رقم البحث (16)

PRO AND ANTI-INFLAMMATORY GENES EXPRESSION OF BOVINE OVIDUCT EPITHELIUM CELLS IN RESPONSE TO CORTISOL .

BY

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ABSTRACT

Bovine oviduct epithelium play pivotal role in the immunoregulation for early pregnancy recognition and maintenance. Cytokines are the primary factors that regulate the immune effector function. This study was designed to check the immune response of the bovine oviduct epithelial cells (BOECs) to different levels of cortisol. BOECs were isolated from bovine oviducts collected from slaughterhouse. This were cultured in DMEM media and kept in humidified incubator at 38C^{o} and 5% CO2. After confluent, the cells were subjected to 0, 5, 50 and 500 nmol/ml of hydrocortisol. After 24 hr incubation, the cells were harvested and subjected to mRNA extraction and RT-PCR for quantification of some pro and anti- inflammatory cytokines gene expression. The results revealed significant ($p < 0.05$) increases in the expression of mRNA for TLR2, IL-4 and IL-10 in cortisol exposed cells at the dose of 50 and 500 nmol/ml. On the same manner, 5 nmol/ml cortisol significantly increased TLR4 expression. On contrary, TNF-α and IL-1β genes were down regulated significantly in BOECs exposed to 50 or 500 nmol/ml cortisol. These data open the way to determine the potential role of glucocorticoids in the immunomodulation of bovine oviduct during the reproductive events.

Key words: BOEC, cortisol, cytokines, immunity.

INTRODUCTION

Oviduct is a small tube responsible for the transient hosting of gametes and embryos. It orchestrates a series of complex actions, all of which need to be precisely initiated and completed for achievement a proper reproductive events in early pregnancy (Yaniz et al., 2000; Kenngott and Sinowatz 2007). The presence of gametes and embryos initiate and modulate local mechanisms at the molecular level, which represent the first exchange of signals with the maternal environment (Georgiou et al., 2007; Koelle et al., 2010;Holt and Fazeli, 2010). The oviduct exhibits an extraordinary flexibility that is hormonally driven and exactly timed according to the embryonic stage and site where it directly contacts the epithelium (Spilman et al., 1978 and Abe and Hoshi, 2008; Nakahari et al., 2011). Besides peritoneal as well as follicular fluid (FF), which enters the oviduct mainly during the peri-ovulation period, the main components of oviduct fluid passively find their way into the oviduct via the blood and lymphatic vessels by transudation and actively by de novo synthesis (Henault and Killian, 1993; Buhi 2002; Kenny et al., 2002; Hugentobler et al., 2007, 2008; and Goncalves et al.,2008). The volume and components of tubal fluid are subjected to cyclic changes (Bauersachs et al. 2003), which finally determine the functional (Aguilar and Reyley, 2005; Hugentobler et al., 2010 and Avile´ s et al., 2010) and physical (Nichol et al., 1997 and Hunter et al., 2011) characteristics of the fluid.

Glucocorticoids (GC) are involved in the regulation of various physiological processes including female reproduction (Alfaidy et al., 2003; Tetsuka et al.,2003 and Lee etal., 2007). Although cortisol influence on the function of myometrium, placenta and fetal membranes during the peri-parturient period is well established (Brann &Mahesh, 1991and Michael et al., 2003), its role during the estrous cycle and early pregnancy is still controversial. Recent studies have shown that glucocorticoid receptors and 11β-HSD genes and proteins are expressed in the bovine (Lee et al., 2007 and Lee et al., 2009) and ovine endometrium (Yang et al., 1996 and Simmons et al., 2010) during the estrous cycle and early pregnancy. Several studies showed that stressors activate the hypothalamic-pituitary-adrenal axis and enhance the secretion of glucocorticoids that can mediate suppression of reproductive processes (Lee et al., 2007) . Cortisol is not produced *de novo* by the ovaries (Omura & Morohashi, 1995) but transport takes place from the adrenal glands through the circulation in response to the mid-cycle surge of gonadotrophins. The preovulatory follicle reduces the expression of 11-HSD type 2, while 11-HSD type 1 is up-regulated favoring enhanced cortisol production at the

expense of inactive cortisone (Tetsuka et al., 1997). The fluid of preovulatory follicles collected immediately before ovulation exhibits levels of free cortisol exceeding the serum levels as seen in hyper activity of adrenal gland (Yding Andersen & Hornnes 1994, Harlow *et al.* 1997). A precise physiological function of these high levels of biologically active cortisol is not yet clear. While, Espey and Lipner (1994) reported its possible ovulatory role , Yding et al.,(1992) mentioned that the follicular fluid(FF) cortisol could be expelled and a part is taken by the oviduct , and the remainder is found in the peritoneal cavity in the vicinity of the oviduct. The oviduct may thus possess some mechanisms that inhibit invasion and/or survival of fertilizable spermatozoa . With this background, it is proposed that the local high concentrations of free cortisol in the oviduct derived from follicular fluid released at ovulation participate in the protection of the oviduct from invasion by leukocytes, allowing fertilization and early embryo development to occur in a proper manner before embryo releases IFN-t (Yanagimachi , 1994) .

Toll-like receptors are part of the IL-1 family. Their function is to distinguish antigens and to initiate an appropriate immune response (Jefferies and O'Neill, 2002; Medzhitov, 2001; Modlin, 2002; Takeuchi and Akira, 2002). To date, 10 TLRs have been identified (Zarember and Godowski, 2002). Although there is some overlap, each has a unique function in antigen detection where TLR2 is known to share in immune tolerance response. In contrast, TLR4 is responsible for gram negative lipopolysaccharide (LPS) recognition and pro-inflammatory cell-signaling. Each leads to the expression of distinct inflammatory genes (Supajatura et al., 2002). Toll-like receptor 4 leads to increased interferon (IFN)-b, tumor necrosis factor (TNF)-a, interleukin (IL)-1b, IL-6, and IL-13. Toll-like receptor 2 leads to increased IL-4, IL-6, IL-8, IL-13, IL-5, and IL-10, but not IL-1b. Other study, demonstrated that, acute phase cytokines in bovine innate neutrophil immunity include TNF-α, IL-1, and platelet activating factor (Carl et al.,2002). Interleukin-1 is necessary for release of IL-8 (essential for neutrophil recruitment) by bovine epithelial cells in airways and intestinal tissues (Boudjellab et al.,2000). Moreover they demonstrated that, the epithelial cells play an important role in immune regulation, by this cytokine induction.

The objective of this study was to elucidate changes in gene expression profile of BOECs in response to different doses of cortisol. Such goal is curcial to confirm our hypothesis concerning the possible role of cortisol in the immune tolerance function of oviduct during establishment of early pregnancy.

MATERIALS AND METHOD

Recovery of oviduct epithelial cells for tissue culture.

Bovine oviducts were obtained from local slaughterhouse and were transported to laboratory in cooling vessels containing PBS 1% with addition of 1% (v/v) penicillin – streptomycine. The oviducts were trimmed from the surrounding connective tissue, closed from their posterior part near the isthmus by artery forceps and the epithelial cells were separated from the lamina propria by light pressure of glass slide on their external surface. 5ml worm PBS were infused into the oviduct by straile needle, the PBS containing suspended cells were collected in falcon tube after release of the forceps. The collected cells were cleared by centrifugation at 1000xg / 15 min, and this clearance process was repeated for 2-3 times. The cells were then cultured on DMEM media (11960- 044, Invetrogen). Seeding the cells on the collagen pre-coated 6 well plate (nunc, Denmark) using 3 ml of DMEM media containing 1 % amphitracin, 0.1% gentamycin and 5 % fetal calf serum (26140-087,GIBCO-BRL, Invetrogen life technology) per each well. They were incubated for 5 days in humidified incubator at 38C^{o} at 5 % CO_2 with medium changing each 2 days. After confluent, the cells were replated into 12 well plate and subjected to cortisol. The previous protocole was conducted according to Way (2006).

Treatment of cell

Each well were exposed to 0, 5, 50 or 500 nmol / ml of hydrocortisol (sigma – Aldrich) in four replicate for each dose followed by incubation for 24 hr.

RNA isolation

Total RNA was extracted following the protocol of Chomczynski and Sacchi (1987) using TRIzol reagent (Invitrogen). The RNA concentration and purity was determined using a NanoDrop® (ND-1000 Spectrophotometer, NanoDrop Technologies Inc, Delaware, USA) at 260 and 280 nm absorbance. The extracted total RNA was stored in RNA storage solution (Ambion, Texas, USA) at -80°C until use for cDNA production.

Formation of cDNA

RNA samples were treated with DNase using the RQ1 RNase-Free DNase kit (Promega. Co., Madison, WI, USA). Two microgram of extracted RNA was incubated for 30 min. at 37 °C with 1 ul RQ1 RNase free DNase 10x reaction buffer and 2 ul of 1ug/ul Rnase – free Dnase respectively. Then 1 ul RQ1Dnase stop solution (20mM EDTA) was added, and

the mixture was incubated for 10 min. at 65°C to terminate the reaction . First strand cDNA synthesis was conducted according to commercial protocol described in superscript™ II reverse transcriptase kit (Invetrogen, Calsbad, Ca, USA). The first mixture was prepared using 2ul total RNA extract from culture cells,1.5 ul of 50 ng/ul random primer (Invitrogen), 1.5 ul of 10 mM PCR Nucleotoid mix(dNTP, Roche diagnostics, Indianapolis, IN, USA) and 12 ul H2O , yielding final volume 18 ul per tube this mix was incubated at 65 °C for 5 min. in thermal cycler (Bio-Rad, Munich, Germany). The samples were kept on ice while the second mixture; 3 ul of 0.1 M DTT (Invetrogen) , 1.5 ul of 40 units/ ul RNasin ™ riboneclease inhibitor (promega, Madison, WI,USA) and 6 ml of 5x first strand buffer(Invitrogen) was added to each tube. The samples were incubated for 2 min at 42 °C, and 0.2 ul of 200 units/ul SuperScript™ II revearse transcriptase was added to each tube. The thermal cycler was programmed at 25°C for 10 min , 42°C for 50 min and then 70°C for 15 min. The synthesized cDNA was stored at -30°C.

Real –time PCR

Real time pcr was performed using a quanti-Tect SyberGreen PCR kit (Qiagen) and a chrom 4 real –time PCR system (Bio-Rad laboratories , Hercules , CA, USA) with the following primers for TLR4 NM AAG3206 Forward: CCGGATCCTAGACTGCAGCTT and Reverse: TCCTTGGCAAATTCTGTAGTTCTTG (annealing temperature , 56°C) (;for TLR2: forward CCACGGAAGGAGCCTCTGA and reverse, GCCATCGCAGACACCAGTT (annealing temperature,54°C) (AF368419);for IL-1B: Forward TTCCTGTGGCCTTGGGTATC and reverse TGGGCGTATCACCTTTTTTCA (annealing temperature , 56°C) (Ito and Kodama (1996) ,for TNF-α Forward: TGGGAAGCTTACCTTTTCCTTTC and Reverse: TTCTTCATGACCCAGATACATCCT (annealing temperature , 56°C) (Bienhoff and Allen (1995) ;for IL- 10: forward CTGCTAACCGACTCCTTAATGC and reverse ATTTCTGGGCCATGCTTCTC (annealing temperature, 58°C); for IL- 4: forward GTCTCACATTGTCAGTGCAAA and reverse TTCAGCTTCAACACTTGGAGT (annealing temperature, 56°C) , and for B – actin NM_001101: Forward: GTGGCATCCACGAAACTACC and Reverse: GTACTTGCGCTCAGGAGGAG (annealing temperature , 58°C).The thermal cycling conditions include activation of hot star Taq DNA polymerase at 95 °C for 15 min. , followed by 55 cycles of denaturation at 94°C for 15 sec; annealing of primers at different temperature 15 sec. and elongation at 72 °C for 30 sec. To quantify the mRNA abundance , standard curves for each gene were generated by serial dilution of a known quantities of purified RT-

PCR products. The relative difference in the initial amount of each mRNA was comparing the Ct values. The expression of each gene relative to that of B actin mRNA was calculated for adjustment of the amount of RNA used in the RT- PCR.

PCRs were quantified using a Light Cycler (Roche Diagnostics, Indianapolis, IN, USA)

Statistical analysis

All data are presented as means ± SEM. The statistical analysis of data were performed using SPSS software version 17 (SPSS Inc., Chicago, IL, USA) The statistical significance of differences about genes expression of mRNA was assessed by one-way ANOVA followed by Bonferroni's multiple comparison test. Probabilities less than 5% ($P < 0.05$) were considered significant.

RESULTS

Pro inflammatory cytokines

mRNA expression of TLR-4, TNF-α and IL-1β were showed in figure (1). TLR-4 mRNA was significantly upregulated at 5nmol / ml hydrocortisol. No effects were recorded at higher concentrations. Treatment of BOEC with 50 o 500 nmol / ml hydrocortisol resulting in down regulation of m RNA for TNF-α and IL-1β.

Anti- inflammatory cytokines

mRNA gene expression for TLR-2 , IL-4 and IL-10 was depicted in figure (2). Treatment of BOECs with cortisol significantly increase the expression of TLR-2. IL-4 and IL-10 gene expression showed the same manner in the response as the result of exposure of BOECs to high doses of cortisol (50 and 500 nmol / ml).

DISCUSSION

The oviduct plays a pivotal role in mammalian reproduction, providing an optimal environment for oocyte maturation, sperm capacitation, fertilization, and transport of gametes and embryos (Ellington 1991, Hunter 2003).

The oviduct epithelium consists of ciliated and secretory cells. Kinocilia are actively involved in the transport of oocytes, sperm, and early embryos, while secretory products are essential for providing optimized microenvironments (Abe & Hoshi 1997, Leese et al.2001).

The results of this study showed significant up regulation of TLR-4 gene on expose of BOEC to 5 nmol/ml cortisol. At higher doses no significant changes than control one was recorded. This data could be referred to the suppressive effects of high levels of glucocorticoids on innate immunity (Jin et al., 2009). The previous report of Jin et al., (2007) indicated that hydrocortisone had a benifacial effects on the innate immunity of corneal epithelial cells through increase the mRNA expression of TLR-2 and TLR-4. This reports could support our data in the BOEC.

In spite of glucocorticoids are not specific ligand of TLR (Jin et a., 2009) but cortisol act as unspecific ligand of it. These hypotheses could explained significant down regulation of pro inflammatory genes (TNF-a nd IL-1B) expression in this results.

It would be documented that, occupation of TLR-4 by its ligand could be resulted in release of many proinflammatory cytokines, IFN-γ, TNF-α, IL-1 and IL-8, that mediate inflammatory reaction through activation of T helper type 1(Nicolai and Leonard., 1997).

The data in these study indicated that physiological dose of cortisol 5 nmol/ ml improve the innate immune response of BOECs through up regulation of TLR-4 and TLR-2 genes expression indicating the pathogen recognition and surveillance. Although, the higher doses could suppress immune status as 50 or 500 nmol/ ml could increase the anti- inflammatory cytokines (Il-10 and IL-4) and decreased the pro inflammatory cytokines (TNF-α and IL-1B). These findings could explain the previous notation of Mark et al.,(2011) who indicated the dose biphasic effects of cortisol on inflammatory response. They added that, glucocorticoid support the activity of defense mechanisms in a permissive manner while higher stress act acutely to suppress inflammation to prevent tissue injury.

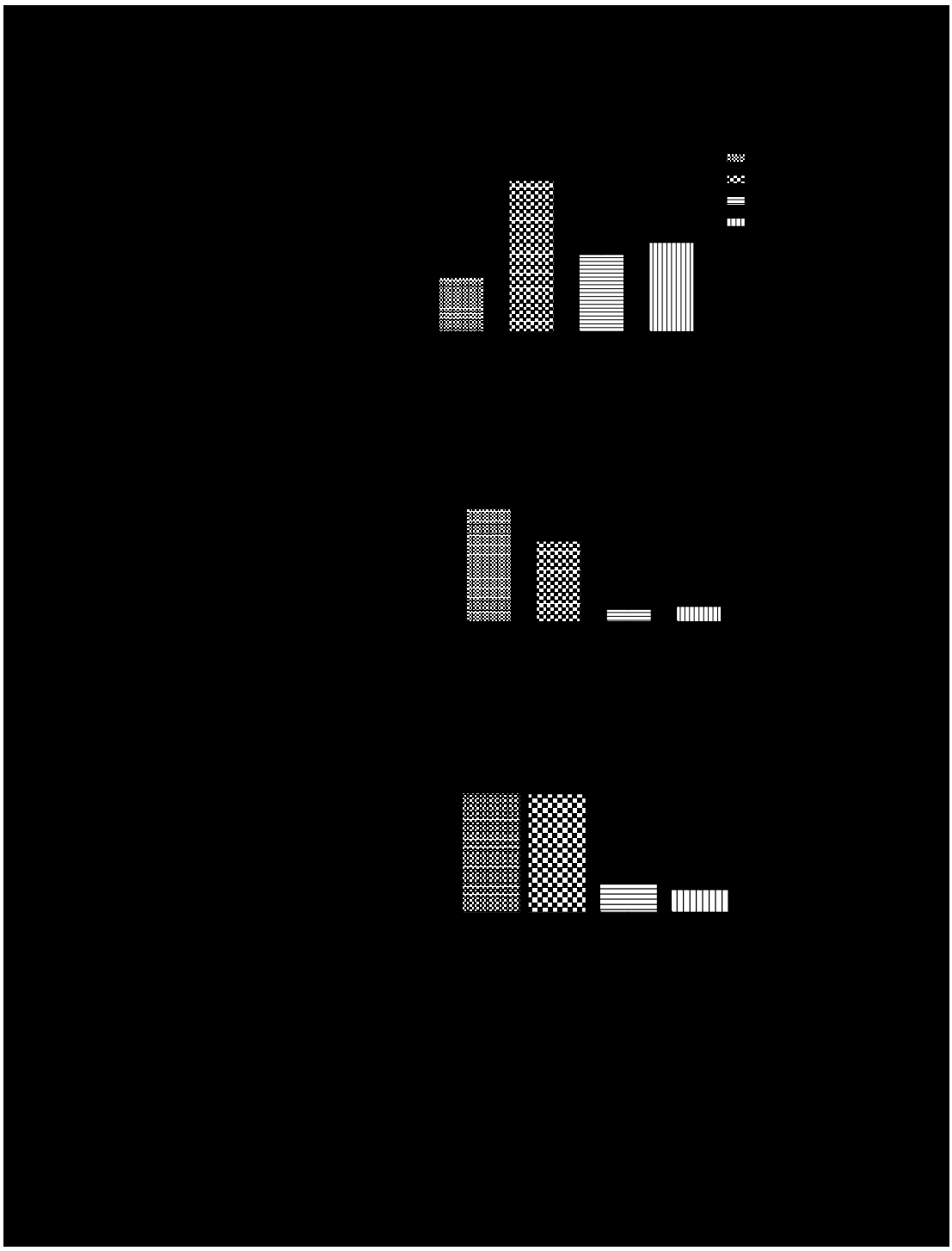
From our data and the other reports of Mark et al., (2011) and Dynes & Araneo., (1989) we can state that, high levels of cortisol support type- 2 helper cell cytokines than type-1 helper cell. These down expression of genes as appeared in figure (2) could be refereed to binding to and activating negative regulatory elements in the promoters of cytokine genes (Ray et al ., 1990 and Mukaida et al., 1992), by inducing the production of I K B ~α ,protein that binds and neutralizes the cytokine transcription factor nuclear factor-kapa which essential in the function transcription of TLR-4 (Scheinman et al ., 1995).

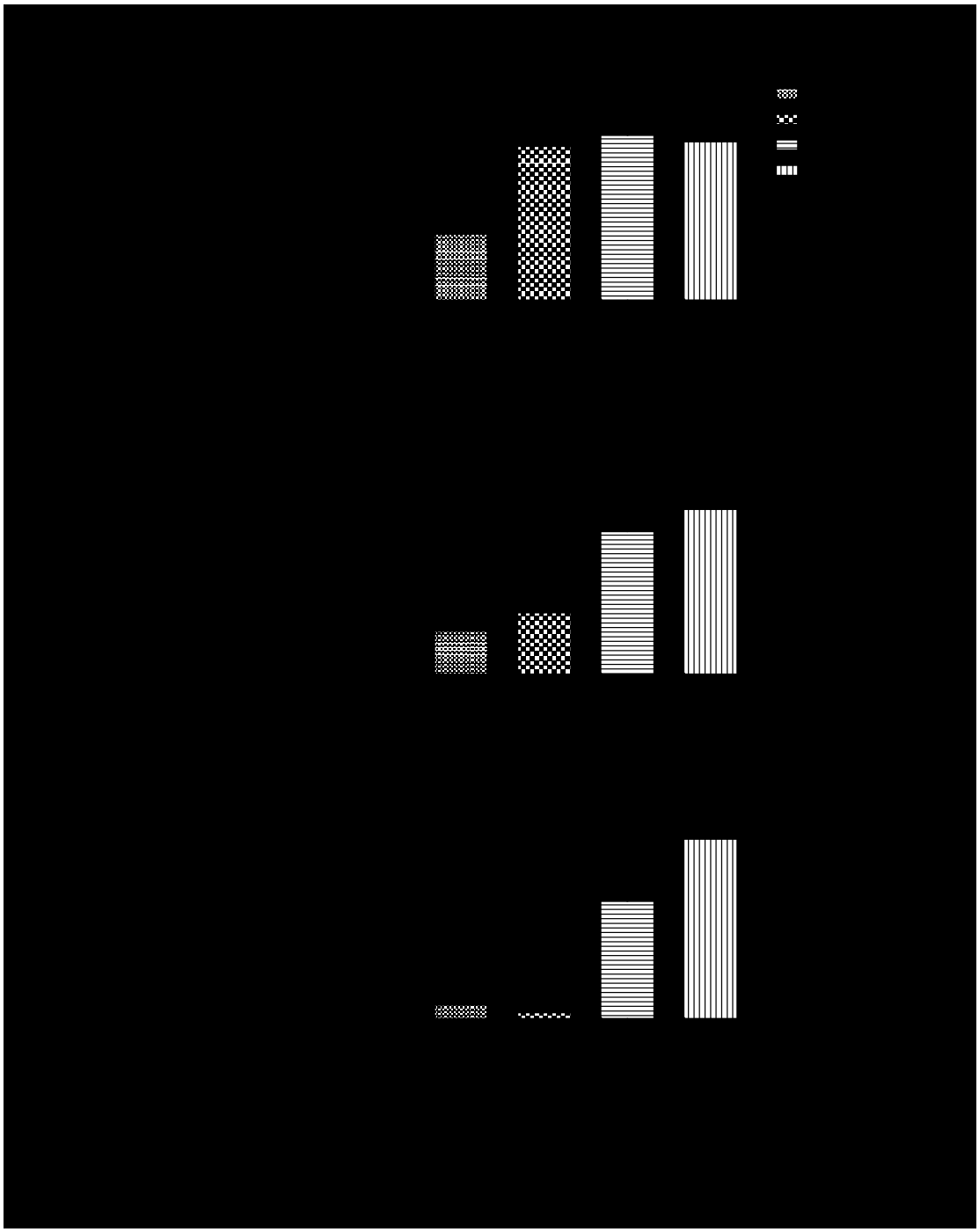
It has been estimated by Andresen., (2002) that, the concentration of free cortisol in pre-ovulatory FF reaches levels ten times higher than the corresponding values in serum. The precise physiological function of these high levels of biologically active cortisol is not yet

clear. However, it may have anti-inflammatory role at area of ovulation (Espey and Lipner 1994). Other possible role as proposed by Andresen., (2002) is the local high concentrations of free cortisol in the oviduct derived from FF released at ovulation might participate in the protection of the oviduct from invasion by leukocytes, allowing fertilization and early embryo development to occur in a proper manner and enhance cross talk between early embryo and dam before INF-t release.

In our data high cortisol levels , 50 and 500 nmol / ml, could inhibit expression of Th-1 cytokines (TLR-4, TNF-a and IL-1B) and stimulate expression of Th-2 ones (TLR-2, IL-10 and IL-4). So, the oviduct immune response may be possess some mechanisms to protect the allogenic sperm and semi allogin embryo from attacked by immune cells around time of ovulation. Thus, the local action of high levels effects of cortisol on BOECs may prove to be an important physiological mechanism by which cortisol affect female reproductive organ and provide a tool for a more detailed understanding of interactions between glucocorticoids and female reproductive organs.

If these hypotheses prove correct, future research is needed to study the interaction between cortisol and pro inflammatory ligands or cortisol and other steroids hormones in order to understand complex action of FF. In addition, it may describe a new therapeutic principle for the physiological actions of cortisol in reproductive events and early pregnancy.





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الملخص العربى

الجينات المحفزة والمثبطة للالتهاب فى النسيج الطلائى لقناة المبيض فى الابقار استجابة للكورتيزون

يوسف الصعيدى

قسم الفسيولوجيا - كلية الطب البيطرى – جامعة المنصورة – المنصورة – مصر

يلعب النسيج الطلائى لقتاة المبيض فى الابقار دورا هاما فى تنظيم المناعة والتعرف على الزيجوت واستمراريته فى بداية فترة الحمل كما تعتبر السيتوكين من العوامل المهمة والمؤثرة فى تنظيم الوظيفة المناعية .

اجريت هذه الدراسة لملاحظة مدى الاستجابة المناعية للخلايا الطلائية لقناة المبيض فى الابقار لمستويات مختلفه من الكورتيزول . تم تجميع الخلايا الطلائية لقناة المبيض من الحيوانات المذبوحة من المجزر وتم زراعتها مستخدما وسط DMEM وحضانة ثانى اكسيد الكربون وبعد النمو الكامل للخلايا تم اضافة مستويات ٠، ٥، ٥٠، ٥٠٠ نانومول /مل من الكورتيزول وبعد التحضين لمدة ٢٤ ساعة تم اخذ الخلايا لفصل الحامض النووى الريبوزومى واجلراء اختبار البلمرة المتسلسل .

اظهرت النتائج زيادة معنوية فى mRNA لكلا من الجينات المثبطة للمناعة (TLR2, IL-4 and IL-10) وذالك فى الخلايا المعرضة لمستويات ٥٠ ، ٥٠٠ نانومول / مل كورتيزول وكذالك بالمثل زيادة معنوية فى TLR4 عند مستوى ٥ نانومول / مل ، من ناحية اخرى فان مستوى ٥٠ ، ٥٠٠ نانومول / مل كورتيزول ادى الى نقص معنوى فى ظهور جينات IL1- β , TNF-α .

هذة النتائج قد تلفت النظر الى دور الجلوكوكورتكويد فى التحور المناعى فى قناة المبيض للابقار اثناء احداث التناسل .