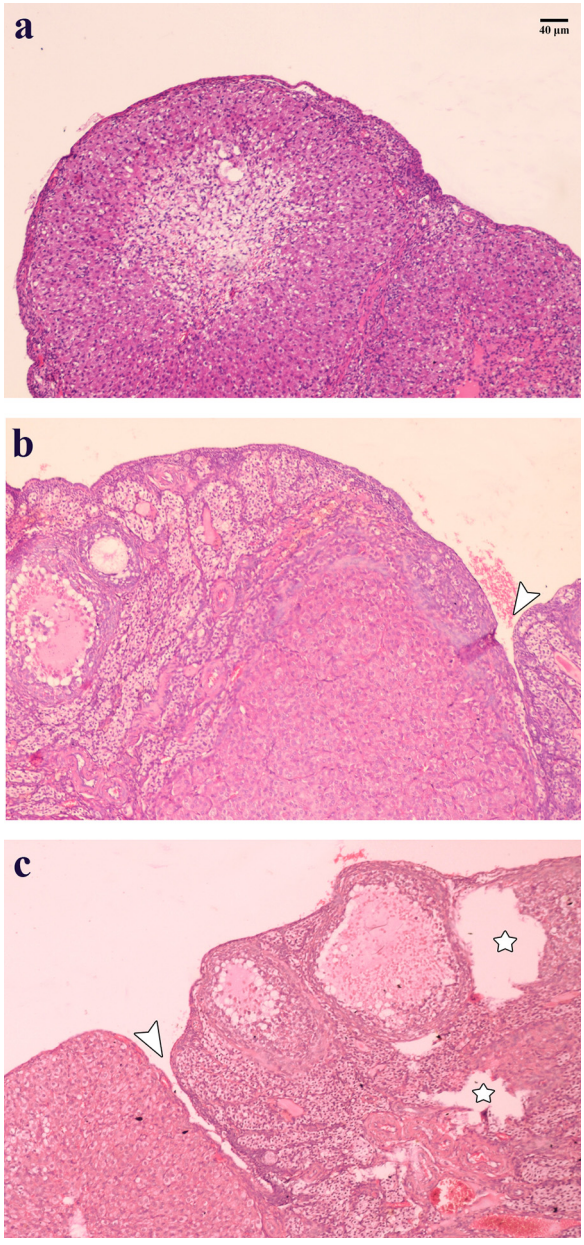

Fig. 1. Representative photomicrographs of the ovaries in various groups. Loss of normal smooth surface of the ovary is observed in the LTZ6 (b) and LTZ12 (c) compared with the control (a). The cortical invagination (arrow head) seen in the LTZ6 group becomes more obvious and deeper in the LTZ12 group which acquires numerous cortical inclusion cysts (asterix).
(H&E (a, b, c) ×200, scale bar = 40 μm).

2.5.2. Serum monocyte chemoattractant protein-1 (MCP-1) assessment

Serum of blood samples of each animal was separated and stored at −80 °C for determination of MCP-1 by an ELISA kit purchased from RayBiotech Inc. (Cat no ELR-MCP1-1, Norcross, USA).

2.6. Statistical analysis

Values were presented as mean ± SEM. One-way ANOVA test was employed for comparisons between groups followed by Tukey