

*Original article*

**Additive effects of inflammation and stress reaction on Toll-like receptor 4-mediated growth of endometrioid stromal cells**

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**Running title:** LPS and HSP70 in endometriosis

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## **Abstract**

**Title:** Additive effects of inflammation and stress reaction on Toll-like receptor 4-mediated **growth of endometrioid stromal cells**.

**Study question:** Is there any combined effect between inflammation and stress reaction on Toll-like receptor 4 (TLR4)-mediated growth of endometriotic cells?

**Summary answer:** A combined effect between local inflammation and stress reaction in pelvic environment may be involved in TLR4-mediated **growth of endometrioid stromal cells**.

**What is known already:** Higher endotoxin levels in menstrual fluid (MF) and peritoneal fluid (PF) and higher tissue concentrations of human heat shock protein 70 (HSP70) in eutopic and ectopic endometria promote TLR4-mediated **growth of endometriotic cells**.

**Study design, size and duration:** This is a case-controlled biological research with prospective collection of sera, MF, PF and endometrial tissues from women with and without endometriosis and retrospective evaluation.

**Participants/materials, setting, methods:** Peritoneal fluid was collected from 43

women with endometriosis and 20 control women during laparoscopy. Sera and endometrial biopsy specimens were collected from a proportion of these women. MF was collected from a separate population of 20 women with endometriosis and 15 control women. HSP70 concentrations in sera, MF, PF and in culture media were measured by enzyme-linked immunosorbent assay (ELISA). Gene expression of HSP70 by endometrial cells in response to lipopolysaccharide (LPS) was examined by qRT-PCR. The individual and combined effects of LPS and HSP70 on the secretion of interleukin-6 (IL-6) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) by PF-derived macrophages (M $\phi$ ) were examined by ELISA and on endometrial cell proliferation by bromodeoxyuridine and [<sup>3</sup>H]-Thymidine incorporation assay.

**Main results and the role of chance:** Concentrations of HSP70 was the maximum in MF, intermediate in PF and the lowest in sera. LPS stimulated gene expression and secretion of HSP70 by eutopic endometrial stromal cells (ESCs) and this effect was abrogated after pre-treatment of cells with anti-TLR4 antibody. This effect was significantly higher for ESCs derived from women with endometriosis than in control women. LPS was able to significantly stimulate the production of IL-6 and TNF $\alpha$  by M

$\phi$  and promoted proliferation of ESCs. Again, exogenous treatment of M  $\phi$  and ESCs with HSP70 stimulated IL-6/TNF $\alpha$  production and cell proliferation and a significant additive effect between LPS and HSP70 was observed. While individual treatment with either polymyxin B, an LPS antagonist or anti- HSP70 antibody was unable to suppress combined LPS+ HSP70-promoted cytokine secretion or cell proliferation, pretreatment of cells with anti-TLR4 antibody was able to significantly suppress their combined effect on IL-6/TNF $\alpha$  secretion and ESCs proliferation. Our findings suggest that both endotoxin and HSP70 were mutually involved in inducing stress reaction and inflammation. A combined effect between local inflammation and stress reaction in pelvic environment may be involved in TLR4-mediated **growth of endometriotic cells**.

**Limitations, reasons for cautions:** Further studies are needed to examine the mutual role between other secondary inflammatory mediators and endogenous stress proteins in promoting pelvic inflammation and **growth of endometrioid stromal cells**.

**Wider implications of the findings:** Since endometriosis is a multi-factorial disease, it is difficult to explain uniformly its growth regulation by a single factor. Our findings may provide some new insights to understand the physiopathology or pathogenesis of

endometriosis and may hold new therapeutic potential.

**Study funding/competing interest(s):** This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Sports, Culture, Science and Technology of Japan. There is no conflict of interest related to this study.

**Trial registration number:** not applicable.

**Key words:** endometriosis / LPS / HSP70 / TLR4 / inflammation / cell growth

## **Introduction**

Endometriosis is an estrogen-dependent chronic inflammatory disease and affects 8-10% of the total women population (Strathy, 1982). The main clinical problems of endometriosis are sufferings from a variable severity of pain, difficulty in achieving pregnancy, and recurrence after medical or surgical treatment (Strathy, 1982; Khan et al., 2008a; Stratton and Berkley, 2011). The exact pathogenesis of endometriosis is still unclear. There are some established hypotheses and regulatory factors supporting the development or maintenance of this disease (Khan et al., 2008a; Burney and Giudice, 2012). However, it is difficult to explain uniformly the pathogenesis of endometriosis by a single factor. Hormonal factors and inflammation

are commonly involved in the growth regulation of endometriosis (Khan et al., 2005a, 2008a; Burney and Giudice, 2012).

Endometriosis induces a variable amount of inflammatory reaction in pelvic environment depending on the staging and morphologic appearance of disease (Halme et al., 1987; Harada et al., 2001; Khan et al., 2004a). As an initial inflammatory mediator, bacterial endotoxin or lipopolysaccharide (LPS) has been recently reported to regulate Toll-like receptor 4 (TLR4)-mediated **growth of endometriotic cells** (Khan et al., 2010a, 2012). Again, endometriosis induces a variable amount of stress reaction in pelvic environment during cyclic menstrual reflux, implantation and invasion of endometrial cells into pelvis (Asea et al., 2000, 2002; Zugel and Kaufmann, 1999; Matzinger, 1998).

In fact, tissue stress reaction in endometriosis may occur in response to several types of stress-related stimuli such as inflammation, physical stress (cell proliferation, invasion), chemical stress (receptor/ligand binding), neurogenic stress, pain sensation, and oxidative stress (Khan et al., 2008b). As a marker of tissue stress reaction, we recently demonstrated that human heat shock protein-70 (HSP70) regulates

TLR4-mediated **growth of endometriotic cells** (Khan et al., 2008b). However, information on the combined role of inflammation and stress reaction in women with endometriosis is lacking.

Therefore, in the first part of this study, we aim to measure HSP70 levels in sera, menstrual fluid (MF) and peritoneal fluid (PF) collected from women with and without endometriosis. Secondly, we examined the ability of LPS in the production of HSP70 by eutopic endometrial stromal cells (ESCs) in primary culture. Thirdly, we investigated single and combined effects of LPS and HSP70 on the production of cytokines by peritoneal macrophages ( $M\phi$ ) and in the proliferation of eutopic/ectopic ESCs derived from women with and without endometriosis.

## **Material and Methods**

**Reagents.** Culture media: RPMI-1640 medium for macrophages and Dulbecco's Modified Essential Medium (DMEM):Hams F12 medium for stromal cells and were supplemented with 100 IU/mL of penicillin G, 50 mg/mL of streptomycin, 2.5  $\mu$ g/mL of amphotericin B (GIBCO, Grand Island, NY). Fetal bovine serum (FBS), lipopolysaccharide (LPS, derived from *Escherichia coli*, serotype 0111:B4), polymyxin

B ( $1 \mu\text{g/ml}$ ) and anti-HSP70 neutralizing antibody ( $10 \mu\text{g/ml}$ ) were all purchased from Sigma Chemical Co. (St. Louis, MO). Anti-TLR4 neutralizing antibody (HTA-125,  $10 \mu\text{g/ml}$ ) was purchased from HyCult Biotechnology, PA, USA, and highly purified recombinant human HSP70 ( $10 \mu\text{g/ml}$ ) (low endotoxin, ESP-555) was purchased from Stressgen, Victoria, Canada. GnRH agonist, (GnRHa,  $10^{-8}\text{M}$ ) (Leuplin: leuprolide acetate), was kindly provided by Takeda Pharmaceutical Co. (Tokyo, Japan). The concentrations of LPS, HSP70, polymyxin B, anti-HSP70 antibody, anti-TLR4 antibody and GnRHa were chosen based on previously published literatures (Khan et al., 2008b, 2010a, 2010b; Liu et al., 2010).

***Patient samples.*** The subjects in this study were women of reproductive age.

Peritoneal fluid (PF) was collected from 43 women with endometriosis and cycle matched to 20 women without endometriosis. Women with endometriosis aged between 20 and 42 years were recruited by either elective laparoscopy for infertility or diagnostic laparoscopy for dysmenorrhea and subsequently confirmed by histology. The control group, between 18 and 32 years old, consisted of fertile women and were operated on for dermoid cyst. All patients included had laparoscopies to confirm presence or



absence of endometriosis.

The staging and the morphological distribution of peritoneal lesions were based on the revised classification of the American Society of Reproductive Medicine (r-ASRM) (1997). Neither the study group nor the endometriosis-free group had been on hormonal medication in the 3 months prior to the surgical procedure. All control women and women with endometriosis had regular menstrual cycles (28-32 days). The phases of the menstrual cycle was determined by histological dating of eutopic endometrial samples taken simultaneously with pathological lesions derived from these women. The distribution of patients in different revised-ASRM staging of endometriosis and in different phases of the menstrual cycle is shown in Table 1. Sera and biopsy specimens were collected from a proportion of these women before and during laparoscopy.

All body fluids and biopsy specimens were collected in accordance with the guidelines of the Declaration of Helsinki and were approved by the Institutional Review Board of Nagasaki University. An informed consent was obtained from all women.

***Collection of menstrual blood.*** Under strict aseptic measure, we collected

menstrual blood from a separate population of 20 women with endometriosis and 15 women without endometriosis on day 1 to day 3 of the menstrual cycle and as described previously (Kamiyama et al., 2004; Khan et al., 2010). The materials obtained were transferred into heparinized endotoxin-free plastic containers, processed, centrifuged, and stored. All samples (sera, MF and PF) were stored at -80°C for subsequent analysis.

***Isolation of M  $\phi$  and eutopic/ectopic ESCs.*** Macrophages from the PF and stromal cells from the eutopic and ectopic (peritoneal lesions) endometria were collected from six women each with or without endometriosis. The detail procedures of the isolation of M  $\phi$  (Rana et al., 1996; Khan et al., 2005b) and stromal cells (Osteen et al. 1989; Sugawara et al., 1997) were described previously. A brief description of each isolation procedure is mentioned in Supplementary data 1.

***Treatment of stromal cells and macrophages.*** ESCs ( $10^4$  cells/ml) derived from eutopic and ectopic endometria were plated in 96 well microtitre plate and culture media was collected in a time-dependent fashion (0, 6, 12, 24, 48, 72hrs) to examine basal (non-treated) secretion of HSP70 in the culture media. At the pre-confluent stage, eutopic ESCs from women with and without endometriosis were treated with various

doses of LPS (0, 5, 10, 100ng/ml) in serum free DMEM medium and incubated for another 24 hour. Culture media and ESCs were collected for HSP70 assay and gene expression. A neutralizing experiment was performed with anti-TLR4 antibody (10  $\mu$ g/ml) 20 minutes prior to treatment with LPS in order to examine any change in the secretion and gene expression of HSP70.

The isolated peritoneal M  $\phi$  derived from women with and without endometriosis were cultured in triplicate (10<sup>5</sup> cells per well) for 24 hours to assess basal production of IL-6 and TNF $\alpha$ . To evaluate the LPS/ HSP70-stimulated secretion of IL-6/TNF $\alpha$ , after initial culture with serum containing RPMI medium, M  $\phi$  were serum starved for 24 hours and then serum free M  $\phi$  were cultured for another 24 hours with highly purified recombinant human HSP70 (10  $\mu$ g/ml) and LPS (10ng/ml). A blocking experiment was performed with anti-HSP70 antibody (10  $\mu$ g/ml), polymyxin B (1  $\mu$ g/ml) and anti-TLR4 antibody (10  $\mu$ g/ml) 20 minutes prior to LPS+ HSP70 treatment in order to examine any change in the secretion of cytokines in culture media without washing the pre-incubated antibodies. After 24 hours, the cultured media were collected in triplicate, pooled, and frozen at -70°C until testing. Possible contamination of

endotoxin with HSP70-treated cells was examined by measuring endotoxin levels in the culture media by the limulus amoebocyte lysate test (Endotoxin-Single Test; Wako-Jun-Yaku Co. Ltd., Tokyo, Japan), pre-treatment of cells with polymyxin B (1  $\mu$ g/ml), and by heat treatment (65°C) of HSP70-treated cells.

**Gene expression of HSP70 in ESCs.** Total RNA was isolated from LPS- or anti-TLR4 antibody-treated and -non-treated ESCs using RNeasy Mini Kit (Qiagen, Tokyo, Japan). RNA (1  $\mu$ g) was added to reverse the transcription reaction (RT-PCR), and cDNA (1  $\mu$ L) was subjected to real-time qPCR using an ABI 7900HT system (Applied Biosystems, Warrington, U.K). Primers used for HSP70 gene expression were as follows: forward, 5'-CGACCTGAACAAGAGCATCA-3' and reverse, 5'-ATGACCTCCTGGCACTTGTC-3'. Gene expression of  $\beta$ -actin was used as internal control. All primers and probes were designed as described previously (Khan et al., 2008b; Liu et al., 2010). The gene expression level of HSP70 were calculated and normalized by dividing the corresponding values of  $\beta$ -actin. PCR conditions were as follows: For HSP70, 40 cycles at 95°C for 10 sec, 64°C for 10 sec, and 72°C for 12 sec; for  $\beta$ -actin, 30 cycles at 95°C for 10 sec, 60°C for 10 sec, and 72°C for 10 sec. All PCR

conditions were followed by melting curve analysis.

Each PCR product was purified with a QIAEX II gel extraction kit (QIAGEN), and their identities were confirmed using an ABI PRISM™ 310 genetic analyzer (Applied Biosystems, Foster city, CA).

***HSP70/cytokine assays in the body fluids/culture media.*** The culture media of basal (non-treated) and stimulated (treated) ESCs and M  $\phi$  were prospectively collected in triplicate and assays were performed retrospectively. The concentrations of HSP70 in sera/MF/PF and culture media were measured in duplicate by using a commercially available sandwich ELISA (StressXpress™, EKS-700, Stressgen Victoria, Canada) according to the manufacturer's instructions. The concentrations of IL-6 and TNF  $\alpha$  in culture media were measured in duplicate using ELISA developed by R & D system in a blind fashion (Quantikine, R & D system, Minneapolis, MN). The antibodies used in HSP70, IL-6, and TNF  $\alpha$  determination do not cross-react with other cytokines. The limits of detection were 200pg/ml for HSP70, 0.70 pg/ml for IL-6, and 4.4 pg/ml for TNF  $\alpha$ . Both the intra-assay and inter-assay coefficients of variation were <10% for all these assays.

**Terminal deoxy-UTP-biotin nick end-labeling (TUNEL) assay.** The detail procedure of TUNEL assay was described previously (Dmowski et al., 2001). **A brief description of TUNEL assay is mentioned in Supplementary data 2.**

Quantitative analysis of the apoptotic cells was performed with a cytometer under x400 magnification using Olympus (model DP20) microscope. The apoptotic index was defined as the number of apoptotic cells per 10mm<sup>2</sup> unit area.

**BrdU incorporation assay.** The detail procedure of 5-Bromo-2-deoxyuridine (BrdU) incorporation assay was described previously (Takagi, 1993; Khan et al., 2005b). **A brief description of BrdU incorporation assay is mentioned in Supplementary data 3.**

We examined the proliferation of ESCs in response to HSP70, LPS, and anti-HSP70 antibody, polymyxin B and anti-TLR4 antibody and the differences in cell proliferation were expressed as the percentage of controls. The absorbance values correlated directly to the amount of DNA synthesis and thereby to the number of proliferating cells in culture.

**[<sup>3</sup>H]-Thymidine incorporation assay.** To evaluate effect of HSP70 and LPS on

DNA synthesis in eutopic and ectopic ESCs,  $5 \times 10^4$  cells were plated into each well of 24-well-multiplates and were incubated for 48 hr with fresh medium or fresh medium containing HSP70, LPS, and anti-HSP70 antibody, polymyxin B and anti-TLR4 antibody. Eight hours before the desired incubation period, cells were treated with 14.8kBq/ml of [ $^3$ H]-thymidine and were incubated again at 37°C until cells were harvested. Radioactivity (cpm/well) was quantified in a Packard Tri-Carb 2100 liquid scintillation analyzer. (Packard Instrument Co., Frankfurt, Germany).

***Statistical Analysis.*** The clinical characteristics of the subjects were evaluated by one-way analysis of variance. All results are expressed as either mean  $\pm$  SD or mean  $\pm$  SEM. The concentrations of HSP70 in different body fluids were not distributed normally and non-parametric test such as Mann-Whitney U-test and Student's t-test were used to analyze differences between groups. For comparison among groups, the Kruskal-Wallis test was used. A box plot analysis of HSP70 levels in different body fluids was performed using the medians and inter-quartile range (IQR). A *P* value  $<0.05$  was considered statistically significant.

## **Results**

There were no significant differences in clinical characteristics between women with or without endometriosis (Table 1). As an initial study, we also examined six women with endometriosis but without infertility. We did not find any difference in cytokine profile or cell growth in response to LPS/HSP70 in these two groups of women with endometriosis with and without infertility. Therefore, we represented our combined data here in women with and without infertility.

***HSP70 concentrations in body fluids.*** As an initial inflammatory mediator, we previously reported that endotoxin (LPS) levels in MF and PF were significantly higher in women with endometriosis than in non-endometriosis (Khan et al., 2010, 2012). Here, we found that as a marker of potentially active stress protein, the concentrations of HSP70 were the highest in the MF, intermediate in the PF and the lowest in the sera. In fact, HSP70 levels in the MF and PF was significantly higher in women with endometriosis ( $p < 0.05$  for MF or PF) than in control women (Figure 1, A, B). No difference in HSP70 levels of PF was observed between r-ASRM stage I-II and stage III-IV endometriosis or in sera between women with and without endometriosis (Figure 1, A, B). Kruskal-Wallis test indicated that HSP70 level in PF was the highest



in the menstrual phase among all phases of menstrual cycle ( $p < 0.05$  vs. other phases, Figure 1, C).

***TLR4-mediated production of HSP70 by LPS in ESCs.*** A substantial amount of HSP70 secretion in the culture media of basal eutopic and ectopic ESCs was observed. The basal secretion of HSP70 by ESCs was predominant at 12hr to 48hr incubation and significantly higher for cells derived from ectopic endometria than from eutopic endometria of women with endometriosis at these incubation times ( $p < 0.05$  for 12hr, 24hr, 48hr, Figure 2, A). Further incubation of ESCs showed a decline in basal HSP70 secretion. A significantly less amount of basal HSP70 secretion was observed by eutopic ESCs derived from control women than from endometriosis women (data not shown).

LPS dose-dependently stimulated the production of HSP70 by eutopic ESCs derived from women with and without endometriosis (Figure 2, B). This effect of LPS was significantly higher in women with endometriosis than in control women ( $p < 0.05$  for each of 5, 10 and 100ng/ml). Pretreatment of ESCs with anti-TLR4 antibody (10  $\mu$ g/ml) significantly abrogated LPS-promoted HSP70 secretion in the culture media

( $p < 0.05$  vs. anti-TLR4 antibody-non-treated cells, Figure 2, B). The neutralizing experiment of anti-TLR4 antibody on HSP70 gene expression was performed and we found that similar to protein secretion, blocking of TLR4 significantly suppressed LPS-stimulated HSP70 gene expression ( $p < 0.05$  vs. anti-TLR4 antibody-non-treated cells). This effect was equally observed for ESCs derived from women with and without endometriosis (Figure 2, C).

***TLR4-mediated production of IL-6 and TNF  $\alpha$  by HSP70- and LPS-treated***

***M  $\phi$*** . According to our initial time-dependent and dose-dependent study, we found a maximum increase in the levels of different macromolecules and cell growth at 24 to 48 hr and in response to 10  $\mu$ g/ml of HSP70 and 10-100ng/ml of LPS. Therefore, here we represented all our experimental data in response to 10  $\mu$ g/ml of HSP70 and 10ng/ml of LPS with an incubation period of 24 hr.

Exogenous treatment with HSP70 and LPS significantly increased the secretion of IL-6 and TNF  $\alpha$  in the culture media of M  $\phi$  derived from women with endometriosis than in non-endometriosis (Figure 3). A prominent additive effect between HSP70 and LPS in further secretion of IL-6 and TNF  $\alpha$  was observed. This

effect was less observed for TNF  $\alpha$  secretion in control women. While polymyxin B (1  $\mu$  g/ml), a potent LPS antagonist and anti- HSP70 antibody (10  $\mu$  g/ml) were unable to suppress combined LPS+HSP70-stimulated IL-6/TNF  $\alpha$  secretion in M  $\phi$  culture media, pretreatment of cells with anti-TLR4 antibody (10  $\mu$  g/ml) was able to significantly decrease LPS+HSP70-stimulated production of IL-6 and TNF  $\alpha$  (Figure 3). The neutralizing effect of anti-TLR4 antibody on IL-6 secretion was observed for M  $\phi$  derived from women with and without endometriosis but this effect on TNF  $\alpha$  secretion was observed only for M  $\phi$  derived from women with endometriosis (Figure 3). It can be noted that only IL-6 secretion and not TNF  $\alpha$  secretion by non-treated M  $\phi$  was significantly higher in women with endometriosis than in control women.

***Effect of HSP70 on TUNEL-positive ESCs cells and apoptotic index.*** ESCs

derived from proliferative phase endometria of women with endometriosis showed increased number of TUNEL-positive cells (Figure 4, b, c, d of A) and increased apoptotic index in response to GnRHa ( $10^{-8}$ M)(Figure 4, B). Exogenous treatment with HSP70 (10, 50  $\mu$  g/ml) was found to significantly decreased GnRHa-induced TUNEL-positive cells (e, f of A) and apoptotic index (Figure 4, B). This anti-apoptotic

effect of HSP70 was not observed with lower doses (1, 5  $\mu$ g/ml). GnRHa- and HSP70-non-treated ESCs showed minimal apoptotic cells.

***Effect of HSP70 and LPS on the proliferation of ESCs.*** In vitro exposure of both eutopic and ectopic ESCs to HSP70 (10 $\mu$ g/ml) significantly incorporated BrdU into these cells and ESCs proliferation in response to HSP70 was significantly higher when compared with HSP70-non-treated cells ( $p < 0.05$  for both eutopic and ectopic ESCs). Both of these ESCs proliferation derived from eutopic and ectopic endometria of women with endometriosis was significantly suppressed after pretreatment of cells with anti-TLR4 antibody ( $p < 0.05$  for each of ESCs) (Figure 5, A). Although less BrdU incorporation, a similar pattern of suppression effect was observed for ESCs derived from eutopic endometria of control women (data not shown).

When we examined single and combined effect of HSP70 and LPS on BrdU incorporation into ESCs derived from ectopic endometria, we found that in addition to their individual significant effect on cell proliferation, an additive effect between HSP70 and LPS was observed in further promoting ESCs proliferation ( $p < 0.05$  vs. single treatment). While individual treatment with polymyxin B or anti-HSP70 antibody

was unable to suppress combined LPS+HSP70-promoted cell proliferation, pretreatment of cells with anti-TLR4 antibody was able to significantly suppress this combined effect on cell proliferation ( $p < 0.05$  vs. LPS+HSP70 treatment) (Figure 5, B).

In order to strengthen the results of BrdU incorporation assay in cell proliferation, we also performed [<sup>3</sup>H]-thymidine incorporation assay to examine DNA synthesis of eutopic/ectopic ESCs in response to HSP70, LPS and other neutralizing antibodies. We found a similar pattern of cell proliferation of eutopic/ectopic ESCs as shown in Figure 6 (A, B).

***Exclusion of endotoxin contamination with HSP70-treated cells.*** In order to exclude the possible contamination of endotoxin with HSP70-treated cells, both M  $\phi$  and endometrial stroma, we repeatedly measured endotoxin level in the culture media. We could not detect any endotoxin in the culture media of HSP70-treated M  $\phi$  or HSP70-treated stromal cells. Pre-treatment of M  $\phi$  with polymyxin B (1  $\mu$  g/ml) failed to decrease the levels of any of these macromolecules in the culture media of HSP70-treated cells (data not shown). Since LPS is heat stable and HSP70 is heat labile (Wallin et al., 2002), we further excluded endotoxin contamination by heat treatment

(65°C) of HSP70-treated cells for 20 minutes. We could not detect any cytokine in the culture media or cell proliferation of HSP70-treated cells, possibly due to degradation of HSP70 after heat treatment.

## **Discussion**

We demonstrated here for the first time that a mutual inflammatory reaction and stress reaction occur in the pelvic environment of women with endometriosis. When there is inflammatory reaction in intrauterine environment or in pelvis as evidenced by the elevated endotoxin (LPS) levels in MF and PF (Khan et al., 2010a, 2012), LPS induces an internal tissue stress reaction and pro-inflammatory response as we confirmed here by LPS-stimulated cytokine production and increased HSP70 production in the culture media of basal and LPS-stimulated ESCs. Again, as a marker of stress reaction, HSP70 significantly stimulated the secretion of IL-6 and TNF $\alpha$  by M $\phi$  as well as markedly promoted ESCs proliferation. An additive effect between LPS and HSP70 was observed in cytokine production and ESCs proliferation. These effects of LPS and Hsp70 were more remarkable in cells derived from women with endometriosis than in control women.

It can be noted that M  $\phi$  from control women were less responsive to single and combined treatment with HSP70 and LPS in the secretion of TNF $\alpha$  when compared with IL-6 secretion from similar control women. This could be due to the variation in the secretion ability or receptor-ligand binding affinity in M  $\phi$  of control women for the secretion of IL-6 or TNF $\alpha$ . This may explain why there was no significant reduction in TNF $\alpha$  after pre-treatment of control M  $\phi$  with anti-TLR4 antibody.

We previously reported that LPS and HSP70 alone was able to exhibit TLR4-mediated increased cytokine production and growth of endometriotic cells (Khan et al., 2008b, 2010a). In our current study, we demonstrated that pretreatment of M  $\phi$  and ESCs with either polymyxin B or neutralizing antibody against HSP70 was unable to abrogate combined LPS+HSP70-promoted cytokine production and cell proliferation. In contrast, cytokine production and growth promoting effect of combined LPS+HSP70 were significantly suppressed when the biological function of TLR4 was blocked with anti-TLR4 antibody. This indicates that LPS- and HSP70-mediated inflammatory reaction and growth of endometriotic cells may be mediated by TLR4 in pelvic environment. Here, we confirmed the validity of BrdU incorporation assay in ESCs

proliferation by an additional [3H]-thymidine-based cell proliferation assay that showed a similar pattern of ESCs proliferation in response to LPS and HSP70.

Toll-like receptor (TLR4) is an essential receptor for bacterial endotoxin or LPS recognition (Akira and Takeda, 2004; Takeda and Akira, 2005). In addition to *E.coli*-derived LPS, there are other exogenous (F protein from respiratory syncytial virus, chlamydial HSP60) and endogenous ligands (fibronogen, fibronectin, heparan sulphate, hyaluronic acid and HSP60) including HSP70 that can also transmit signal through TLR4 (Kiechl et al., 2002; Khan et al., 2009). Therefore, we presume that blocking of TLR4 may be more effective in reducing inflammatory response and growth of endometriotic cells in pelvis than to block a single ligand of TLR4. The gene and protein expression level of TLR4 in peritoneal M  $\phi$  and eutopic/ectopic endometrial cells are reported elsewhere (Fazeli et al., 2005; Hirata et al., 2005; Khan et al., 2008b, 2010a).

As a source of endotoxin (LPS) in pelvis, we recently reported significantly higher colony formation of *E.coli* in menstrual blood derived from women with endometriosis than in control women (Khan et al., 2010a). We further established that



higher prostaglandin E<sub>2</sub> in MF was responsible for *E.coli* contamination of menstrual blood that regulates down-stream cascade of LPS/TLR4/NF-kB in the development or maintenance of endometriosis (Khan et al., 2012). In a recent study, Hayashi et al., (2013) demonstrated a positive correlation between TLR4 and mPGES-1 gene expression in endometriotic lesions. As a rate-limiting metabolizing enzyme, mPGES-1 may participate in the production of PGE<sub>2</sub> in MF and PF in response to LPS or other inflammatory mediators in women with endometriosis.

Different physical stress, chemical stress, neurogenic stress, painful stimuli and oxidative stress may trigger variable degrees of tissue stress reaction in the pelvis of women with endometriosis (Khan et al., 2008b, Gill et al., 2010). In a previous study (Khan et al., 2008b), we observed remarkable endogenous tissue stress reaction in active blood-filled opaque red lesions than in transparent/translucent lesions or other less active peritoneal lesions. LPS itself may stimulate M $\phi$  for the production of TLR4-mediated reactive oxygen species (Khan et al., 2009). Therefore we presume that there is a close relationship among LPS, HSP70 and oxidative stress. Increased expression of HSP70 or inflammatory response in pelvis might be related to oxidative

stress induced by TLR4 stimulation (Gill et al., 2010).

Here we further demonstrated that as a potential marker of tissue stress reaction, soluble Hsp70 level was significantly higher in the MF and PF of women with endometriosis than in control women. Persistent endogenous stimulation in pelvis with LPS or HSP70 may change the cell membrane permeability causing efflux of different cytokines out of immune cells or may cause shifting of resting cells (S0) to proliferative phenotype (S2) in cell cycle. This may also result in increasing cytokine levels by M $\phi$  or increase in ESCs proliferation after exogenous exposure to LPS or HSP70. The possible contamination of endotoxin in the study of HSP70-treated cells during bio-culture procedure was a matter of concern in different reports (Byrd et al., 1999; Triantafilaou et al., 2001, 2004; Wallin et al., 2002). We carefully excluded the possible contamination of HSP70-treated cells with endotoxin by serial exclusion experiments.

Increased HSP70 gene and protein expression in the eutopic/ectopic endometria of women with endometriosis carries some biological significance. As a molecular chaperon, HSP70 inhibits apoptosis of host cells by preventing recruitment of caspases to the apoptosome complex (Beere et al., 2000). Therefore, increased

production of HSP70 by peritoneal M  $\phi$  and ESCs in pelvis may contribute to increased survival of endometrioid cells in women with endometriosis. HSP70-promoted survival of endometrial cells during the menstrual period and its retrograde entry into pelvis may facilitate their ectopic survival and implantation. This was further supported by the anti-apoptotic effect of HSP70 in our current study.

Our current findings provide us new information that a vicious cycle between inflammatory reaction and stress reaction is constantly occurring in the pelvis of women with endometriosis. Endotoxin (LPS) and HSP70 are mutually involved in inducing stress reaction and inflammation in pelvis. We conclude that a crosstalk between local inflammation and tissue stress reaction in pelvic environment may be involved in TLR4-mediated growth of endometriotic cells. Targeting TLR4 or source of initial inflammatory mediator either in intrauterine environment or within vaginal cavity could be a potential therapeutic approach to effectively reduce pelvic inflammation and growth of endometriotic cells. Future studies regarding investigating the relationship between secondary inflammatory mediators and other endogenous stress proteins or between ovarian steroids and inflammation in the pelvis of women with endometriosis

may strengthen our current findings.

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**Figure legends:**

**Figure 1.** Concentrations of human heat shock protein-70 (HSP70) in the menstrual fluid (MF), peritoneal fluid (PF) and sera derived from women with and without endometriosis and were measured by enzyme-linked immunosorbent assay. HSP70 levels in MF and PF were significantly higher in women with endometriosis than in control women ( $p < 0.05$  for each of MF and PF) (**A**, **B**). No difference in HSP70 levels in the PF was observed between revised-ASRM stage I-II and III-IV endometriosis (**B**). Kruskal-Wallis test indicated the highest HSP70 levels in the PF during the menstrual phase when compared with other phases of the menstrual cycle (**C**). Boxes represent the distance (interquartile range) between the first (25%) and third (75%) quartiles, and horizontal lines in the boxes represent median values. \* $p < 0.05$  vs. proliferative phase and secretory phase. The results are expressed as mean  $\pm$  SEM of duplicate measurements of each sample.

**Figure 2.** Time-dependent effect of LPS (**A**) on the production of HSP70 in the culture media of eutopic (white bar) and ectopic (black bar) endometrial stromal cells (ESCs) derived from women with endometriosis and was measured by

enzyme-linked immunosorbent assay. A maximum secretion of HSP70 in the culture media was observed at 12hr to 48hr and this secretion was significantly higher by ESCs derived from ectopic endometria than from eutopic endometria (\*p<0.05 for each indicated time).

LPS was able significantly stimulate secretion of HSP70 in a dose-dependent fashion (**B**) and this secretion was significantly higher in eutopic ESCs derived from women with endometriosis (black bar) than in control women (white bar) (\*p<0.05 for each indicated dose). Pretreatment of ESCs with anti-TLR4 antibody significantly decreased HSP70 secretion (\*p<0.05 vs. anti-TLR4 antibody-non-treated cells at indicated doses) (**B**). The relative HSP70 gene expression as analyzed by real-time qPCR in eutopic ESCs was also significantly suppressed after pretreatment of cells with anti-TLR4 antibody (black bar) (**C**). \*p<0.05 vs. without anti-TLR4 antibody (white bar). The results are expressed as mean  $\pm$  SEM of triplicate experiments of six different patients.

**Figure 3.** Single and combined effects LPS and HSP70 on the production IL-6 and TNF  $\alpha$  in the culture media of macrophages ( $M\phi$ ) derived from the PF of

control women (white bar) and women with endometriosis (black bar) and was measured by enzyme-linked immunosorbent assay. Besides individual significant effects (\* $p < 0.05$  vs. controls), a significant additive effect between LPS and HSP70 was observed in the production of these cytokines (\*\* $p < 0.05$  vs. single effect of HSP70 or LPS). While polymyxin B, a potent LPS antagonist and anti-HSP70 antibody were unable to suppress combined LPS+HSP70-promoted IL-6/TNF  $\alpha$  secretion, pretreatment of M  $\phi$  with anti-TLR4 antibody significantly decreased LPS+HSP70-stimulated cytokine production (# $p < 0.05$  for each of IL-6 and TNF  $\alpha$ ). A significantly higher basal secretion of IL-6 by HSP70/LPS-non-treated M  $\phi$  was observed in women with endometriosis than in control women (left panel) and this difference in basal effect was not observed for TNF  $\alpha$ . The results are expressed as mean  $\pm$  SEM of triplicate experiments of six different patients.

**Figure 4.** Dose-dependent effect of HSP70 on the pattern of apoptosis in endometrioid stromal cells (ESCs) (A) and apoptotic index (number of apoptotic cells/10mm<sup>2</sup> area) (B) and as measured by Terminal deoxy-UTP-biotin nick end-labeling (TUNEL) assay. We used GnRH agonist (GnRH<sub>a</sub>, leuprolide acetate,

$10^{-8}$ M) as a pro-apoptotic agent. HSP70 was able to significantly suppressed GnRHa-induced TUNEL-positive cells (e, f of A) and apoptotic index at a higher doses (10, 50  $\mu$  g/ml) but not at a lower doses (1, 5  $\mu$  g/ml). GnRHa- and HSP70-non-treated ESCs derived from the proliferative phase endometria of women with endometriosis showed minimal apoptotic change. The results are expressed as mean  $\pm$  SEM of triplicate experiments of six different patients. \* $p < 0.05$  vs. GnRHa-treated ESCs. Magnification of slides in (A), x400.

**Figure 5.** Single and combined effects of HSP70 and LPS on stromal cell proliferation derived from the eutopic and ectopic endometria of women with endometriosis and were measured by bromodeoxyuridine (BrdU) incorporation assay. Comparing to non-treated cells (control), HSP70 significantly stimulated proliferation of stromal cells derived from both eutopic and ectopic endometria ( $p < 0.05$  vs. non-treated cells) (A). This cell proliferation effect of HSP70 was significantly suppressed after pretreatment of cells with anti-TLR4 antibody (A, upper panel). Besides significant individual effect of HSP70 and LPS, an additive effect of HSP70+LPS on the proliferation of stromal cells derived from ectopic endometria was

observed ( $p < 0.05$  vs. single treatment)(**B**). Pretreatment of cells with anti-TLR4 antibody was able to significantly suppress combined LPS+HSP70-promoted cell proliferation ( $p < 0.05$ ) while such effect was not observed with individual pretreatment of cells with either polymyxin B or anti-HSP70 antibody (**B**, lower panel). The results are expressed as mean  $\pm$  SEM of triplicate experiments of six different patients.

**Figure 6.** Single and combined effects of HSP70 and LPS on stromal cell proliferation derived from the eutopic and ectopic endometria of women with endometriosis and were measured by [<sup>3</sup>H]-Thymidine incorporation assay. Radioactivity was expressed as count per minute per well (cpm/well). We found a similar pattern of growth promoting effect of eutopic/ectopic ESCs in response to HSP70 and LPS (A, B) and a similar fashion of anti-proliferative effect in response to anti-HSP70 antibody, polymyxin B and anti-TLR4 antibody (A, B) as shown in Figure 5. The results are expressed as mean  $\pm$  SEM of triplicate experiments of six different patients.



Figure 1

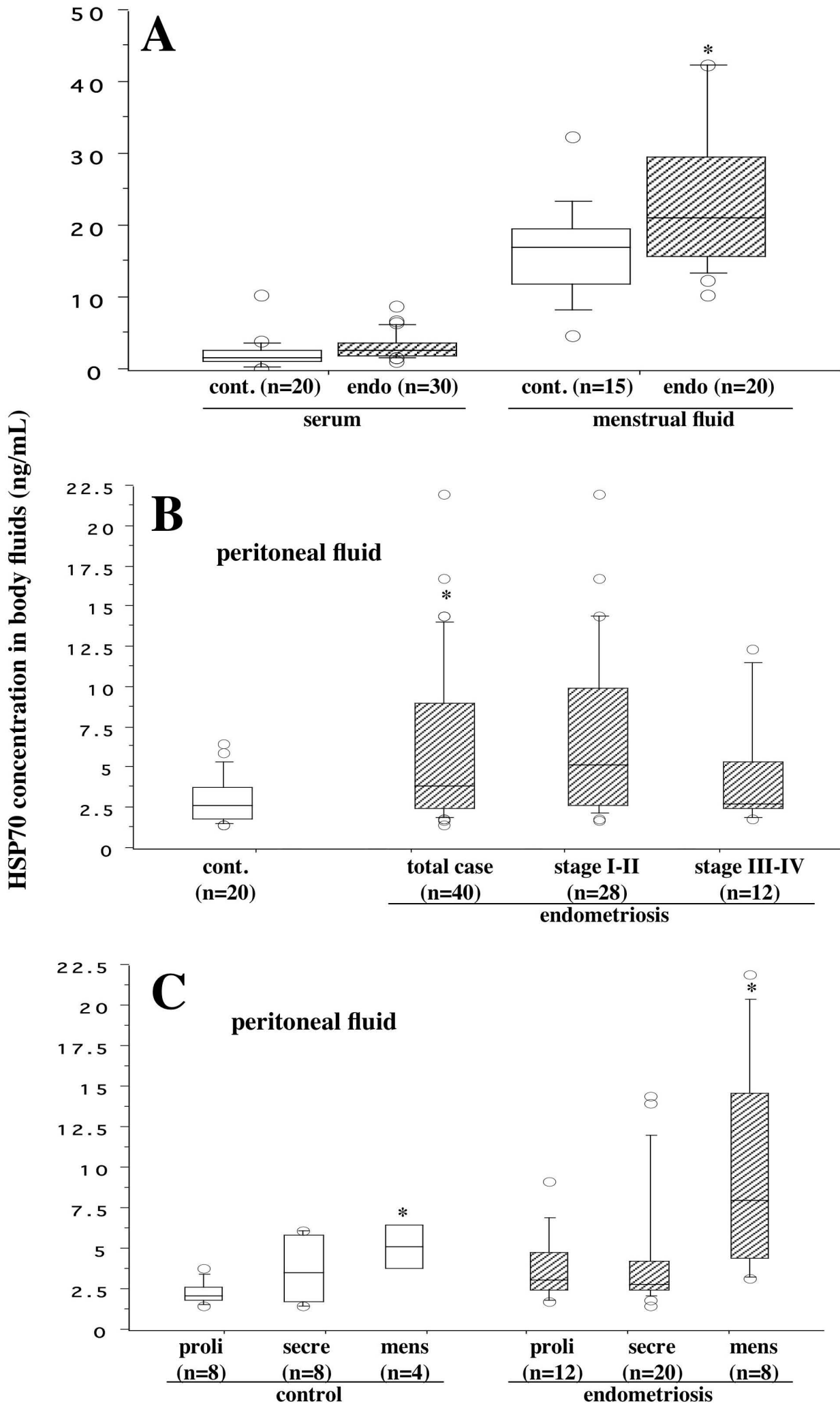


Figure 2

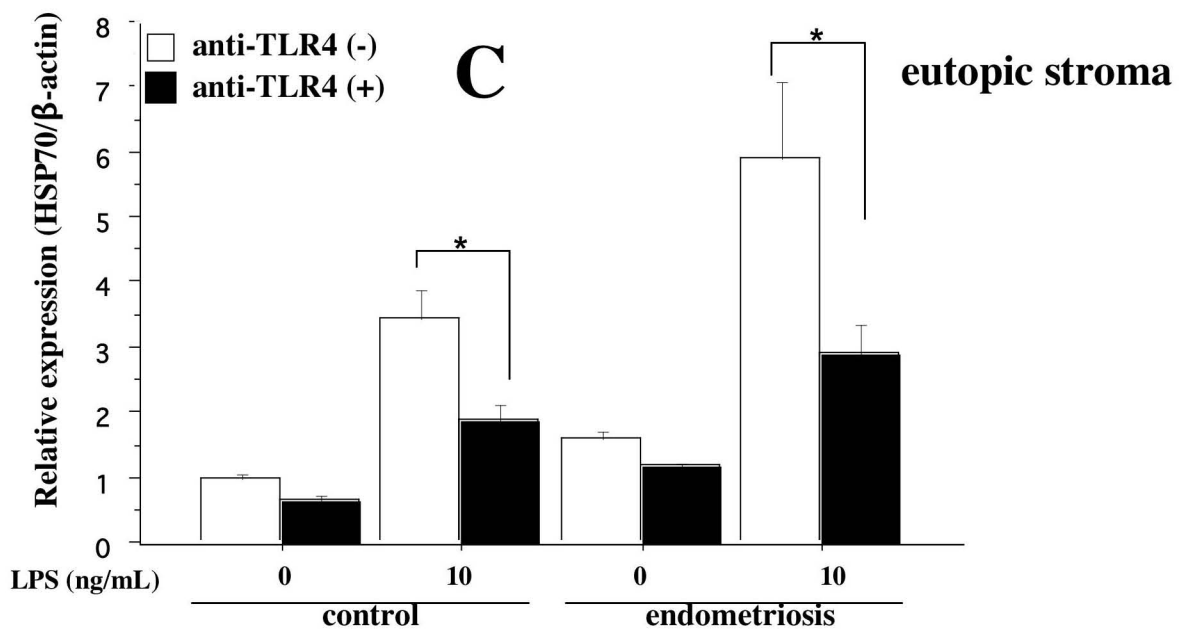
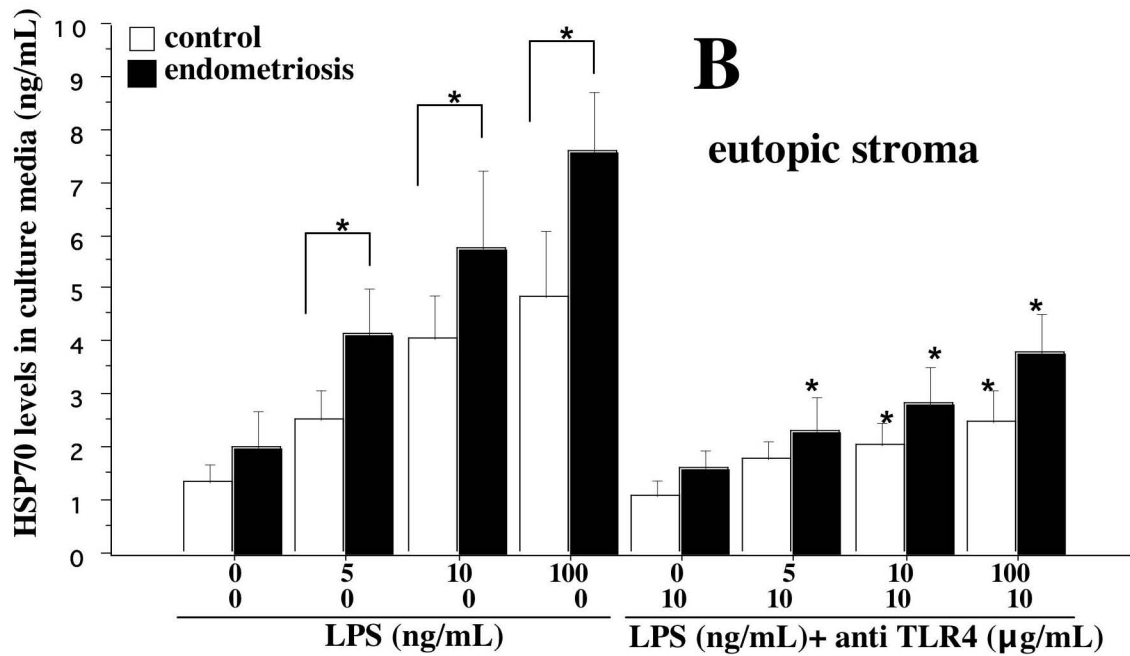
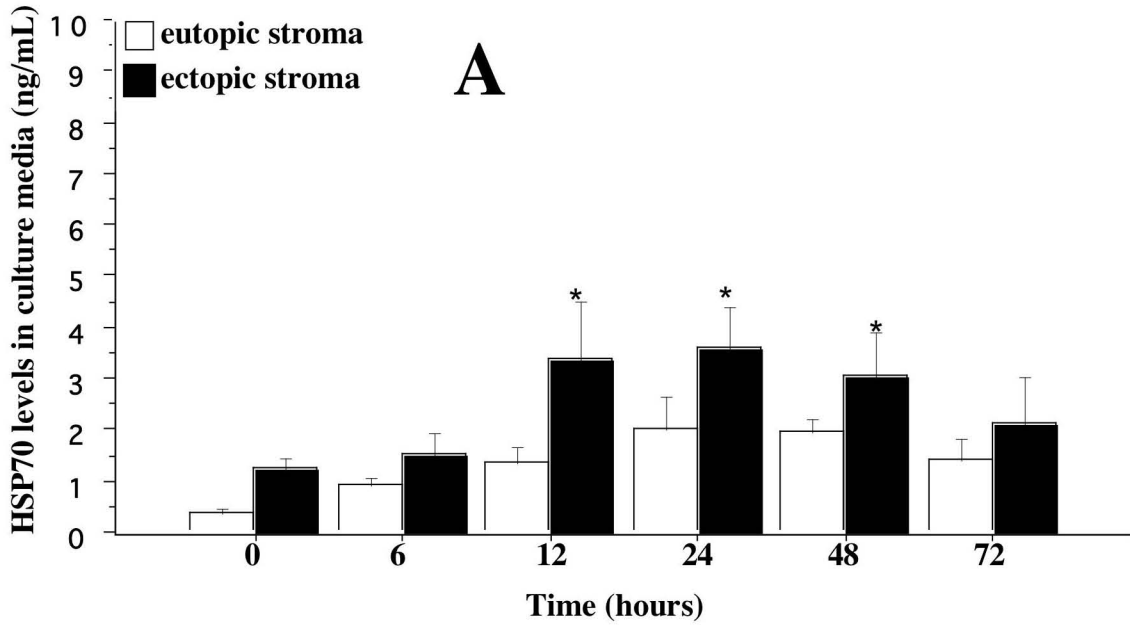


Figure 3

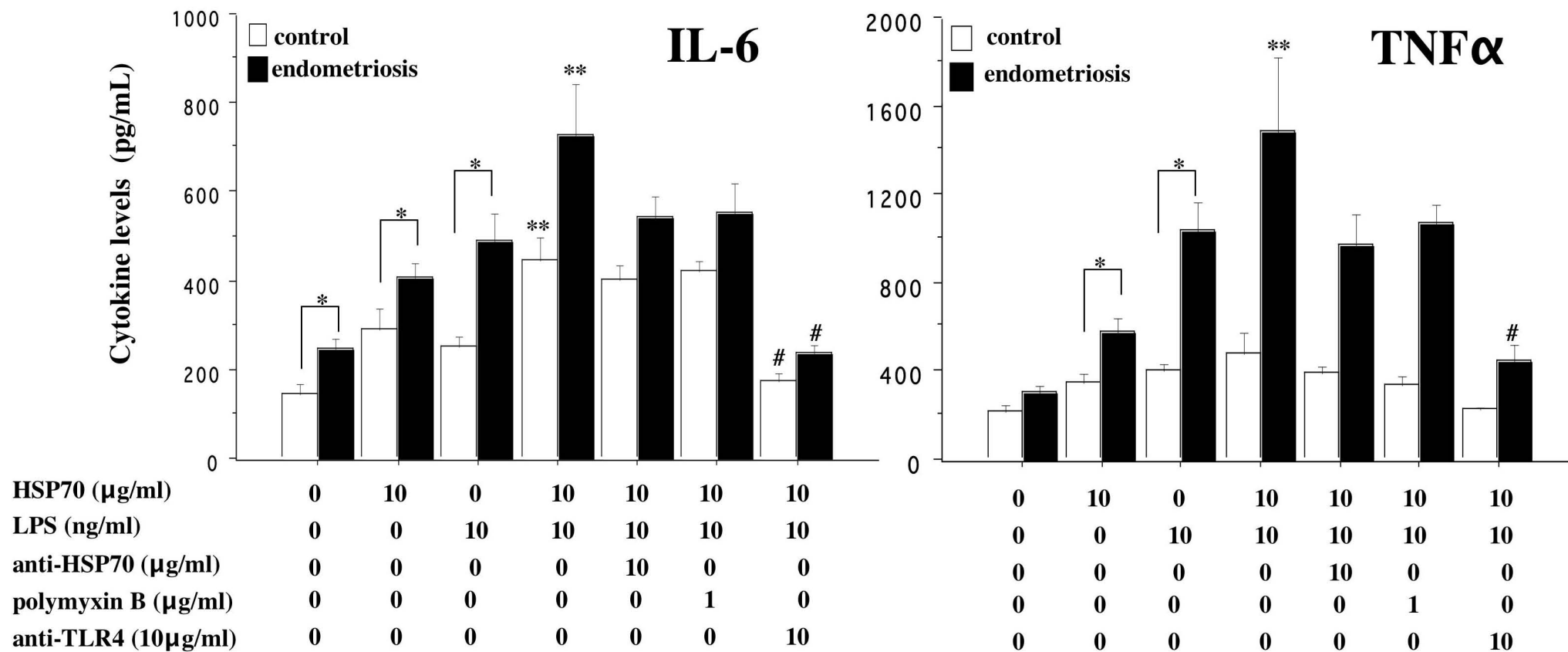


Figure 4

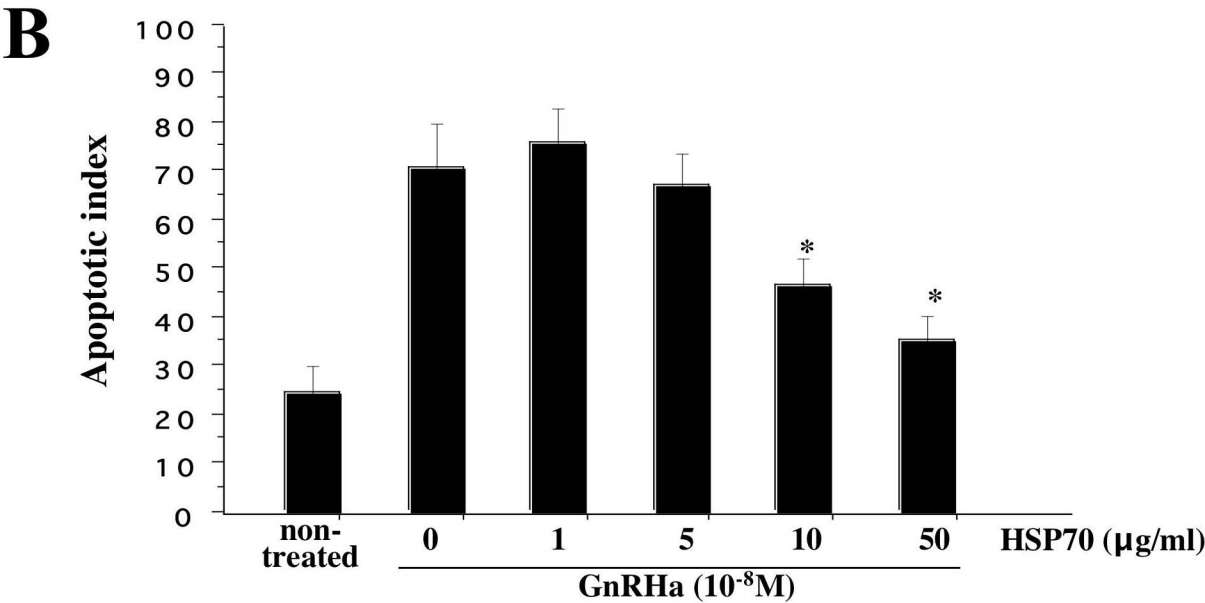
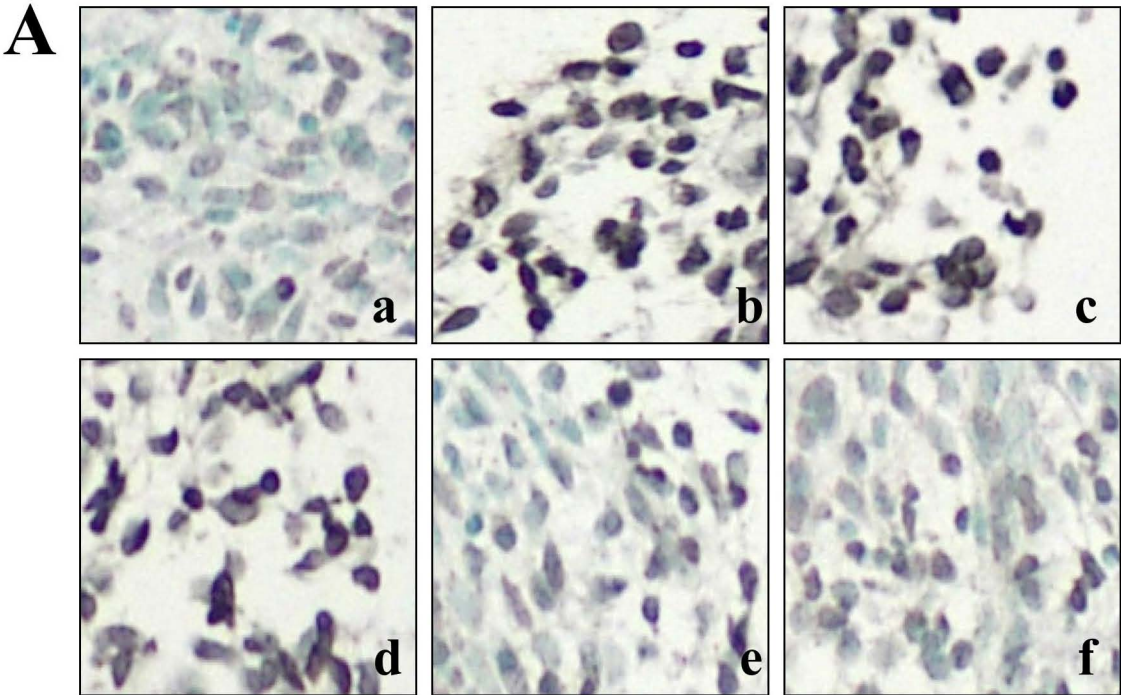


Figure 5

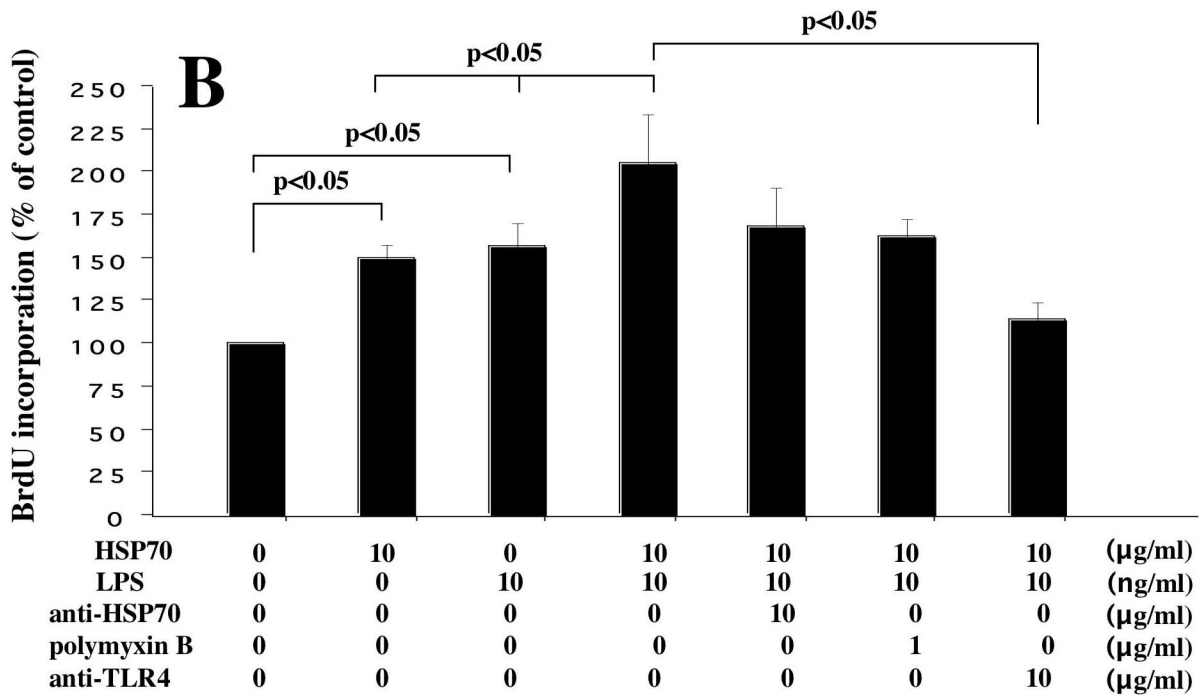
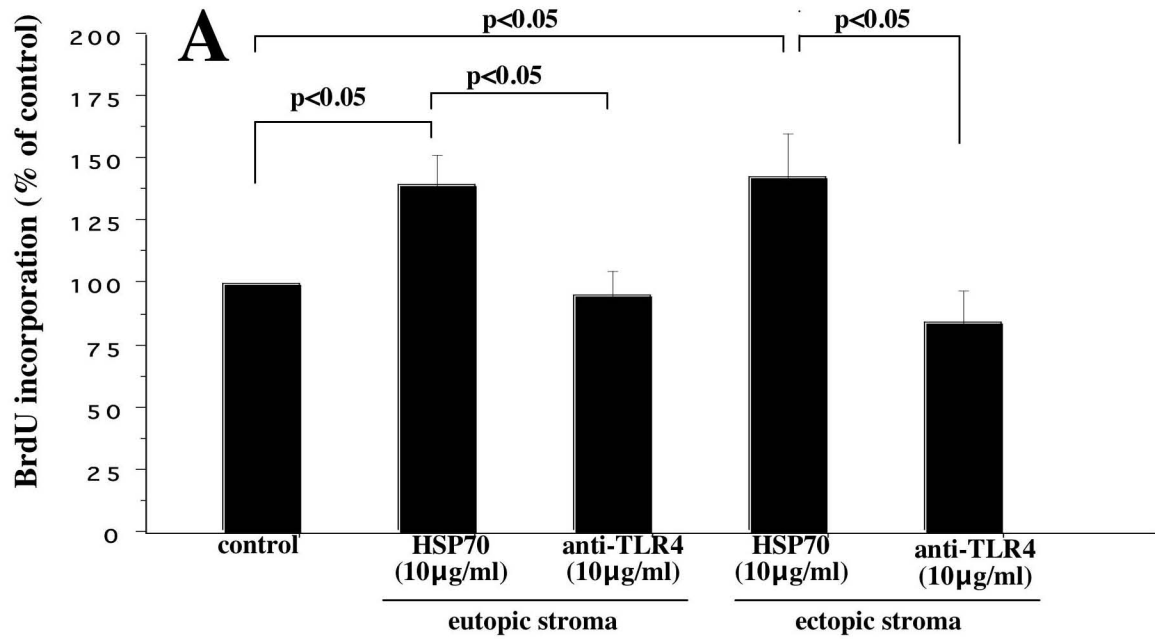


Figure 6

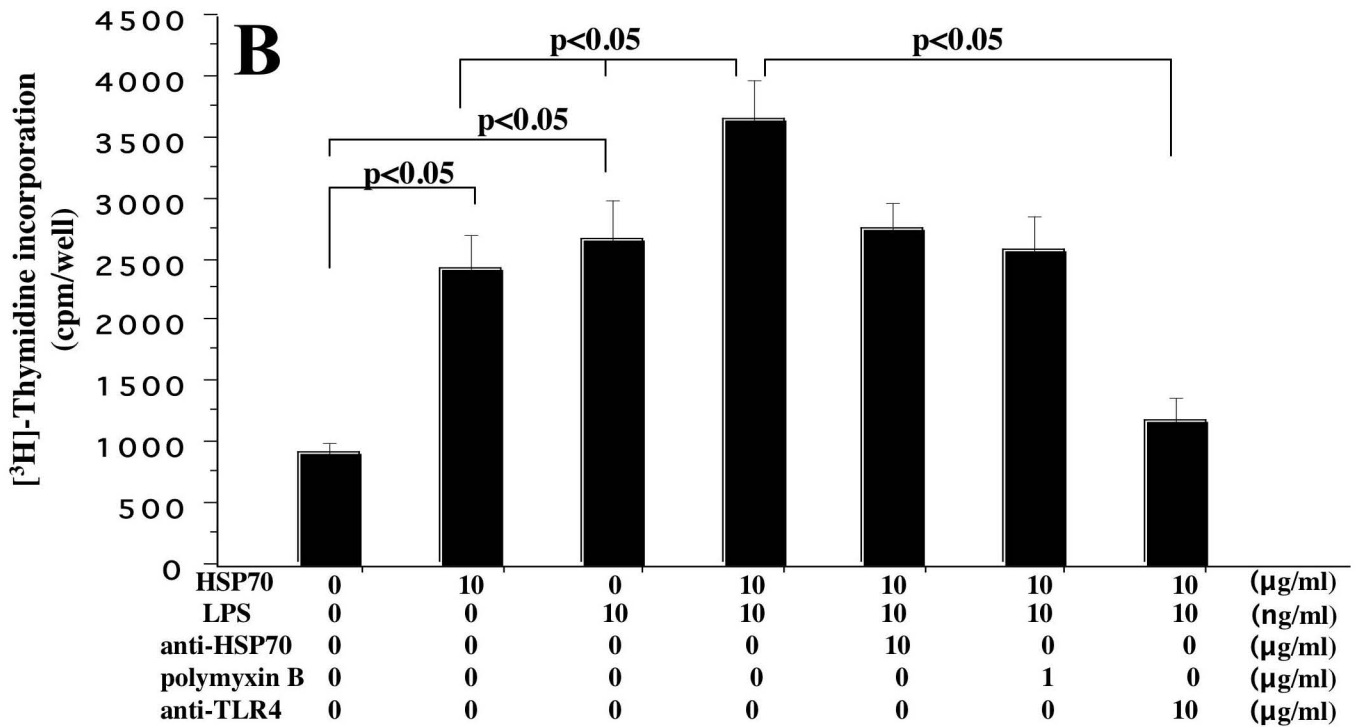
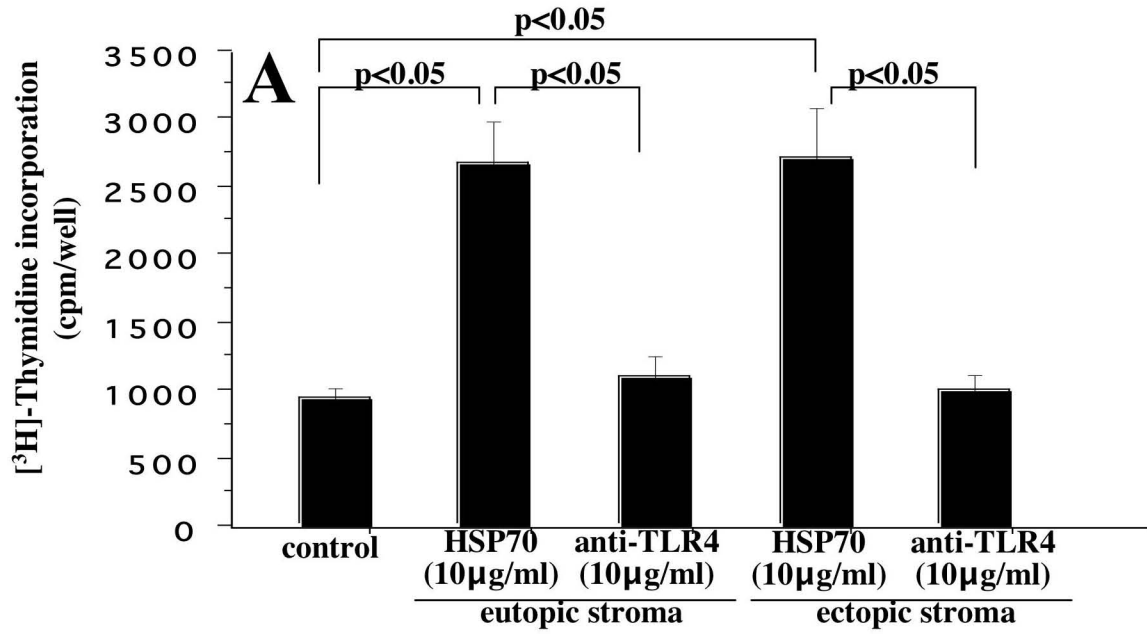


Table 1. Clinical profiles of patients with and without endometriosis..

	control	endometriosis
No. of patients	20	43
Age in years: (mean $\pm$ SD)	28.6 $\pm$ 3.8	29.8 $\pm$ 4.6
Range of age: (years)	18-32	20-42
r-ASRM staging: I-II/III-IV		28/15
Menstrual cycle: P/S/M	8/8/4	12/23/8

The results are expressed as mean  $\pm$  SD. r-ASRM, revised classification of the American Society of Reproductive Medicine. P, proliferative phase, S, secretory phase, M, menstrual phase.