

Full Length Research Paper

Exploratory endometriosis reduction in rats treated with pioglitazone

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The objective of this paper was to assess the effectiveness of the pioglitazone on endometriosis in rats. The study was performed on 24 Wistar rats. Endometriosis induction was performed in 24 rats, which were divided into three groups: group I (model group); and group II, the treatment group, comprising the other 12 rats. After 3 months, pioglitazone was performed in all animals. Pioglitazone treatment seems to be effective in treatment of endometriosis.

Key words: Pioglitazone, endometriosis, uterine implant, NF- κ B p65, I κ B α .

INTRODUCTION

Endometriosis is a chronic disorder defined by the presence of endometrial tissue outside the uterine cavity. It is predominantly found in women of reproductive age and is an established cause of infertility and chronic pelvic pain (Berkley et al., 2005; Olive and Schwartz, 1993). Endometriosis is considered a benign disorder, but it shares some of the characteristics of malignancy, such as abnormal morphology, deregulated cell growth, cellular invasion, and neoangiogenesis. Additionally, endometriosis is closely linked to severely impaired fertility, as it can be diagnosed in 68% of patients suffering from infertility (Koninckx et al., 1991). To date, insights into the pathophysiology of endometriosis, and thus the development of effective treatment strategies, are surprisingly meager, which is attributed in part to the

difficulties in studying the disease in humans. Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily that affect gene expression upon ligand binding (Agic et al., 2006). Such ligands encompass endogenous fatty acids and eicosanoids in addition to synthetic ligands, including nonsteroidal antiinflammatory drugs (Lebovic et al., 2001; Fallah et al., 2011; Zainuddin et al., 2011) and thiazolidinediones (TZDs) such as pioglitazone used in this study.

An ideal treatment would eliminate endometriotic lesions, prevent recurrence, be affordable with few if any side effect, and not impede ovulation. Immune modulating drugs have been studied as candidate treatment options given the implicit role of an abnormal immune response seen with this disease (Barrier et al., 2004; D'Hooghe et al., 2006; Falconer et al., 2006; Brown and Plutzky, 2007; Willson et al., 2000; Sher and Alyemini, 2011; Biswas et al., 2011).

Therefore, the objective of this study, using a rat model

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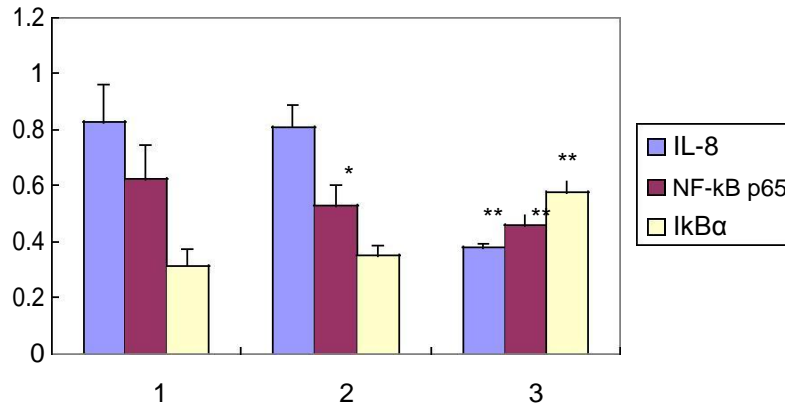


Figure 1. Effect of the pioglitazone on the levels of IL-8, NF-κB p65 and IκBα protein expression in uterine implant. 1. Model control; 2. Pioglitazone I; 3. Pioglitazone II; * $p < 0.05$, ** $p < 0.01$, compared with model control.

of endometriosis, was to assess the ability of pioglitazone to impede the development of endometriosis.

MATERIALS AND METHODS

Animal treatment

Methodology was proposed by Nogueira et al. (2007). Shortly after the midway incision, the uterine horns were identified; fragments of the medium third were resected, immersed in saline solution and cut into 4 × 4 mm fragments. The fragments were sutured to the mesentery adjacent to the artery that irrigates the cecum, with the serosal surface sutured to the peritoneum and the endometrial layer turned to the cavity. After the first surgery, the animals were kept in the laboratory for a period of 21 days. After this period, the rats underwent an additional operation; an inventory of the peritoneal cavity was taken using a digital pachymeter to identify the success of the autotransplantation, followed by a volume calculation using this formula: $[4p \text{ (length/2)} \times \text{(width/2)} \times \text{(height/2)/3}]$ (Kudon et al., 1997). Classification of the experimental endometriosis implant growth was performed according to Quereda et al. (1996), and only those animals that progressed to a growth score III remained in the study (Figure 1).

After the surgical approach, the rats were identified and randomly divided into (pioglitazone-treated Group) and model control groups (MC group). Gavage of pioglitazone at 0, 25 and 0.50 mg/kg d for 15 days was carried out for the pioglitazone groups (n=8 in each group). The MC group received 1 ml daily gavage of 0.9% saline solution for 14 days. After the end of the medical treatments, the rats were euthanized using ketamine anesthesia, and a third laparotomy was performed. After opening the abdominal wall, an inventory of the peritoneal cavity and measurements of autotransplant volumes were performed; the transplant and the middle third of the remaining uterine horn were then removed. The salvaged tissue was rinsed with 0.9% saline solution and stored in 10% formaldehyde buffer for later anatomopathological analysis. Paraffin tissues were sectioned in 5-mm widths and placed in a warm bath; the slides were incubated with Meyer albumin and dried afterwards. Tissue sections were stained with hematoxylin–eosin (HE), and histological analysis was performed by one pathologist. The estrous cycles were analyzed in the middle third of the uterine horn remaining and drug efficacy was evaluated.

Serum tumor necrosis factor-alpha (TNF-α) level

Serum TNF-α level was measured using ELISA kits.

Immunohistochemistry

The specimens (endometriosis tissue) were cleared with xylene, and treated with 0.3% H₂O₂ in methanol. After washing with PBS, the Interleukin-8 (IL-8) was measured according to S-P immunohistochemistry kits.

Western blot analysis

Protein concentration of the samples (endometriosis tissue) was measured using bovine serum albumin (BSA) as the standard.

Proteins (20 or 40 μl) were separated using SDS-PAGE in 10% polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Richmond, CA), and the plate was blocked with 8% skim milk/TBS for 1 h at room temperature. The membrane was then incubated with the abovementioned anti-rat NF-κB p65, IκBα and β-actin antibodies diluted in 8% skim milk/TBS (1:200) overnight at 4°C followed by another 1 h incubation with horseradish-peroxidase-conjugated secondary antibodies (donkey anti-rabbit IgG; Amersham Pharmacia Biotech Ltd., Arlington Heights IL) and rabbit anti-goat IgG (Cappel, Aurora, OH)). The protein bands were visualized by ECL plus Western blotting detection system (Amersham Pharmacia Biotech Ltd., Arlington Heights, IL) followed by a brief exposure to Hyperfilm (Amersham Biosciences UK Ltd.). Quantity One v3.0 software (PDI, Inc, NY, USA) was used to quantitate the band intensities.

RESULTS

Table 1 shows the effect of pioglitazone on uterine implant volume in the experimental rats. There was insignificant difference in initial implant volume between groups. The value of uterine implant volume was found to be significantly decreased in the pioglitazone-treated rats

Table 1. Effect of pioglitazone on uterine implant volume, volume change and inhibition rate in the experimental rats.

Group	Implant volume (mm ³) before treatment	Implant volume (mm ³) after treatment	Volume change (mm ³)	Inhibition rate (%)
Model control	38.57±13.84	40.85±16.84	-2.28±4.29	-
Pioglitazone I	39.42±15.08	27.91±11.29 **	11.51±10.04 **	29.06
Pioglitazone II	40.13±22.14	17.04±7.39 **	23.09±12.65 **	57.39

** $p < 0.01$, compared with model control.

Table 2. Effect of the pioglitazone on spleen index, serum and peritoneal fluid TNF- α levels in experimental animals.

Group	Spleen index (mg/g)	Serum TNF- α (ng/L)	peritoneal fluid TNF- α (ng/L)
Model control	1.832±0.121	35.04±6.93	99.48±4.29
Pioglitazone I	2.104±0.159	21.21±6.91 **	73.82±2.85 **
Pioglitazone II	2.475±0.362 **	14.83±5.02 **	50.14±3.01 **

* $p < 0.05$, ** $p < 0.01$, compared with model control.

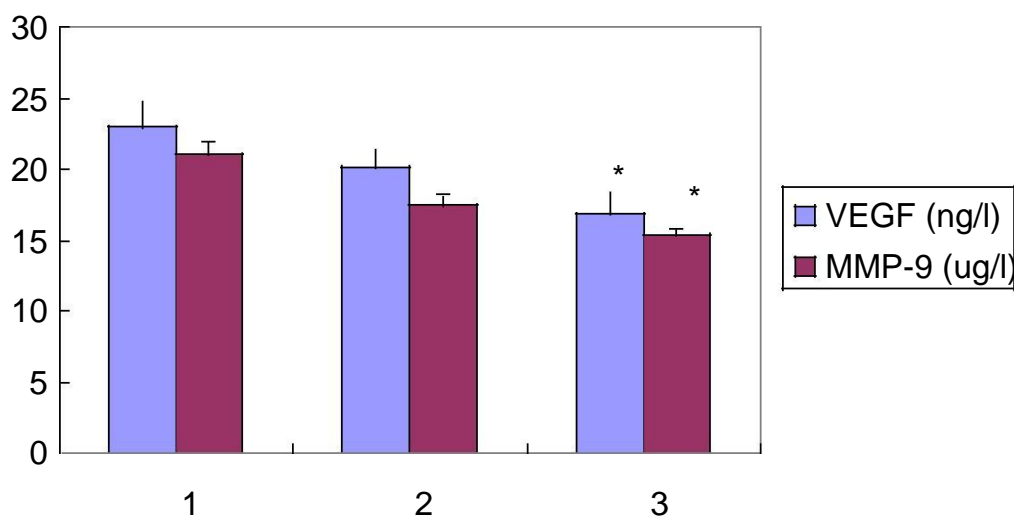


Figure 2. Effect of the pioglitazone on peripheral blood VEGF and MMP-9 levels. 1. Model control; 2. Pioglitazone I; 3. Pioglitazone II, * $p < 0.05$, compared with model control

when compared to model control rats. There was significant difference in volume change between model control group and pioglitazone-treated groups. Inhibition rate increased with the increasing medicine concentration.

The spleen index in the pioglitazone-treated animals was significantly higher than that of the corresponding model controls. However, there was a significant decrease in serum and peritoneal fluid TNF- α levels of the animals treated with pioglitazone compared to the corresponding model control animals ($p < 0.01$) (Table 2).

The levels of IL-8, and NF- κ B p65 in uterine implant of pioglitazone-treated animals were significantly lower than that of the corresponding model control (Figure 1). However, I κ B α in uterine implant of pioglitazone-treated

animals were significantly higher than that of the corresponding model control.

Figure 2 shows that the peripheral blood vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9) levels were significantly lower in the pioglitazone-treated groups than in the model control group ($p < 0.05$, $p < 0.01$). The effect was dose-dependent.

Figure 3 shows that the monocyte chemotactic protein-1 (MCP-1) and intercellular adhesion molecule 1 (ICAM-1) expression levels were significantly lower in the pioglitazone-treated groups than in the model control group ($p < 0.05$, $p < 0.01$). The effect was dose-dependent.

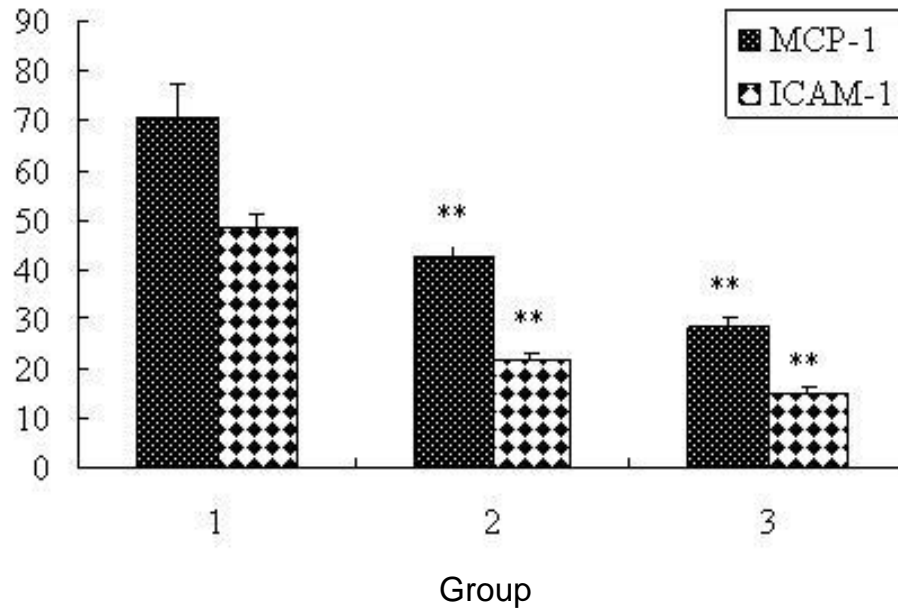


Figure 3. Effect of the pioglitazone on MCP-1 and ICAM-1 expression levels.

DISCUSSION

Endometriosis is one of the most common benign gynaecological conditions. These studies have indicated consistently that nulliparous women and women reporting short and heavy menstrual cycles are at an increased risk (Missmer and Cramer, 2003); other factors have been studied but the data are less consistent. These epidemiological findings support the retrograde reflux hypothesis—just one of several theories (others include the induction theory, the celomic metaplasia theory, and the embryonic rests theory (Oral and Arici, 1997; Witz, 1999) that have been put forward to explain the pathogenesis of endometriosis. Although not all of these alternative theories have been abandoned, at present, retrograde menstruation is considered the *primum movens* responsible for the development of the disease, at least in its form of peritoneal implants (Matarese et al., 2003).

In our study, we found that pioglitazone treatment inhibited uterine implant volume. To clarify the mechanisms involved in this inhibition, we assessed spleen index, serum and peritoneal fluid TNF- α levels, IL-8, NF- κ B p65 and I κ B α in uterine implant, and peripheral blood VEGF and MMP-9 expression levels after administration of pioglitazone. Spleen index was increased compared with model control rats. In addition, serum and peritoneal fluid TNF- α levels were decreased in the pioglitazone treatment rats.

Increased concentrations of neutrophils in the peritoneal fluid of patients with endometriosis are possibly related to increased local production of neutrophil-specific chemotactic factors. Indeed,

endometriosis has been reported to be associated with elevated concentrations of peritoneal IL-8 (Arici et al., 1998). In our study, we also found that pioglitazone treatment inhibited levels of IL-8, NF- κ B p65 in uterine implant. I κ B α level in uterine implant was increased with increasing pioglitazone concentration. Therefore, it is plausible that accumulation of neutrophils in the pelvic cavity of patients with endometriosis is at least partially due to IL-8, possibly derived from ectopic endometrioid tissue (Bersinger et al., 2008). Increased numbers of neutrophils in the peritoneal cavity of patients with endometriosis may also be related to their increased survival rate owing to antiapoptotic activity of the peritoneal fluid (Kwak et al., 2002).

The proliferation and resistance of EcC to apoptosis contribute to endometriosis development (Garcia-Velasco and Arici, 2003; Beliard et al., 2004; Harada et al., 2004). The role of NF- κ B as a proliferative and antiapoptotic factor has been proven in many studies (Yamamoto and Gaynor, 2001; Beg et al., 1995; Grumont et al., 1998; Slee et al., 1999; Karin and Lin, 2002; Doosti et al., 2011; Zhao et al., 2011). However, NF- κ B may have different effects on apoptosis in different cell types (Kasibhatla et al., 1999; O'Connor et al., 2000). A recent study showed the predictive value of NF- κ B/p65 activation and PR-B immunoreactivity for ovarian endometrioma recurrence, implicating these biomarkers in endometriosis recurrence and identifying these proteins as therapeutic targets to prevent recurrence (Shen et al., 2008).

Vascular endothelial growth factor (VEGF) facilitates the development of endometriotic lesions (Shifren et al., 1996; Van Langendonck et al., 2008). In a rat endometriosis model, a significant reduction in

microvessel density was achieved by inhibiting the NF- κ B pathway (Celik et al., 2008). Members of the metalloproteinase (MMP) family are structurally related proteins that degrade the extracellular matrix and components of the basement membrane (Hulboy et al., 1997). The 92-kD type IV collagenase (MMP-9) is an important enzyme for degradation of the basement membrane (primarily collagen type IV) and is crucial to the invasive ability of trophoblast cells (Librach et al., 1994; Behrendtsen et al., 1992) and to the progression of human neoplasms, as well as endometriosis (Fishman et al., 1997). Matrix metalloproteinase-9 plays an important role in the pathogenesis of endometriosis (Chung et al., 2001). In our present study, pioglitazone treatment significantly decreased the peripheral blood VEGF and MMP-9 expression levels in animals with endometriosis.

The use of pioglitazone led to a significant decrease of the uterine implant in experimentally induced endometriosis in rats. Moreover, the inhibition rate increased with increasing dose of pioglitazone. In addition to evidence that changes in inflammation, immunology and oxidative stress are somehow involved in the physiopathology of endometriosis, pioglitazone can act upon these diverse processes. Combined with our results, these conclusions suggest that pioglitazone could be a promising alternative for treating endometriosis.

REFERENCES

- Agic A, Xu H, Finas D, Banz C, Diedrich K, Hornung D (2006). Is endometriosis associated with systemic subclinical inflammation? *Gynecol. Obstet. Invest.*, 62:139–147
- Arici A, Seli E, Zeyneloglu HB, Senturk LM, Oral E, Olive DL (1998). Interleukin-8 induces proliferation of endometrial stromal cells: a potential autocrine growth factor. *J. Clin. Endocrinol. Metab.*, 83: 1201–1205.
- Barrier BF, Bates GW, Leland MM, Leach DA, Robinson RD, Propst AM (2004). Efficacy of anti-tumor necrosis factor therapy in the treatment of spontaneous endometriosis in baboons. *Fertil. Steril.*, 81(1): 775–779.
- Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D (1995). Embryonic lethality and liver degeneration in mice lacking the RelA component of NF κ B. *Nat.*, 376:167–170.
- Behrendtsen O, Alexander CM, Werb Z (1992). Metalloproteinases mediate extracellular matrix degradation by cells from mouse blastocyst outgrowths. *Dev.*, 114:447–456.
- Beliard A, Noel A, Foidart JM (2004). Reduction of apoptosis and proliferation in endometriosis. *Fertil Steril.*, 82:80–88.
- Berkley KJ, Rapkin AJ, Papka RE (2005). The pains of endometriosis. *Science*, 308:1587–1589.
- Bersinger NA, Frischknecht F, Taylor RN, Mueller MD (2008). Basal and cytokine-stimulated production of epithelial neutrophil activating peptide-78 (ENA-78) and interleukin-8 (IL-8) by cultured human endometrial epithelial and stromal cells. *Fertil. Steril.*, 8 (5): 1530–1536.
- Biswas SK, Chowdhury A, Das J, Zahid Hosen SM, Uddin R, Shahedur Rahaman M (2011). Literature review on pharmacological potentials of *Kalanchoe pinnata* (Crassulaceae). *Afr. J. Pharm. Pharmacol.*, 5(10): 1258–1262.
- Brown JD, Plutzky J (2007). Peroxisome proliferator-activated receptors as transcriptional nodal points and therapeutic targets. *Circulation*, 115:518–533.
- Celik O, Hascalik S, Elter K, Tagluk ME, Gurates B, Aydin NE (2008). Combating endometriosis by blocking proteasome and nuclear factor κ B pathways. *Hum. Reprod.*, 23:2458–2465.
- Chung HW, Wen Y, Chun SH, Nezhat C, Woo BH, Lake Polan M (2001). Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-3 mRNA expression in ectopic and eutopic endometrium in women with endometriosis: a rationale for endometriotic invasiveness. *Fertil. Steril.*, 75:152–159.
- D'Hooghe TM, Nugent NP, Cuneo S, Chai DC, Deer F, Debrock S, Kyama CM, Mihalyi A, Mwenda JM (2006). Recombinant human TNFRSF1A (r-hTBP1) inhibits the development of endometriosis in baboons: a prospective, randomized, placebo- and drug-controlled study. *Biol. Reprod.*, 74:131–136.
- Doosti A, Dehkordi PG, Davoudi N (2011). RA p53 codon 72 polymorphism associated with breast cancer in Iranian patients. *Afr. J. Pharm. Pharmacol.*, 5(10): 1278–1281.
- Falconer H, Mwenda JM, Chai DC, Wagner C, Song XY, Mihalyi A, Simsa P, Kyama C, Cornillie FJ, Bergqvist A, Fried G, D'Hooghe TM (2006). Treatment with anti-TNF monoclonal antibody (c5N) reduces the extent of induced endometriosis in the baboon. *Hum. Reprod.*, 21:1856–1862.
- Fallah E, Farshchian M, Khanmohammadi M (2011). Molecular and seroepidemiological study of *Leishmania infantum* infection among humans, dogs and wild canines from Azarshahr (new endemic focus), Iran. *Afr. J. Microbiol. Res.*, 5(10): 1237–1242.
- Fishman DA, Bafetti LM, Banionis S, Kearns AS, Chilukuri K, Stack MS (1997). Production of extracellular matrix-degrading proteinases by primary cultures of human epithelial ovarian carcinoma cells. *Cancer*, 75:1457–1463.
- Garcia-Velasco JA, Arici A (2003). Apoptosis and the pathogenesis of endometriosis. *Semin Reprod. Med.*, 21:165–172.
- Grumont RJ, Rourke IJ, O'Reilly LA, Strasser A, Miyake K, Sha W, Gerondakis S (1998). B lymphocytes differentially use the Rel and nuclear factor kappaB1 (NF- κ B1) transcription factors to regulate cell cycle progression and apoptosis in quiescent and mitogenactivated cells. *J. Exp. Med.*, 187:663–674.
- Harada T, Kaponis A, Iwabe T, Taniguchi F, Makrydimas G, Sofikitis N, Paschopoulos M, Paraskevaidis E, Terakawa N (2004). Apoptosis in human endometrium and endometriosis. *Hum. Reprod. Update*, 10:29–38.
- Hulboy DL, Rudolph LA, Matrisian LM (1997). Matrix metalloproteinase as mediators of reproductive function. *Mol. Hum. Reprod.*, 3:27–45.
- Karin M, Lin A (2002). NF- κ B at the crossroads of life and death. *Nat. Immunol.*, 3:221–227.
- Kasibhatla S, Genestier L, Green DR (1999). Regulation of fas-ligand expression during activation-induced cell death in T lymphocytes via nuclear factor κ B. *J. Biol. Chem.*, 274:987–992.
- Koninckx PR, Meuleman C, Demeyere S, Lesaffre E, Cornillie FJ (1991). Suggestive evidence that pelvic endometriosis is a progressive disease, whereas deeply infiltrating endometriosis is associated with pelvic pain. *Fertil. Steril.*, 55:759–765.
- Kudon M, Susuki Y, Ideyama Y, Nanya T, Mori M, Shikama H (1997). Inhibitory effects of a novel aromatase inhibitor, YM511, on growth of endometrial explants and insulin-like growth factor – I gene expression in rats with experimental endometriosis. *J. Steroid Biochem. Molec. Biol.*, 63(1–3):75–80.
- Kwak JY, Park SW, Kim KH, Na YJ, Lee KS, (2002). Modulation of neutrophil apoptosis by plasma and peritoneal fluid from patients with advanced endometriosis. *Hum. Reprod.*, 17: 595–600.
- Lebovic DI, Mueller MD, Taylor RN (2001). Immunobiology of endometriosis. *Fertil Steril* 75:1–10.
- Librach CL, Feigenbaum SL, Bass KE, Cui TY, Verastas N, Sadovsky Y (1994). Interleukin-1 b regulates human cytotrophoblast metalloproteinase activity and invasion in vitro. *J. Biol. Chem.*, 269:17125–17131.
- Matarese G, De Placido G, Nikas Y, Alviggi C (2003). Pathogenesis of endometriosis: natural immunity dysfunction or autoimmune disease?

- Trends Mol. Med., 9: 223–228.
- Missmer SA, Cramer DW (2003). The epidemiology of endometriosis. *Obstet. Gynecol. Clin. North Am.*, 30: 1–19.
- Nogueira NJ, Torres OJM, Borges MOR (2007). Changes in the volume and histology of focus of endometriosis in rats treated with simvastatin. *Rev. Bras. Ginecol. Obstet.*, 29 (8):396–402.
- O'Connor L, Huang DC, O'Reilly LA, Strasser A (2000). Apoptosis and cell division. *Curr. Opin. Cell. Biol.*, 12:257–263.
- Olive DL, Schwartz LB (1993). Endometriosis. *N. Engl. J. Med.*, 328:1759–1769.
- Oral E, Arici A (1997). Pathogenesis of endometriosis. *Obstet. Gynecol. Clin. North Am.*, 24: 219–233.
- Quereda F, Barroso J, Acien P (1996). Individual and combined effects of triptoreline and gestrinone on experimental endometriosis in rat. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, 67:35–40.
- Shen F, Wang Y, Lu Y, Yuan L, Liu X, Guo SW (2008). Immunoreactivity of progesterone receptor isoform B and nuclear factor kappa-B as biomarkers for recurrence of ovarian endometriomas. *Am. J. Obstet. Gynecol.*, 199:486.e1–10.
- Sher H, Alyemni MN (2011). Evaluation of anti-diabetic activity and toxic potential of *Lycium shawii* in animal models. *J. Med. Plants Res.*, 5(15): 3387-3395.
- Shifren JL, Tseng JF, Zaloudek CJ, Ryan IP, Meng YG, Ferrara N (1996). Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. *J. Clin. Endocrinol. Metab.*, 81:3112–3118.
- Slee EA, Adrain C, Martin SJ (1999). Serial killers: ordering caspase activation events in apoptosis. *Cell Death Differ.*, 6:1067–1074.
- Van Langendonck A, Donnez J, Defrere S, Dunselman GA, Groothuis PG (2008). Antiangiogenic and vascular-disrupting agents in endometriosis: pitfalls and promises. *Mol. Hum. Reprod.*, 14: 259–268.
- Willson TM, Brown PJ, Sternbach DD, Henke BR (2000). The PPARs: from orphan receptors to drug discovery. *J. Med. Chem.*, 43:527–550.
- Witz CA (1999). Current concepts in the pathogenesis of endometriosis. *Clin. Obstet. Gynecol.*, 42: 566–585.
- Yamamoto Y, Gaynor RB (2001). Therapeutic potential of inhibition of the NF- κ B pathway in the treatment of inflammation and cancer. *J. Clin. Invest.*, 107:135–142.
- Zainuddin BSM, Musa M, Zainuddin ZF (2011). Surface display of multi-epitopes synthetic gene of *Mycobacterium tuberculosis* on *Salmonella typhi* Ty21a using a pgsA anchor protein. *Afr. J. Microbiol. Res.*, 5(10): 1137-1146.
- Zhao YT, Bao YZ, Shi WY, Wang XD, Zhong XH (2011). Protective effects of baicalin on lipopolysaccharide (LPS)-induced implantation failure and the uterine endometrium in mice. *Afr. J. Pharm. Pharmacol.*, 5(14): 1661-1668.