Microscopic Changes of Ovaries in Relation to Inflammatory Mediators of Blood Plasma in Superovulated Rats

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ABSTRACT - ***This study was aimed to evaluate the microscopic changes that occur within the ovary and to assess the level of inflammatory mediators in blood plasma in rats that are superovulated. Eighteen female 12 weeks old Sprague Dawley rats were used in this study whereby histological sections of ovaries were examined to study the morphology of the ovary and blood analysis was carried out to analyse the inflammatory mediators in the blood plasma. The number of large follicles and healthy follicles were significantly increased (P<0.05) in superovulated rats but the diameter of the follicles were indifferent (P>0.05) when compared to control rats. The levels of Interleukin 8 (IL-8) was up regulated (P<0.05) at 8 hours after human Chorionic Gonadotropin (hCG) treatment but the Prostaglandin E_2 (PGE_2) and Nerve Growth Factor (NGF) showed insignificant differences (P>0.05) from control rats. It can be concluded from this study that IL-8 indicates increased level of inflammation in superovulated rats. The finding of this study in the increased level of IL-8 in superovulated rats is useful in further studies addressing problems in the superovulation treatment.***

Keywords: Superovulation, Interleukin 8, Nerve Growth Factor, Prostaglandin E_2, Inflammation

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1. INTRODUCTION

Superovulation is a treatment used to induce multiple follicular developments in animals to produce two fold or more than the normal number of oocytes produced during normal ovulation [1]. Superovulation is very useful in research which requires large number of oocytes especially for the generation of transgenic and cloned animals [2] as well as in the animal breeding industry [3]. However, serious problems were encountered when superovulation was carried out which includes the inability of the oocyte to be fertilized or to undergo a normal development into embryo. Furthermore, the use of exogenous gonadotropin reduced fertility in rats when those rats were mated naturally [4] and the high doses of gonadotropin exhibits undesired effects on hormonal profiles, ovulation, fertilisation, embryo recovery and viability rates [5]. These problems could arise probably from the inflammatory event that happens during ovulation. In order to address these problems, the underlying physiology that occurs during superovulation treatment should be well understood. The inflammatory process that takes place in superovulated rats are worth researching as ovulation has been widely reported as an acute inflammatory process [6] but the inflammatory response in superovulated rats are relatively unclear.

Many physiological and biochemical changes takes place throughout the oestrous cycle to regulate the process that occurs in the ovary in preparation for ovulation to take place. Follicle hyperaemia, large production of Prostaglandins

(PG) and hyaluronan-rich matrix synthesis are the common characteristics indicating inflammation and wound repair which could be observed during the process of ovulation [6]. Other inflammatory-like changes that are linked with ovulation includes the presence of inflammatory mediators such as interleukin 1β (IL-1β), Interleukin 6 [7], Interleukin 10, Interleukin 8 (IL-8), Tumour Necrosis Factor [8], histamine, serotonin, bradykinin and prostaglandins [9]. Four inflammatory mediators had been highlighted in this study which has a prominent role in ovulation whereby Interleukin1β, an early gene response to inflammation and triggers the production of Prostaglandin E_2 (PGE_2) [9]. Prostaglandin E_2, a key component in ovulation results in the inability of the ovary to ovulate when this mediator was inhibited. In addition to these, Interleukin-8 which functions by recruiting neutrophils that is required during follicular development and luteolysis [10] and Nerve Growth Factor (NGF) which has a profound effect in tissue remodelling and wound healing [11] were also included in this study.

This study was carried out to fully understand the underlying physiology of the ovulation process when the superovulation treatment was carried out. The objectives are to a) evaluate the microscopic changes in the ovary due to the superovulatory treatment and to compare it with the ovary of normal rats b) assess the inflammatory mediators in the blood plasma of normal and superovulated rats.

2. MATERIALS AND METHODS

2.1 Animals

Eighteen female 12 weeks old Sprague Dawley rats with a 5-day oestrus cycle were used. The rats were kept in individual Lab style rodent breeder cages (30.2cm X 18.4cm X 15.2cm) with stainless steel wire top and heavy duty polypropylene cage base. The rats were subjected to natural day and night cycle and had free access to commercial feed and water ad libitum. The vaginal smear was conducted daily at 0800 hour to determine the stage of the oestrous cycle. Rats that showed two successive 5-day oestrous cycles were used for the experiment. This experiment was carried out after approval was obtained from the Animal Care and Use Committee of Universiti Putra Malaysia (Reference number: UPM/FPV/PS/3.2.1.551/AUP-R105).

2.2 Hormone treatment and histology

Eighteen rats were randomly divided into three treatment group where 12 rats were given 0.15mL (30i.u) of intramuscular injection of serum gonadotrophin (FOLLIGON® Intervet; Australia), during their dioestrus phase and 52 hours later 0.25mL (25i.u) of Chorulon (CHORULON® Intervet; Australia, with active ingredient of human chorionic gonadotrophin) was administered intramuscularly [12]. Blood samples were carried out by cardiac puncture before the rats were euthanized. The three treatment group includes i) Control rats given intramuscular injection of normal saline (n=6) and euthanized accordingly after confirming that those rats were in the oestrus phase of the oestrus cycle, ii) 8 hours (n=6) and iii) 18 hours (n=6) post Chorulon injection. Blood plasma samples were stored at -20ºC until assayed. The ovaries were weighed and the left ovaries were fixed in 10% buffered formalin, processed and then embedded in paraffin, serially sectioned at 4µm and alternate five slides were stained with Haematoxylin and Eosin.

2.3 Follicle classification

The diameters of the follicles appearing the largest in the serial sections were measured in micrometer at two right angles of the follicle. The follicles were classified as small follicles (mean diameter ≤450µm) and large follicles (mean diameter >450µm) as stated by [1]. Follicles with the diameters smaller than 80µm were excluded. The follicles were classified as healthy unless at least one of these characteristics of unhealthy follicles was observed: the presence of scattered pyknotic nuclei in the granulosa cell layer, intense eosinophilic staining of oocytes [13], detachment of granulosa cell layer from basal membrane [14] and the presence of cell debris in the antrum of the follicle [15].

2.4 Assay of rat inflammatory mediators (IL-1β, Rat IL-8, PGE_2 and NGF) in blood plasma

Concentrations of IL-1β, IL-8, PGE_2 and NGF were determined using Enzyme Linked Immunosorbent Assay (ELISA) kit (CUSABIO, China). Standard curves generated in the range of 62.5-4000pg/mL (IL-1β), 18.75-1200pg/mL (IL-8), 0.4-100pg/mL (PGE_2) and 0.78-50pg/mL (NGF). The blood plasma was diluted 1:200 for NGF assay and was used directly for the other assays. The minimal detectable dose was 15.63pg/mL (IL-1β), 5pg/mL (IL-8), 0.25pg/mL (PGE_2) and 0.2pg/mL (NGF). The intra-assay variation were 3% (IL-8), 6% (PGE_2) and 7% (NGF).

2.5 Statistical data analysis

Data obtained from the experiment were analysed using statistical analysis (SPSS) version 16.0. Data were expressed as mean ± standard error mean and analysed with one way ANOVA and Duncan homogenous subset for the number of follicles, levels of IL-8 and NGF. The healthy and unhealthy follicle scoring and level of PGE_2 was analysed with the non-parametric test of Kruskal Wallis and confirmed with Mann-Whitney U test to compare differences present between each groups. A confidence interval of 5% or less was considered to be statistically significant.

3. RESULTS

Table 1 shows the ovarian weight of rats. The weight of both left and right ovaries of superovulated rat 8 hours post hCG were significantly heavier (P<0.05) than 18 hours post hCG or control. But the weight was not significantly different (P>0.05) between left and right for all the groups. Ovaries of rats 8 hours post hCG recorded the highest weight, followed by rats in 18 hours post hCG and control group.

Table 1: The weight (mean ± se) of ovaries in superovulated rats 8 hours or 18 hours post hCG and control rats

	8 hours post hCG	Control	18 hours post hCG
n	6	6	6
Left ovary	0.122 ± 0.020^{bx}	0.069 ± 0.003^{ax}	0.083 ± 0.001^{ax}
Right ovary	0.119 ± 0.019^{bx}	0.065 ± 0.003^{ax}	0.091 ± 0.006^{abx}
Total	0.241 ± 0.038^{a}	0.134 ± 0.003^{b}	0.174 ± 0.007^{a}

[ab] Mean values within the same row with different superscript are significantly different (p<0.05).

[xy] Mean values within the same column with different superscript are significantly different (p<0.05).

Table 2 shows the mean number of large, small and total follicles counted in the left ovaries of control, 8 hours post hCG and 18 hours post hCG rats. The number of large follicles were significantly higher (P<0.05) in 8 hours post hCG rats compared to control rats. However, the total number of follicles of both of these groups were not significantly different (P>0.05). The number of large follicles were significantly reduced (P<0.05) in 18 hours post hCG rats but it is still significantly higher (P<0.05) than the control group.

Table 2: The number (mean ± se) of large, small and total follicles in ovaries of superovulated rats 8 hours or 18 hours post hCG and control rats

Treatment	n	Number of follicles		Total
		Large	Small	
8 hours post hCG	6	40.8 ± 2.020^{a}	54.7 ± 2.030^{a}	95.5 ± 0.720^{a}
Control	6	10.7 ± 1.840^{b}	83.2 ± 2.630^{b}	93.8 ± 2.700^{a}
18 hours post hCG	6	28.8 ± 1.620^{c}	52.7 ± 3.320^{a}	81.5 ± 3.780^{b}

[ab] Mean values within the same column with different superscript are significantly different at p<0.05.

Table 3 shows the mean diameter of large and small follicles in the treatment group and control rats. Data showed no significant difference (P>0.05) in the diameter of large follicles in both groups of treated rats and control group. The diameter of large follicles in 18 hours post hCG (758.94 ± 32.328 µm), was larger than the diameter of large follicles in 8 hours post hCG rats (715.31 ± 10.972 µm).

Table 3: The diameter (mean ± se) of large and small follicles in ovaries of superovulated rats at 8 hours or 18 hours post hCG and control rats

Treatment	n	Diameter of follicles (µm)	
		Large	Small
8 hours post hCG	6	715.31 ± 10.972^{a}	273.75 ± 4.751^{a}
Control	6	681.45 ± 60.187^{a}	281.41 ± 13.255^{a}
18 hours post hCG	6	758.94 ± 32.328^{a}	259.13 ± 7.4755^{a}

[ab] Mean values within the same column with different superscript are significantly different (p<0.05)

Table 4 shows the number of large healthy and unhealthy follicles as well as small healthy and unhealthy follicles in the ovary of rats. The data recorded indicates that there were significant increase (P<0.05) in the number of healthy large follicles in the superovulated rats compared to control rats. The large healthy follicles were reduced in 18 hours post hCG rats compared to 8 hours post hCG rats but it was still significantly higher (P<0.05) than control rats. However, the number of unhealthy follicles in 8 hours post hCG rats and 18 hours post hCG rats were not significantly different (P>0.05).

Table 4: The number (mean ± se) large follicles (healthy, unhealthy) and small follicles (healthy, unhealthy) in ovaries superovulated of rat at 8 hours or 18 hours post hCG and control rats (n=6 each group).

Treatment	Large Follicle		Small Follicle	
	Healthy	Unhealthy	Healthy	Unhealthy
8 hours post hCG	33.2 ± 1.200[a]	7.7 ± 1.050[a]	54.67 ± 2.028[a]	0[a]
Control	8.2 ± 1.800[b]	2.5 ± 0.670[b]	83.17 ± 2.626[b]	0[a]
18 hours post hCG	18.8 ± 1.640[c]	10.0 ± 0.630[a]	52.67 ± 3.323[a]	0[a]

[ab] Mean values within the same column with different superscript are significantly different ($p<0.05$)

Figure 1 (A) shows ovary of control rats during oestrus where a few large follicles and corpus luteum were observed; (B) 8 hours post hCG ovaries where there was a high number of large follicles and corpus luteum present and (C) 18 hours post hCG ovaries where many corpus luteum were present.

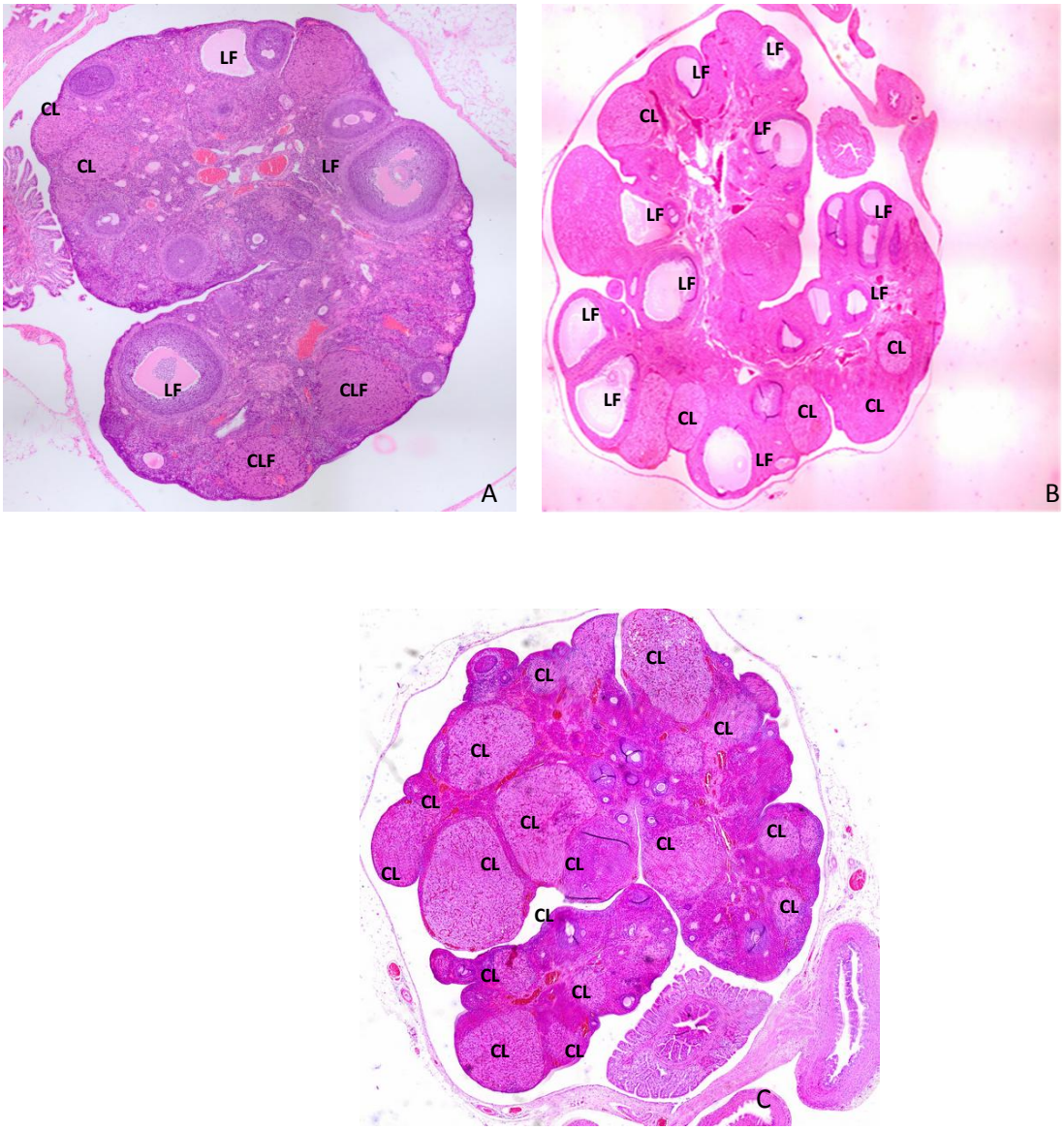

Figure 1: Section of ovaries of (A) normal rats (Oestrus), (B) superovulated rat 8 hours post hCG (C) superovulated rat 18 hours post hCG. LF Large follicles, CL corpus luteum. H&E staining at X4 magnification with the setting of 5X5 vertical image, with 10% overlap and 50% transparency using Multiple Image Alignment Software

The concentration of interleukin 1β in the blood plasma of rats could not be detected by the ELISA kit as the concentration falls below the detection range of the ELISA kit.

Figure 2 shows that the concentrations of IL-8 in 8 hours post hCG rats (19.37±2.725 pg/mL) is more than twice the concentration in control rats (8.44 ± 3.267pg/mL). The level of IL-8 in 18 hours post hCG rats were however the lowest, recording only 2.85 ± 0.126pg/mL.

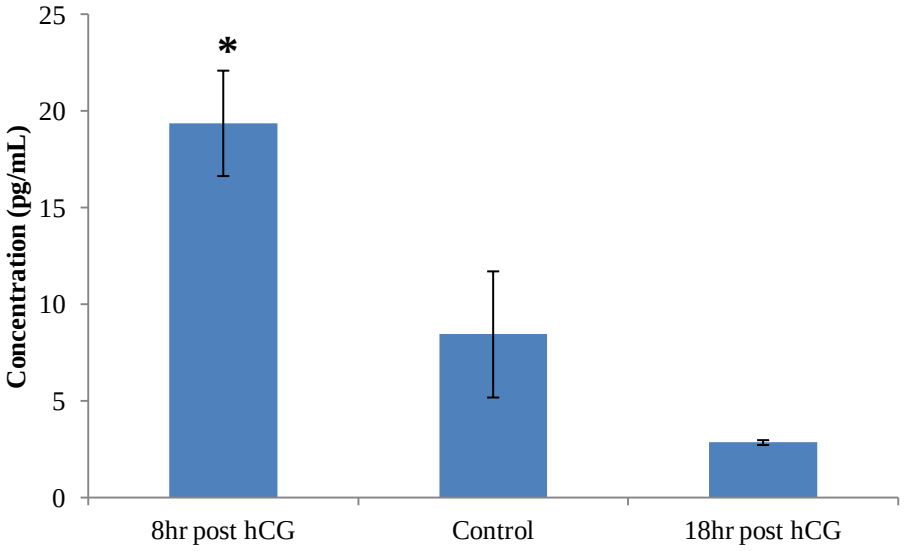

Figure 2: Levels of blood plasma Interleukin-8 of superovulated rats at 8 hours or 18 hours post hCG and control rats. *P<0.05 vs. control group.

Figure 3 indicates that the Prostaglandin E_2 level was highest in 8 hours post hCG rats (0.75 ± 0.130 pg/mL) and showed an insignificant decrease in 18 hours post hCG rats (0.61 ± 0.065 pg/mL). The level of PGE_2 in control rats (0.51 ± 0.102pg/mL) however was the lowest. The readings were not significantly different from each other.

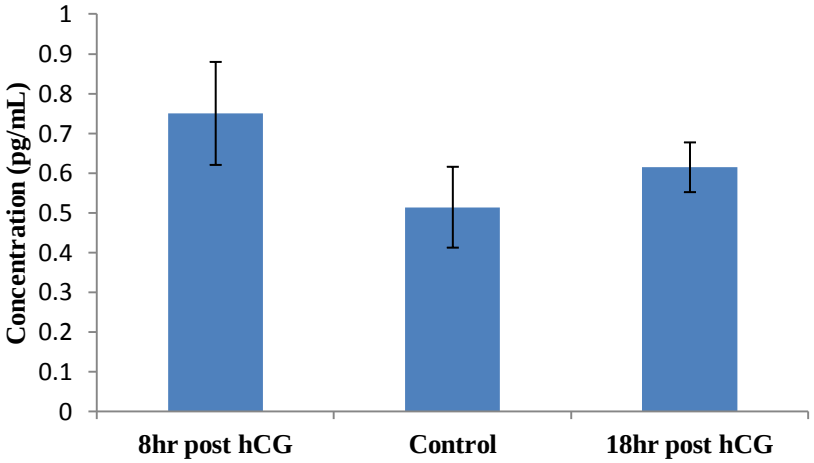

Figure 3: Levels of blood plasma Prostagladin E_2 of superovulated rats at 8 hours or 18 hours post hCG and control rats. * P < 0.05 vs. control group

Figure 4 shows that the level of NGF 8 hours post hCG (1101.8 ± 139.17 pg/mL) is significantly lesser than levels at 18 hours post hCG (1627.0 ± 85.46 pg/mL). However the level of NGF in 18 hours post hCG rats and control rats (1660.8 ± 29.43 pg/mL) were not significantly different.

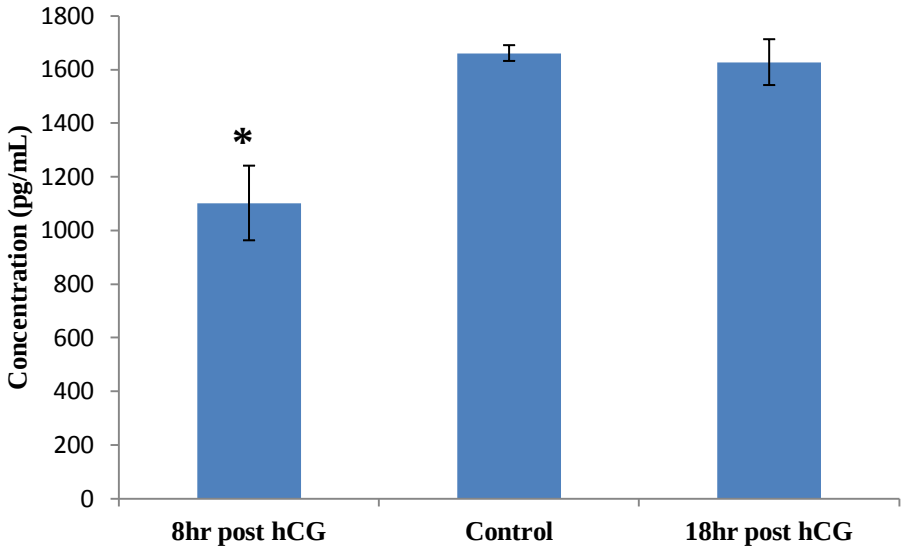

Figure 4: Levels of blood plasma Nerve Growth Factor of superovulated rats at 8 hours or 18 hours post hCG and control rats. $^{*}P < 0.05$ vs. control group

4. DISCUSSION

Our results on the ovarian weight were similar to that reported by [16] whereby increased weights of the ovaries were caused by ovarian tissues being greatly stimulated when they were induced by gonadotropin. Greater ovarian weight were observed in gonadotropin stimulated rats may reflect that greater number of follicles ovulating prematurely, leading to the formation of more corpus luteum which contributes disproportionally to the total weight of the ovaries. Corpus lutea were formed as a result of ovulation and they make up a significant portion of weight of each ovary [17].

A reduced ovarian weight was observed in rats, euthanised 18 hours post hCG compared to 8 hours post hCG rats because many large follicles were reported to have completed ovulation 16 hours following the hCG injection [9]. Decreased ovarian weight in this group could also be associated with a deduced number of developing follicles and increased atresia of the remaining follicles [18].

Repeated events of proliferation, differentiation and transformation of cells as well as the formation and regression of the corpus luteum could be observed during the development of follicles. The final maturation of follicles and corpus luteum functions are regulated mainly by gonadotropins and growth hormones. High numbers of large follicles were present in gonadotropin treated rats as the treatment induces the small follicles to undergo rapid maturation and development stages to form large follicles [19]. Pregnant Mare Serum Gonadotropin stimulates the follicular growth in rats by rescuing the follicle atresia, causing those atretic follicles to reach ovulation [20]. Furthermore, the preantral follicles are highly responsive to endogenous and exogenous gonadotropins [18]. The gonadotropin only affected the large follicles resulting in a higher number of large follicles but does not show difference in the total number of follicles. The reduction in the number of large follicles at 18 hours post hCG from the time of 8 hours post hCG rats is probably because most matured follicles would have ruptured within this time period. Earlier studies have stated the ovary undergoes rupture to release its fertile egg 16 hours after the hCG injection [9]. However, a few large follicles were still present at this point as ovulation might still be taking place and had not been completed.

Even though the number of large follicles was increased by the superovulation treatment, the sizes of both large and small follicles were relatively the same. In a study on hamster [21] hCG treated follicles diameter were not significantly increased in 2 days after hCG injection, but increased significantly on day 3 and 4 after hCG injection. Even though there was no significant increase in the size of large follicles in 8 hours and 18 hours post hCG rats, the latter group recorded a higher mean diameter probably because the follicles had reached its maximum size in order to ovulate [22]. The diameters of the small follicles were relatively unaltered among the different groups probably because the gonadotropin only affects the development of large follicles and not the small follicles [23].

The superovulation treatment by gonadotropins induces multiple follicular developments by preventing atresia in small antral follicles and thus leading to its development into large follicles [20]. Previous study by [21] showed that there was a notable difference of the reduced incidence of atresia in hCG rats compared to control rats. Many healthy large follicles would have ovulated at 18 hours post hCG resulting in lower number of healthy large follicles in this group compared to 8 hours post hCG, whereby ovulation could be observed 16 hours post hCG [9]. A reduced number of healthy small follicles were observed in the ovaries of superovulated rats is probably because many small follicles were recruited to undergo maturation to develop into large follicles under the influence of exogenous gonadotropin as the role of FSH in increasing the number of antral follicles and preventing the incidence of atresia in the follicles are more prominent in the small size follicles.

The levels of Interleukin-1β in the blood plasma of rats were not detected by the ELISA kit. This is probably because the IL-1β concentration was very low in the blood plasma and was below the sensitivity of the kit. A study by [24] reported the expression of IL-1β mRNA in the thecal layer of large preovulatory follicles in rats treated for superovulation was significantly increased at 6 hours after hCG injection. Furthermore, the study concluded that IL-1 served an important function in the ovulation process in the superovulatory cycle. In this study however, the inability of the kit to detect the level of IL-1β was probably because the circulatory level of this mediator was very low. Interleukin 1β which is produced at the site of ovulation by the granulosa cells and surrounding theca interna [25] probably caused the concentration of this mediator in the blood might be very low. Furthermore, cytokines were reported to be produced in a very low concentration where in some cases only a few dozen receptors were needed to be activated per cell to elicit the effect of these mediators [26]. Inflammatory mediators are also produced when there is a demand to its presence and travels only over a short distance causing it to be not reliable for the serum level to reflect the actual event on the site of activation [26]. Therefore, any form of increase in the IL-1β in the blood plasma of stimulated rats compared to the normal rats could not be identified as the level this mediator was still below the detectable range of the kit.

Interleukin-8 plays a crucial role in ovulation as it serves as neutrophil attractant in follicular development whereby inflammatory events were observed during the point of ovulation and luteolysis [10]. Interleukin-8 has been implicated in follicular growth, the process of ovulation and oocyte maturation [27] and it is produced by a number of ovarian cells such as granulosa cells, theca cells and other stromal cells [28]. In this study, IL-8 has high values in 8 hours post hCG rats probably because the expression of IL-8 messenger RNA (mRNA) and production of IL-8 proteins in the granulosa-luteal and stromal cells in the ovary were increased by the administration of hCG [29]. The outcome of this study shows similarities to the study by [30] whereby the study showed that the cytokine which is localised to the theca compartment in the rat ovary has peak values 6 hours following hCG. Furthermore, [27] reported that after the LH surge, the granulosa cells in the ovary naturally produces IL-8 in the follicular fluid, as it is an important angiogenic factor to induce neovascularisation when the ovarian follicles are developing. In addition, the production of IL-8 in granulosa cells were increased by FSH stimulation (PMSG administration) [31]. The level of IL-8 was reduced after ovulation as this chemokine is no longer produced by the cells. This result is similar to the study on rabbits by [32], where the IL-8 concentration peaked at 4 hours following hCG injection and decreased gradually within 9 hours, reached a second peak when ovulation was observed and drops thereafter, reaching its baseline 24 hours after the hCG injection.

Prostaglandin E_2 carries out function in the reproductive system during the development of follicles and ovulation whereas $PGF_{2\alpha}$ regulates the corpus luteum in the process of luteolysis and the absence of $PGF_{2\alpha}$ receptors leads to the failure in parturition as a lack of luteal regression could be observed [33]. The level of PGE_2 is high in 8 hours post hCG rats probably because it was produced in high amount at this period of time. The ovarian PGE_2 level begins to rise 5 hours after the LH surge/hCG injection [34]. This elevated level of PGE_2 is maintained up to the point of ovulation [35]. The level then reduces at 18 hours post hCG rats as there are fewer preovulatory follicles. The preovulatory ovaries of rats treated with PMSG/hCG synthesize Prostaglandin of the E and F series [36]. The level of this mediator in 8 hours post hCG rats was approximately 1.5 fold higher concentration than control rats which showed a different phenomenon in site of production which is the ovaries where [37] reported that the concentration of PGE_2 in LH stimulated ovaries could reach four fold higher concentration than in the non-stimulated ovaries. This is probably because the concentration of this mediator was low in the blood plasma than in the actual site of PGE_2 production. The level of this mediator is then reduced at 18 hours post hCG rats probably because the PGE_2 were rapidly inactivated in the lungs and liver. As much as 80-98% of prostaglandin in blood is removed during a single passage through the pulmonary circulation [38].

The expression of NGF was observed at the later stage of the ovulation process as the 18 hours post hCG rats showed higher levels compared to the 8 hours post hCG group. This is probably because NGF is a vital component of wound healing and tissue repair which takes place at the later stage of the ovulation cycle. The low expression of blood plasma NGF preceding ovulation is probably because there are fewer sites of tissue rupture and inflammation. The follicular rupture during ovulation is an inflammatory like process whereby during this process the tissue was usually involved in damage and repair [9]. The repair phase was characterised by the proliferation of fibroblasts whereby nerve growth factor was cited to be expressed in fibroblasts and the mRNA of NGF was reported to be increased in ovulatory rat follicle in the thecal tissue and ovarian stroma that contains fibroblast [36]. In this study, the level of NGF in 18 hours post-hCG

rats are not significantly different from control rats probably because the expression of this mediator is not influenced by the number of site for repair and wound healing.

5. CONCLUSION

This study clearly indicates that the superovulation treatment leads to rapid development of the small follicles into large follicles by preventing atresia, resulting in increased number of large follicles without increasing the number of total follicles and the diameter of the follicles. The inflammatory mediators screened in this study indicated that only IL-8 had increased expression in blood plasma while PGE_2 and NGF remained unaltered. Therefore, further research on the circulatory level of other inflammatory mediators that play important role in ovulation should be carried out. Furthermore, researches emphasising on IL-8 and the possible effects that could be caused by the up regulation of this mediator should be carried out in future.

6. ACKNOWLEDGEMENTS

This project was supported by Universiti Putra Malaysia under the Fundamental Research grant (02-10-07-306FR) and is greatly appreciated.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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