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Blockade of endothelin receptors with bosentan limits ischaemia/reperfusion-induced injury in rat ovaries

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ABSTRACT

Objective: To investigate the role of endothelin receptors in ovarian ischaemia/reperfusion (I/R) injury in rats using the endothelin receptor antagonist bosentan.
Study design: Group 1: sham operation; Group 2: sham operation and bosentan 60 mg/kg; Group 3: bilateral ovarian ischaemia; Group 4: 3-h period of ischaemia followed by 3 h of reperfusion; Groups 5 and 6: bosentan 30 and 60 mg/kg, respectively, with bilateral ovarian ischaemia applied 30 min later; the bilateral ovaries were removed after 3 h of ischaemia; Groups 7 and 8: 3 h of bilateral ovarian ischaemia was applied, with bosentan 30 and 60 mg/kg, respectively, administered 2.5 h after the induction of ischaemia; following the 3-h period of ischaemia, 3 h of reperfusion was applied, after which the ovaries were removed.
Results: Ischaemia and I/R decreased superoxide dismutase (SOD) activity and the level of glutathione (GSH) in ovarian tissue, but increased the level of malondialdehyde (MDA) significantly compared with the sham operation group. Bosentan 30 and 60 mg/kg before ischaemia and I/R decreased the MDA level and increased SOD activity and the GSH level in the experimental groups. The serum levels of the inflammatory cytokines interleukin (IL)-1β, IL-6 and tumour necrosis factor-α were also measured in the I/R injury model in rat ovaries. The levels of these cytokines were significantly higher in the ischaemia and I/R groups compared with the sham operation and sham operation plus bosentan groups. The histopathological findings also demonstrated the protective role of bosentan against I/R-induced injury in rat ovaries.
Conclusion: Administration of bosentan protects the ovaries against oxidative damage and I/R-induced injury.

1. Introduction

Ovarian torsion is a condition in which an ovary twists or turns on its supporting ligament, causing occlusion of the ovarian vein or artery and resulting in obstruction of venous and lymphatic drainage. Many studies have been undertaken to investigate the use of medications that prevent ischaemia/reperfusion (I/R) injury in ovarian torsion [1–4].

Due to torsion of the adnexa, blood flow in the ovary decreases and eventually results in ischaemia [5,6]. Maintaining the circulation of the ovary after detorsion deteriorates the injury developed during reperfusion [7,8]. Restoration of blood supply to ischaemic tissues can cause additional damage due to the release of reactive oxygen species (ROS). Production of ROS is an important mechanism of injury in the I/R process which is characterized by oxidative stress. Moreover, ROS have been implicated in the pathogenesis of tissue injury during reperfusion [9].

It has been suggested that activation of neutrophils, accumulation of released cytokines and nitric oxide, platelet activation and apoptosis also play a paramount role in I/R injury [10]. The formation of cytokines and ROS is stimulated by endothelin (ET) [11]. ET-1, a potent vasoconstrictor peptide with 21 amino acids, is

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produced by a large variety of cells from a wide range of species including endothelial cells, leukocytes, macrophages and monocytes [12–14]. ET receptor type A (ETA) and type B (ETB), which have different molecular and pharmacological characteristics, are present in the ovaries [15–17]. In general, the two dissimilar ET receptors are expressed constitutively but are spatially confined to specific cell types in the ovary; a number of cells express both receptors, whereas other cells express one form or the other [18].

ET plays a role in the modulation of microcirculatory blood flow disturbances during ischaemia, and is one of the most important mediators in I/R [19–23]. Many studies have reported the release of ET during I/R injury [24,25], and there has been growing interest in the roles of ET receptor antagonists in I/R injury. ET antagonists have been reported to have beneficial effects in several I/R models including the heart, liver, lung, kidney and small bowel [26–28]. Bosentan is a potent, non-peptide, oral ETA and ETB antagonist, with higher affinity for the ETA subtype [29]. The ET antagonist bosentan has been reported to protect and improve microcirculatory blood flow in several splanchnic organs and in peripheral tissues [29]. The protective effect of bosentan in myocardial I/R injury [30], I/R-induced endothelial dysfunction [31] and I/R injury in rat skeletal muscle [32] has been observed previously in a number of experimental models. To date, however, no studies have shown a link between ET antagonists and ovarian I/R injury.

The aim of this study was to evaluate the possible role of ET-1 in the pathogenesis of ovarian I/R injury. This study investigated whether a specific ET receptor antagonist, bosentan has a protective effect against ovarian I/R injury by evaluating alterations in the oxidant–antioxidant system, generation of cytokines and histopathological examinations.

2. Materials and methods

2.1. Animals

Animals were housed in accordance with international guidelines, and the study was approved by and conducted in accordance with the Institutional Animal Care and Use Committee. In total, 64 adult female Wistar albino rats were used in this study.

2.2. Surgical technique

The rats were randomized into eight groups and anaesthetized with intraperitonal thiopental sodium 25 mg/kg. The area of the abdomen to be operated was prepared and cleaned using betadine. A longitudinal incision (2.5 cm) was performed in the midline area of the lower abdomen. A small peritoneal incision was made, and the uterine horns and adnexa were located. In Group 1, a sham operation was performed. These rats only underwent laparotomy. In Group 2, rats were administered bosentan 60 mg/kg by oral gavage, and the bilateral ovaries were surgically removed 6 h later. In Group 3, bilateral ovarian ischaemia was created by applying vascular clips below the ovaries in the rats. Atraumatic vascular clamps were used in this study to produce ovarian ischaemia. The incision was closed with 4/0 nylon sutures, and the bilateral ovaries were surgically removed after 3 h. In Group 4, a 3-h period of ischaemia was followed by 3 h of reperfusion, following which the bilateral ovaries were removed. In Groups 5 and 6, rats were administered bosentan 30 and 60 mg/kg, respectively, by oral gavage before 30 min of ischaemia, and then bilateral ovarian ischaemia was created by applying vascular clips below the ovary in both groups. After a 3-h period of ischaemia, the bilateral ovaries were surgically removed. In Groups 7 and 8, a 3-h period of bilateral ovarian ischaemia was created by applying vascular clips below the ovary. Rats were administered bosentan 30 and 60 mg/kg, respectively, by oral gavage 2.5 h after the induction of ischaemia. At the end of 3-h period of ischaemia, the bilateral vascular clips were removed and 3 h of reperfusion was applied. The ovaries were subsequently removed for histological and biochemical examination.

2.3. Biochemical investigations

2.3.1. Cytokine serum measurements

Sera from the eight groups were separated and stored at −80 °C until they were thawed for the assay. Interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α from each sample were measured with highly sensitive enzyme-linked immunosorbent assay (ELISA) kits [BMS630, eBioscience (USA); KRC0061, Invitrogen (USA); and BMS622, eBioscience (USA); respectively]. Kits were specifically designed for rat cytokines, and all measurements were performed according to the manufacturers' instructions.

2.3.2. Biochemical investigation of ovarian tissues

After macroscopic analyses, rat tissues were kept at −80° C. Tissues weighing approximately 50 mg were homogenized with TissueLyser II (Qiagen) in 1 ml of an appropriate buffer. The tissues were then centrifuged at 4° C. Supernatants were used to determine the levels of glutathione (GSH) and malondialdehyde (MDA), and the enzyme activity of superoxide dismutase (SOD). All assays were carried out at room temperature.

For the biochemical investigation, the activity of SOD and the levels of MDA and GSH from each supernatant were measured in duplicate using highly sensitive ELISA kits [Cayman-706002 (Ann Arbour, MI, USA), Cell Biolabs-STA-330 (San Diego, CA, USA) and Cell Biolabs-STA-312, respectively], which were specifically designed for rat tissue, according to the manufacturers' instructions.

2.3.3. Histological examination

Ovaries were rapidly fixed in 10% buffered formalin for 24–48 h for histological examination. After fixation, each ovarian tissue sample was routinely processed and embedded in paraffin. Five-micrometre-thick sections were cut as paraffin-embedded tissue samples. After deparaffinization and rehydration, sections were stained with haematoxylin and eosin. All sections were studied and photographed using a light photomicroscope.

For semi-thin sections, ovaries were fixed in buffered 3% glutaraldehyde in 0.1 M phosphate. Glutaraldehyde-fixed tissues were postfixed in 1% osmium tetroxide. Each ovarian tissue sample was dehydrated in a graded ethanol series and transferred to propylene oxide. After dehydration, each ovarian tissue sample was embedded in Araldite CY 212. Glutaraldehyde-fixed tissue was postfixed in 1% osmium tetroxide and embedded in thin viscosity resin (Spurr kit, Sigma Chemical Co., St. Louis, MO, USA). Semi-thin sections (1 μm) obtained with an ultramicrotome were stained with Toluidine blue. All sections were studied and photographed using a light photomicroscope.

2.3.4. Statistical analyses

Data for the serum cytokine levels were measured by ELISA, and oxidant and antioxidant enzymes were subjected to one-way analysis of variance using Statistical Package for the Social Sciences Version 18.0 (SPSS Inc., Chicago, IL, USA). Differences between the groups were analyzed using Duncan's multiple range test, and were considered significant at $p < 0.05$. All data were expressed as mean ± standard deviation (SD) in each group.

3. Results

3.1. Results of biochemical investigations

SOD activity and the levels of MDA and GSH were studied in the I/R injury model in rat ovaries (Table 1). In rats exposed to