European Journal of Pharmacology ■ (■■■■) ■■■–■■■

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European Journal of Pharmacology

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

Endocrine pharmacology

# Regression of endometrial implants treated with vitamin $D_3$ in a rat model of endometriosis

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ARTICLE INFO

*Article history:*
Received 23 January 2013
Received in revised form
4 June 2013
Accepted 16 June 2013

*Keywords:*
Endometriosis
Vitamin $D_3$
Cholecalciferol
Rat surgical model

ABSTRACT

Endometriosis is one of the most frequent gynecological diseases. In addition to their side effects, available medical therapies may decrease fertility. Current understanding of endometriosis focuses on the role of the immune system in its pathophysiology. Recent research shed light on the immunomodulatory effect of vitamin $D_3$. Thus, this study was designed to study the effect of vitamin $D_3$ on regression of endometriotic implants in a rat surgical model. Vitamin $D_3$ reduced cyst cross sectional area by 48.8%. Histologically, vitamin D treatment produced fibrosis as well as apoptosis in the stroma. The results of the present study suggest that vitamin $D_3$ administration may have a beneficial effect in treating endometriosis.

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## 1. Introduction

Endometriosis is a frequent gynecological disease. In fact, 10–15% of women in reproductive age and 40–50% of women with infertility are affected by endometriosis (Nothnick and Zhang, 2009). Since endometriosis is an estrogen-dependent disease, temporary atrophy of lesions can be achieved by drugs that suppress ovarian steroids (Yao et al., 2005). This hypoestrogenic state may compromise fertility temporarily (Panay, 2008). This issue is of special concern in reproductive age women. In addition, these therapies cannot be used for prolonged periods because of their severe secondary side effects (Rice, 2002). Furthermore, the chronic nature of the disease necessitates long-term or repeated courses of treatment. During therapy, the efficacy of treatment is high. However, recurrence of symptoms is common (Panay, 2008). Thus, there is a definite need to develop new effective drugs as an alternative to the currently used medical therapies (Grummer, 2006).

Many studies demonstrated that immunomodulators and anti-inflammatory agents are effective in treating endometriosis (Keenan et al., 1999). Vitamin $D_3$ exhibited pronounced immunomodulatory properties in both innate and adaptive immune systems (O'Brien and Jackson, 2012; Casteels et al., 1995). It prevented autoimmune diseases in different experimental models e.g. autoimmune diabetes in non-obese diabetic mice (Mathieu et al., 1995), inflammatory bowel disease (Cantorna et al., 2000), collagen-induced arthritis (Larsson et al., 1998) and prolonged graft survival e.g. pancreatic islets, heart, liver, skin grafts (Mathieu and Adorini, 2002). It has been proposed that vitamin $D_3$ might influence the local activity of immune cells which play important pathogenic roles in the development and maintenance of endometriosis (Casteels et al., 1995). In fact, different types of immune cells (monocytes, T and B lymphocytes) express vitamin D receptor. Furthermore, they possess $1\alpha$-hydroxylase activity; the enzyme that catalyzes the synthesis of vitamin $D_3$ (Van Etten et al., 2003). Moreover, there was a higher 1-$\alpha$-hydroxylase expression in the endometrium of women with endometriosis compared to healthy controls (Agic et al., 2007). Based on these findings, this study was designed to investigate the efficacy of vitamin $D_3$ in treating endometriosis.

## 2. Materials and methods

### 2.1. Animals

Animals under experimental work were handled and treated in a humane manner in accordance with the Declaration of Helsinki. Virgin female rats (*Rattus norvegicus* UJ-1) strain weighing 180–250 g were obtained from The University of Jordan animal house. They were allowed to acclimatize to the laboratory conditions for at least two weeks before use. Food and water were provided ad

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http://dx.doi.org/10.1016/j.ejphar.2013.06.016

libitum. The temperature was maintained between 18 and 25 °C. Light/dark cycle was 14 h of light and 10 h of dark. Vaginal smears were performed as in (Marcondes et al., 2002). Only those females which exhibited a regular estrous cycle during an observation period of 4–5-cycles underwent surgery.

### 2.2. First surgery

For the induction of endometriosis, a 3 cm ventral incision was performed aseptically under chloralhydrate anesthesia (300 mg/kg given intraperitoneally) (i.p). Resection of the right uterine horn was achieved after ligation at the utero-tubal junction and the utero-cervical junction. The excised horn was immersed in sterile normal saline and the endometrium was exposed by lengthwise incision. Two squares of $4 \times 4$ mm$^2$ of open uterus (including both endometrium and myometrium) were prepared. Two fragments were sutured to abdominal wall with the endometrium facing the peritoneal cavity. The fragment was always fixed over a large blood vessel (Fig. 1). A single stitch using 4/0 nylon suture (Hospital & Homecare Imp. & Exp. Co., Ltd., China) was applied at two opposite sides of the "square". In all experiments about 2 ml of sterile normal saline were added to the abdominal cavity to prevent dehydration and to minimize adhesions. The abdominal wall was closed in two layers using 4/0 nylon suture.

### 2.3. Second surgery

Second surgery was performed 28 days after the first one. Briefly, under aseptic conditions a 3 cm ventral incision was made for each rat under chloralhydrate anesthesia. Then, the size of the implant (the two major diameters) was measured by a caliper (accuracy = 0.05 mm). Then the abdominal wall was closed in two layers using 4/0 nylon suture and 2 ml of sterile normal saline were added to the abdominal cavity. After the second surgery, animals were randomly divided into different groups. Group 1 received a daily single dose of 42 μg/kg cholecalciferol (vitamin $D_3$) (Sigma-Aldrich, USA) in dimethyl sulfoxide (DMSO) i.p for 21 days. The selected dose was 1/1000 of the oral $LD_{50}$ of vitamin D. Group 2 received only the vehicle. Group 3 underwent ovariectomy in which the two ovaries were excised after cyst development. Any animal that developed any complication after the first and/or second surgery was excluded (e.g wound infection). At least 7 animals were used per group. The cross sectional area was calculated using the formula for an ellipsoid [length × width × (π/4)] (Eltern, 2008). Animals were randomly divided into different groups.

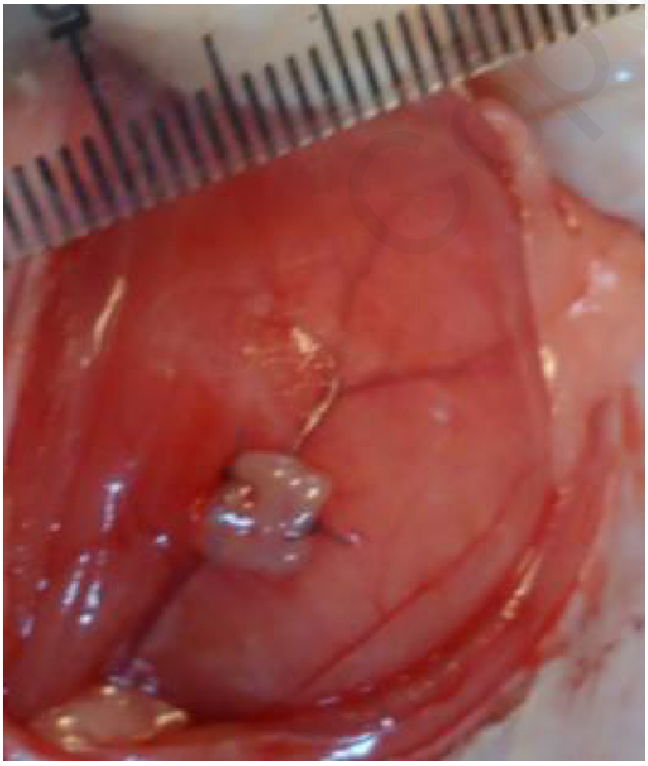

**Fig. 1.** Transplantaion of an endometrial implant. A $4 \times 4$ mm square of open uterus was fixed over a blood vessel using a surgical nylon blue suture with the endometrium facing the peritoneal cavity.

### 2.4. Light microscopic Studies

After 21 days of treatment, endometrial explants (cysts) were stored in 10% formalin solution for histological evaluation. Five micometer-thick sections were prepared and stained with haematoxylin and eosin stain (H&E). In addition, sections were stained with Masson Trichrome stain to evaluate the degree of fibrosis.

### 2.5. TUNEL assay

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay was done using the DeadEnd™ Colorimetric TUNEL System from Promega, USA according to the manufacturer's instructions. Menzel-Glaser SuperFrost Plus slides (Germany) were used for attaching sections.

### 2.6. Statistical analysis

The statistical analyses were performed using Statistical Package for Social Sciences (SPSS) version 17. The effect of treatments on cyst regression was evaluated by one-way analysis of covariance (ANCOVA) using Sidak corrected post hoc comparisons. In all tests a $P$ value $< 0.05$ was considered significant.

## 3. Results

### 3.1. Effect of vitamin $D_3$ on regression of endometrial explants

At the end of the treatment period, 42 μg/kg vitamin D3 resulted in 48.8% reduction in cyst cross sectional area ($P < 0.05$) (Table 1). Histological analysis of the endometrial implants in the control group revealed that the luminal epithelium was well-preserved (Fig. 2). The stroma was infiltrated by inflammatory cells. For vitamin $D_3$ treated group, fibrosis was noticed as a ring all over the cyst directly under luminal epithelium (Figs. 3 and 4).

**Table 1**
Cross sectional area before and after treatment.

| Treatment | Number of rats/group | Cross sectional area before treatment (mm$^2$) | Cross sectional area after treatment (mm$^2$) |
|---|---|---|---|
| vitamin $D_3$ | $N=9$ | $17.0 \pm 2.0$ | $8.3 \pm 1.8$ |
| Negative control (vehicle only) | $N=8$ | $15.8 \pm 1.8$ | $13.6 \pm 1.7$ |
| Ovariectomy | $N=7$ | $18.7 \pm 5.3$ | $0 \pm 0$ |

Values are mean ± SEM, $P < 0.05$.

Please cite this article as: Abbas, M.A., et al., Regression of endometrial implants treated with vitamin $D_3$ in a rat model of endometriosis. Eur J Pharmacol (2013), http://dx.doi.org/10.1016/j.ejphar.2013.06.016

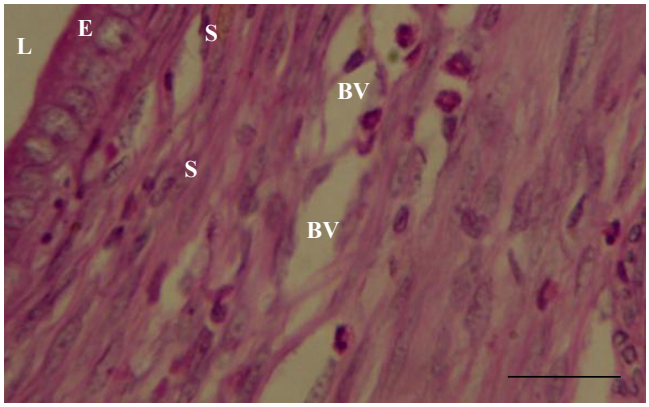


**Fig. 2.** Light microscopic view of a control rat cyst. Luminal epithelium was well preserved and the stroma was highly vascularized (H&E stain). BV: blood vessel; E: luminal epithelium; G: glandular tissue; L: cyst lumen; S: stroma. Scale bar=25 μm.

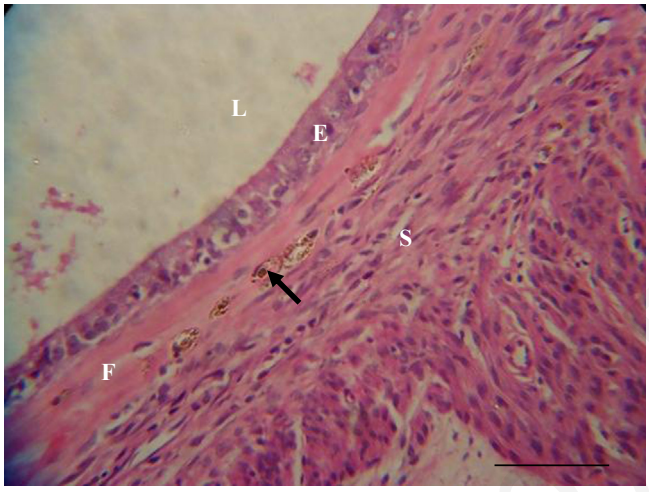


**Fig. 3.** Light microscopic view of a cyst of a vitamin $D_3$-treated rat. Fibrosis and hemosiderin-laden macrophages are seen in this section (arrow). Both are positive signs of healing process (H&E stain). E: luminal epithelium; F: fibrosis; L: cyst lumen; S: stroma. Scale bar=25 μm.

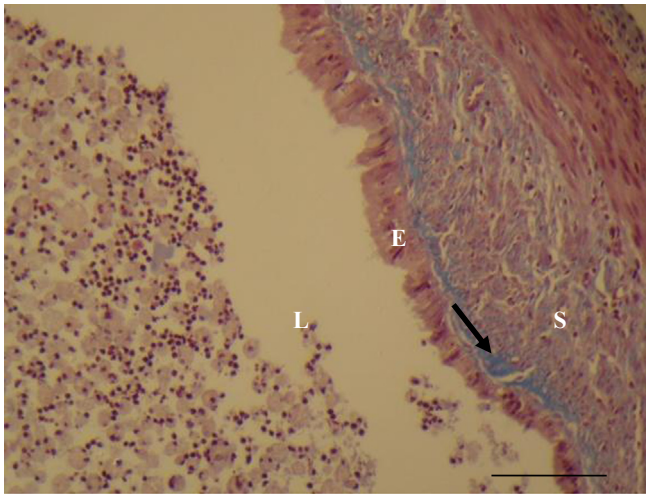


**Fig. 4.** Fibrosis in the stroma of a cyst of a vitamin $D_3$-treated rat. Fibrosis was concentrated directly under luminal epithelium and appears as a blue line (arrow). Fibrosis is usually present in healed foci of endometriosis (masson trichrome stain). E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar=100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### 3.2. TUNEL assay

Very few apoptotic cells in luminal epithelium were seen in control group. For vitamin $D_3$-treated group some cells in the stroma as well as some cells in luminal epithelium were apoptotic (Fig. 5B).

## 4. Discussion

Using a rat surgical model, vitamin $D_3$ reduced cross sectional area of endometriotic cysts by 48.8%. Up to our best knowledge, this is the first report demonstrating that vitamin $D_3$ had such an effect. However, the exact mechanism by which it functions needs more investigation.

It was reported that gonadotropin releasing hormone agonists used as a drug therapy for endometriosis reduce bone mass by about 2–5% when used for 6 months (Seko et al., 2004). Also, aromatase inhibitors decrease local estrogen synthesis in endometriosis. So, they may require estrogen add-back therapy to protect bones (Chwalisz et al., 2002). In fact, vitamin $D_3$ is given

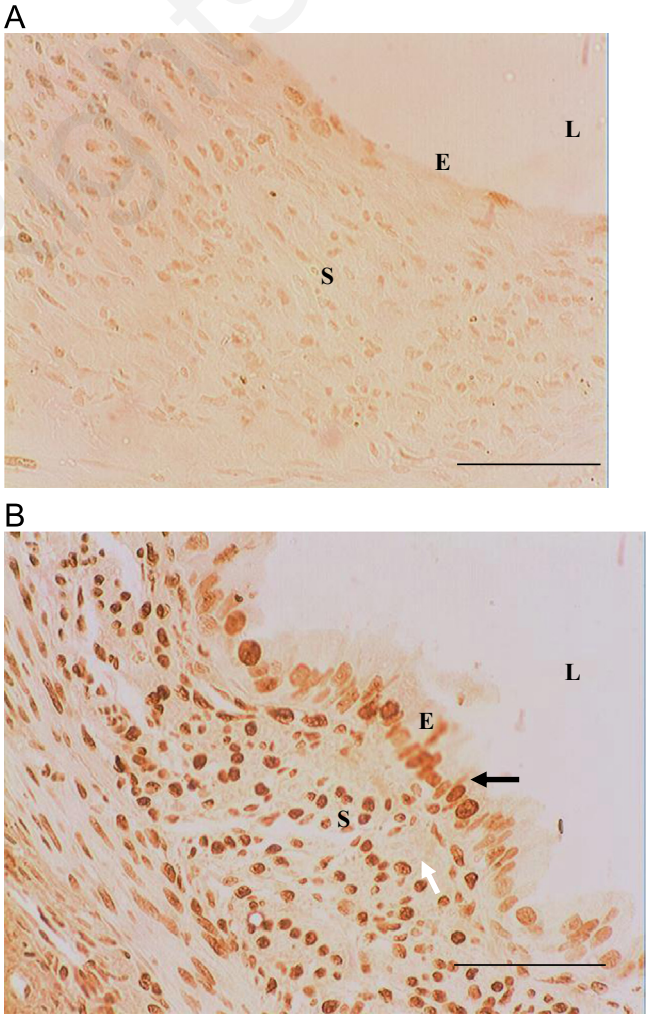


**Fig. 5.** Results of TUNEL assay. (A) control group: The cells in this section failed to take the dark brown color in TUNEL assay which indicates a negative result for apoptosis. (B) Vitamin $D_3$-treated group: some cells in luminal epithelium (black arrow) were positive in TUNEL assay (dark brown staining) as well as some cells in the stroma (white arrow) which means that they underwent apoptosis. E: luminal epithelium; L: cyst lumen; S: stroma. Scale bar=100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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sometimes in endometrioisis as a supplement to prevent osteoporosis (Crosignani et al., 2006).

The doses needed to produce the immune effects of vitamin $D_3$ are high and result in severe hypercalcaemia and accelerated bone remodeling. This constitutes a major problem with its in vivo use (Van Etten et al., 2002). Fortunately, synthetic analogs of vitamin $D_3$ that have similar immunomodulatory activity, but do not have its effects on calcium or bone metabolism (Bouillon et al., 1995). These analogs may prove useful in treating inflammatory diseases such as endometriosis in future.

## 5. Conclusion

The results of the present study indicate that vitamin $D_3$ may have a beneficial effect in treating endometriosis. So, its use may prove to be effective in humans without interference with fertility.

## Acknowledgment

This project was totally financed by the Deanship of Scientific Research at the University of Jordan, Amman.

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