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Evaluation of the Possible Role of Cyclooxygenase-2 in Endometriosis

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Abstract: *Endometriosis is a common, complex and chronic disease related to ectopic implantation and growth of endometrial tissue. Endometriosis is characterized by the presence and/or the growth of endometrial tissue (both glands and stroma) outside the uterine cavity that causes inflammation inside or outside the pelvis. This study aimed to investigate the possible role of COX-2 in endometriosis and its relationship with leptin, lipid peroxidation, nitric oxide and TNF-α. Materials and methods: Endometriosis was surgically induced in* 16 *female albino rats. Animals were divided into three groups. Group 1 (normal control group) was given only the solvent (PEG:saline) of celecoxib. Group 2 (endometriosis induced) was given PEG:saline Group 3 treated endometriosis with celecoxib. Results: endometriosis group showed significant increase in serum levels of leptin, lipid peroxidation (MDA), nitric oxide and highly significant elevation in serum level of TNF-α. Administration of celecoxib in endometriosis showed significant reduction in the serum levels of leptin, lipid peroxidation (MDA), nitric oxide and highly significant decrease in serum level of TNF-α. In conclusion: the current study suggests that selective COX-2 inhibitor celecoxib could ameliorate endometriosis.*

Key words: *cytokines, endometriosis, selective COX-2 inhibitors and cyclooxygenase-2.*

1. INTRODUCTION

Endometriosis is a chronic disease characterized by the presence of endometrial stromal and glandular tissue outside of the uterus, whose major clinical manifestations are infertility and chronic pelvic pain (Giudice, 2010). The pathophysiology of endometriosis is complex, involving many cytokines and pro-inflammatory mediators (Giudice, 2010) and possibly free oxygen radicals (Olivares et al., 2008). Hull and his associate (2005) theorized that inhibition of the prostaglandin pathway was likely to reduce the survival and growth of endometrium at an ectopic site. Cyclooxygenase (COX) -2 has been implicated in inflammatory, angiogenic, proliferative and estrogenic cellular processes (Dannenberg et al., 2001) and is likely to be influential in several of the processes that lead to ectopic endometrial development. Cyclooxygenase-2 is overexpressed in eutopic and ectopic endometrial tissue (Fagotti et al., 2004). Inflammatory mediators such as tumor necrosis factor- α (TNF- α) and interleukin-1b up regulate COX-2 mRNA and protein expression in cultured peritoneal macrophages (Wu et al., 2002). Tumor necrosis factor-α is secreted from activated macrophages and it has potent inflammatory, cytotoxic and angiogenic effects (Kilic et al., 2007).

Leptin is the product of the *ob* (*obese*) gene (Zhang et al., 1994) and initially was mainly associated to the regulation of food intake and energy expenditure (Friedman and Halaas, 1998). More recently, it has been recognized as an important mediator of immune regulation, angiogenesis and inflammation (Styer et al., 2008 and Babaei et al., 2011). The pro-inflammatory and neo-angiogenic properties of leptin have been shown to be important for the formation and persistence of endometriotic lesions (Babaei et al., 2011).

Nitric oxide is a known free radical that is involved in various physiological and pathophysiological processes in different organs, including the human female reproductive tract (Rosselli et al., 1998 and Dong et al., 2001). In the presence of oxidative stress, reactive oxygen species might increase growth and adhesion of endometrial cells in the peritoneal cavity, leading to endometriosis and infertility (Jackson et al., 2005).

Hormonal therapy with agents such as danazol, and GnRH analogues are effective treatments for the pain-associated symptoms of endometriosis and for the induction of the endometriotic lesion regression. The adverse effects, however, limit their long-term use; recurrence rates after cessation of therapy are high. Other medical treatments with improved side effect profiles and no limits on long-term use are needed (Ozawa et al., 2006). There is a real need for more effective approaches to the prevention and treatment of endometriosis. Cyclooxygenase -2 inhibitors show great promise in this respect (Ebert et al., 2005).

Since the clinical profile of endometriosis is typical of inflammatory disease, therapy with anti-inflammatory drugs has been proposed (Nothnick, 2001). We hypothesized that a reduction in prostaglandin levels secondary to COX-2 inhibition was likely to disrupt the inflammatory processes evident with endometriosis. So, the current study was designed to explore the contributions of COX-2 inhibitor celecoxib in treatment of endometriosis.

2. MATERIALS AND METHODS

2.1. Animals

Female adult albino rats weighing 150-200 grams at the age of 3.0-4.0 months were used. Animals were obtained from the animal house, Faculty of Medicine, Assiut University and were housed in animal place with room temperature being maintained at 25±2°C. Animals were fed on a commercial pellet diet and kept under normal light/dark cycle. Animals were given food and water *ad libitum*.

2.2. Surgical Induction of Endometriosis and Treatment

Endometriosis-like lesions were induced through transplantation of the right uterine horn to the bowel mesentery as described previously (Bilotas et al., 2010, Olivares et al., 2011, Ricci et al., 2011) regardless of the estrous cycle status of the rats. Briefly, animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) (Olivares et al., 2011). Rats underwent laparotomy by midventral incision to expose the uterus and intestine. The right uterine horn was removed, opened longitudinally, and cut into square pieces measuring 3×3 mm^2. Three equal pieces of full thickness were sutured onto serosal layer with a single 5-0 nylon suture (Supralon, Ethicon) with endometrial tissue facing the serosa. The abdominal layers were closed separately with a 5-0 nylon suture, 40 000 units/kg penicillin was injected into the muscle. Animals were assigned into three different groups: normal control group (8 rats) and endometriosis induced group (8 rats) both of them were given 0.5 ml i.p. of PEG: saline in 2.0:1.0 "solvent of celecoxib" (Paulson et al., 2000). Celecoxib (European Egyptian pharma, inc) in endometriosis induced (group 3) was given i.p. in dose of 200 mg/kg (Olivares et al., 2011). Celecoxib was dissolved in a solution of PEG: saline 2.0:1.0. PEG (400) was obtained from Adwek Co. England. Treatment was administered daily, started in postoperative day 1 and continued during 28 days. After 4 weeks of treatment, animals were anesthetized using ether. Blood samples were collected from the heart and serum was separated by centrifugation and stored at – 80˚C until analysis.

2.3. Biochemical Assessment

leptin content was determined by a sandwich enzyme immunoassay (ELISA) for the quantitative measurement of rat leptin in serum, plasma and tissue culture medium using kit Cat. No: RD 291001200R.

Malondialdehyde, the oxidative stress product of lipid peroxidation, reacts with thiobarbituric acid under acidic conditions at 95°C to form a pink-colored complex with an absorbance at 532 nm (Ohkawa et al., 1979).Nitric oxide concentration in serum was determined with the Greiss method. The Greiss reagent is made up of a 1% solution of sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihycrochloride in distilled water. The protein and phenol red of the serum were deleted using zinc sulfate (6 mg/400 µl). Sodium nitrite (0.1 M) was used for the standard curve, and increasing concentrations of sodium nitrite (5, 10, 25, 50, 75, and 100 µM) were prepared. The Greiss solution was added to all microplates, containing sodium nitrite and serum and was read by ELISA reader in 540 nm (Khazaei et al., 2011).

Tumor necrosis factor-α was measured, using a sandwich enzyme immunoassay kit protocol supplied by the manufacturer of the antibodies (Multisciences Biologic Company, Hangzhou, China) and resultant optical density determined, using a microplate reader (Thermo Multiskan MK3) at 450 nm.

2.4. Statistical Analysis

Statistics was performed using the statistical graph pad prism 5. One way analysis of variables (ANOVA) was used. Significant differences between the groups were determined using a post-hoc Newman-keuls test. Data were expressed as means ± standard error of the mean (SEM) and the level of significance between groups were considered significant (*) at $p<0.05$ and highly significant (**) at $p<0.01$.

3. RESULTS

3.1. Effect of Celecoxib on Leptin in Endometriosis Group

The endometriosis group showed significant increased serum leptin level in comparison with normal control group. The group treated with celecoxib (200 mg/kg. i.p.) had statistically significant reduction in the elevated serum leptin compared to endometriosis group the result is displayed in figure (1).

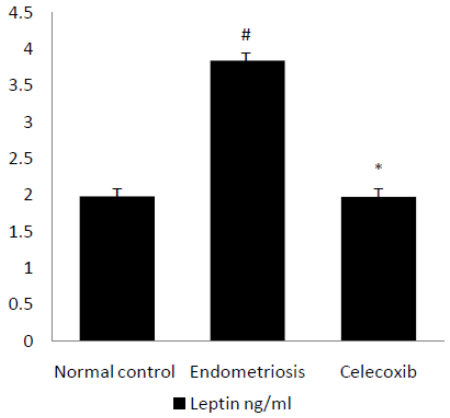

Figure1. *Effect of celecoxib (200 mg/kg. i.p.) on leptin in surgically induced endometriosis in rats*

*Data represent mean ± SE of 8 observations. # Significant result at p<0.05 from normal control * Significant result at p<0.05 from endometriosis*

3.2. Effect of Celecoxib on Lipid Peroxidation in Surgically Induced Endometriosis

After surgical induction of endometriosis in rats, this group showed significant elevation in serum lipid peroxide (MDA) level when compared with normal control group. Administration of celecoxib (200 mg/kg. i.p.) in endometriosis induced group leads to approximate normalization to the abnormal increased MDA as shown in table (1).

Table 1. *Effect of celecoxib (200 mg/kg. i.p.) on lipid perioxide in surgically induced endomeriosis in rats.*

Groups	Lipid perioxide n mol/ml
Normal control	2.74±0.12
Endometriosis	4.35±0.32#
Celecoxib	2.93±0.12*

*Data represent mean ± SE of 8 observations. # Significant result at p<0.05 from normal control * Significant result at p<0.05 from endometriosis*

3.3. Celecoxib Effect on Nitric Oxide after Induction of Endometriosis

Our results showed significant elevation in serum level of NO after surgical induction of endometriosis in rats. Results revealed that the i.p. administration of celecoxib (200 mg/kg) give significant decrease in serum level of NO compared to non-treated endometriosis group (table 2).

Table 2. *Effect of celecoxib (200 mg/kg. i.p.) on nitric oxide in surgically induced endometriosis in rats*

Groups	Nitric oxide µ mol/L
Normal control	19.6±1.8
Endometriosis	39.3±3.1#
Celecoxib	19.8±1.4*

*Data represent mean ± SE of 8 observations. # Significant result at p<0.05 from normal control * Significant result at p<0.05 from endometriosis*

3.4. Modification of Endometriosis Effect on Tumor Necrosis Factor Alpha by Celecoxib

Surgical induction of endometriosis in rats revealed that an increase in serum level of TNF-α which is highly significant when compared with normal control group. Celecoxib administration modified the elevated serum level of TNF-α and produced highly significant decrease in its level (figure 2).

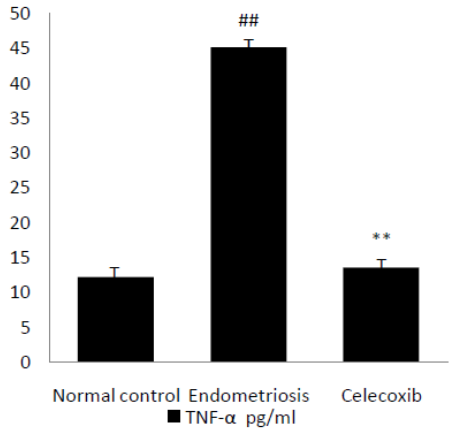

Figure2. *Effect of celecoxib (200 mg/kg. i.p.) on tumor necrosis factor alpha in surgically induced endometriosis in rats*

*Data represent mean ± SE of 8 observations. ## Highly significant result at p<0.01 from normal control and ** highly significant result at p<0.01 from endometriosis.*

4. DISCUSSION

Endometriosis affects a large number of women all over the world, and great efforts are being taken by researchers to give better and longer lasting answers to patients. Treatment for endometriosis is usually performed with surgery and/or medications. Up-to-date treatment options are poor and do not really cure this disease; they aim mainly at reducing pain and endometriotic growth. Nevertheless, the high recurrence rate of this illness is one of the most challenging problems nowadays. So, it is of great importance to study new strategies to treat endometriosis that minimize the process of adhesion, inflammation, angiogenesis that are associated with endometriosis and reduce the rates of recurrence.

In this study, the serum levels of leptin, MDA, NO and TNF-α were compared between normal control group (without endometriosis), endometriosis induced group and celecoxib treated in endometriosis group.

Leptin is a well-recognized pro-inflammatory and mitogenic agent, which plays a pivotal role in the establishment and viability of endometriosis lesions (Styer et al., 2008). These results showing significant elevated serum level of leptin in endometriosis group when compared to normal control group. The present results are consistent with other observations where they found markedly increased levels in leptin mRNA and protein in endometriosis in rats (Wu et al., 2002 and Alvarez et al., 2014). Furthermore, leptin protein levels are increased in peritoneal fluid of endometriosis patients (Bedaiwy et al., 2006). On the other hand, mice lacking functional leptin receptor fail to develop endometriosis-like lesions, and preventive treatment with pegylated leptin peptide receptor antagonist inhibits lesion establishment and development (Styer et al., 2008). Bedaiwy and his associates (2006) found higher levels of leptin in peritoneal fluid from patients with endometriosis compared to patients with idiopathic infertility and those undergoing tubal ligation /reanastomosis. In addition, peritoneal fluid leptin levels correlated positively with the stage of disease. These data suggest that the proinflammatory and neoangiogenic action of leptin may contribute to the pathogenesis of this disease. Moreover, leptin may play a role in the pathophysiology of endometriosis-associated pain. There was no association between peritoneal fluid leptin levels and idiopathic infertility (Bedaiwy et al., 2006).

Other studies evaluating the serum and peritoneal fluid levels of leptin in patients with endometriosis have reported conflicting results. They have been reported unchanged levels (Wertel et al., 2005; Barcz et al., 2008 and Gungor et al., 2009).

Endometriosis has also been associated with significantly higher levels of lipid peroxide-modified rabbit serum albumin, MDA-modified low-density lipoprotein, and oxidized low-density lipoprotein

as measured in serum and compared to tubal ligation cases (Shanti et al., 1999). Furthermore, lipid peroxide concentrations have been reported to be highest in women with endometriosis indicating the involvement of ROS in the development of the disease (Szczepańska et al., 2003). Also, elevated serum level of MDA is in agreement with Yavuz et al., (2014). These observations support the results of the present study where significant increase in serum MDA level in endometriosis in rats is observed. Increased glucose metabolism and defects in the mitochondrial respiratory system are suggested to be the possible sources of excessive reactive oxygen species generation in endometriosis (Jana et al., 2013).

Nitric oxide can be generated independently from NO synthase by reduction of nitrite, which can occur spontaneously under hypoxic and/or acidic conditions (Cortese-Krott et al., 2015).

The results revealed that a significant increase in serum level of NO in surgically induced endometriosis and this is in harmony with Rocha et al., (2015). Rocha and his colleges (2015) showed that women with chronic pelvic pain secondary to endometriosis had significantly elevated plasma NO levels compared with healthy controls and women with chronic pelvic pain secondary to abdominal myofascial pain syndrome. Clinical improvement of chronic pelvic pain after surgical treatment of endometriosis was associated with a reduction of plasma NO levels. However, these findings were not observed in women with myofascial syndrome. This suggests that ablation or excision of endometriosis may be responsible for reducing peripheral levels of NO (Rocha et al., 2015). Furthermore, a significant increase in NO level was also detected in the peritoneal fluid of women with endometriosis (Simons et al., 1999), probably due to increased expression of inducible nitric oxide synthase (iNOS) in peritoneal macrophages (Osborn et al., 2002). Nitric oxide can directly stimulate the production of vascular endothelial growth factor; consequently, it is involved in angiogenesis of the endometrium (Fukumura et al., 2001). Angiogenesis is a critical step in the process of endometriosis progressing (Taylor et al., 2009). Vascular endothelial growth factor, which can be induced by COX-2, is one of the most potent angiogenic cytokines and has been shown to be highly up-regulated in various tumors, including ovary cancer (Gómez et al., 2009), endometrial cancer (Sugimoto et al.,2007), and other benign diseases, such as endometriosis (Taylor et al., 2009).

Tumor necrosis factor-α is a pro-inflammatory cytokine produced mainly by activated macrophages. It promotes the production of other pro-inflammatory cytokines, such as IL-1, IL-6, and additional TNF-α. It is involved in the normal physiology of endometrial proliferation and shedding (Lu et al., 2013). The results showed significant increase in serum TNF- α level in endometriosis group compared to normal control. Other studies are in accordance with our results and shown that peritoneal fluid TNF-α concentration is elevated in women with endometriosis (Bedaiwy et al., 2002; Richter et al., 2005 and Andrei et al., 2015). Tumor necrosis factor-α may play a central role in the local and systematic manifestations of endometriosis, on the basis of evidence showing that it promotes the growth of endometriotic cells. It is known to stimulate the expression of matrix metalloproteinase by endometriotic tissues, and matrix metalloproteinase actively participate in the invasion and matrix remodeling of endometriotic lesions (Kilic et al., 2007).

Moreover, another study explored the association of TNF-α gene polymorphisms and endometriosis, and it seems that some polymorphisms are involved in the pathogenesis of endometriosis (Lee et al., 2008). On the other hand, blocking TNF-α appears to inhibit the development of the disease in animal models (Lu et al., 2013).

The results of this study show significant reduction in serum levels of leptin, MDA, NO and TNF-α after administration of celecoxib in endometriosis group. Ozawa and his associate (2006) suggest that selective COX-2 inhibitors may be effective to suppress the establishment or maintenance of endometriosis partially through their antiangiogenic activity. These findings indicate that COX-2 may be involved in the pathogenesis and progression of endometriosis (Cho et al., 2010). The reduction in cell proliferation and vascular density and the increase in the apoptosis rate are mechanisms implicated are mostly an effect of celecoxib (Olivares et al., 2011). Also, celecoxib attenuated the gene expression of pro-inflammatory mediators, IL-6, IL-1β, and TNF-α as well as nuclear NF-κB (Seung et al., 2014).

Finally, endometriosis is an ambiguous disease with no definitive medical/surgical solutions. To date, standard medical treatments have to deal with the limit of preventing spontaneous ovulation and of recurrence after discontinuation. Since the clinical profile of endometriosis is typical of inflammatory disease, therapy with anti-inflammatory drugs has been proposed (Nothnick, 2001). For this purpose,

COX-2 inhibitors could be used in addition to surgery in adjuvant settings for the treatment and/or prevention of endometriosis-related symptoms.

In conclusion, the possible role of cytokines, pro-inflammatory mediators and oxidative stress in the pathophysiology of endometriosis provides us another point of view for the treatment of the disease. Evidences from the current study demonstrated successful treatment of experimental endometriosis can be accomplished by celecoxib through attenuation of the gene expression of pro-inflammatory mediators and decreased lipid peroxidation that could provide insights into the new endometriosis treatment modalities, which is an area of ongoing research. This preliminary information suggests that celecoxib as a common and cost-saving product that is available can be used as an alternative option in the medical treatment of endometriosis.

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