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IMMUNOHISTOCHEMICAL STUDY OF ESTROGEN AND PROGESTERONE RECEPTORS IN DIFFERENT SIZE CLASSES OF OVARIAN FOLLICLES IN DROMEDARY CAMELS

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ABSTRACT

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The present study aimed to investigate the normal distribution of estrogen (ERα) and progesterone (PR) receptors in the different classes of the ovarian follicles of the she-camel and their relation with the serum and follicular estrogen and progesterone hormonal level. The ERα and PR were detected using an indirect immunohistochemistry method (streptavidin-biotin immunoperoxidase method). Serum and follicular hormone levels were measured by radioimmunoassay. ERα was detected at low amounts in the follicular cells of the primordial, primary follicles and corpora lutea while, detected at moderate in the secondary follicles and high in the oocytes and in the tertiary follicles. On other hand, PR was detected in low reaction in secondary follicles and tertiary follicles. Moreover, it was estimated in a moderate reaction corpora lutea and corpora albicantia and stroma cells and in a strong reaction in the blood vessels. Estrogen concentration in both follicular fluid and serum correlated negatively (not significantly) with the size of the follicle while a positive non significant correlation was found between serum progesterone and the size of the corpus luteum. Serum and follicular fluid estrogen was higher in follicles exceeding 15 mm more than the lesser follicular categories. Slight difference in the concentration of estrogen was found between follicles less than 10 mm in diameter and those between 10 – 15 mm. The expression of ER and PR and the secretion of their specific hormones in the ovary of she-camel were not always correlated with the presence of the hormones.

Key words: *Immunohistochemistry, ER, PR, Camel ovary.*

INTRODUCTION

Steroid hormones are important regulators of reproductive processes in female mammals. Estrogens and progesterone receptors mediate respectively the action of estrogens and progesterone by regulating transcription target genes. Estrogens possess an intrafollicular action by stimulating in synergic manner with FSH the aromatase activity (Adashi *et al.*, 1982 and Fitzpatrick and Richards, 1992). They increase granulosa proliferation (Palter *et al.*, 2001) and are essential to GnRH receptor expression in the growing ovarian follicles (Kogo *et al.*, 1999).

Receptors for estrogen (ER) are expressed as 2 structurally related subtypes in mammals, ERα and ERβ, which are encoded by 2 distinct genes. The existence of these 2 subtypes may partly explain the selective action of estrogen in different target tissues and in the same tissue in different physiological statuses (Conneely, 2001). Studies on several species have confirmed the differential distribution of these 2 receptors in the ovary: ERβ was detected mainly in granulosa cells, whereas ERα was detected in theca cells, stromal cells, and germinal epithelium (Pelletie and Al-afy. 2000; Van den Broeck *et al.*, 2002; Berisha *et al.*, 2002; Amrozi *et al.*, 2004; Sanchez-Criado *et al.*, 2005 and Salvetti *et al.*, 2007). Progesterone plays a major role in controlling ovulation and pregnancy (Graham *et al.*, 1997). The importance of estrogen and progesterone receptors in the female reproductive function was revealed by many authors in different mammalian ovaries. There is a shortage in the studies dealing with the role of these receptors and their distribution in the ovarian follicles of she-camel. Therefore, the present study aimed to investigate the normal distribution of estrogen and progesterone receptors in the different classes of the ovarian follicles in the she-camel and their relation with the serum and follicular estrogen and progesterone hormonal level.

MATERIALS and METHODS

Collection of samples

Ovaries of 35 adult female one-humped camels (*Camelus dromedarius*) slaughtered at a Cairo abattoir, were collected immediately after slaughter. The period from November to April was taken as the peak breeding season, while May-October was considered as the low breeding season.

Pre-slaughter information regarding the nutritional or reproductive status of these camels was not available. After cleaning each ovary off the extraneous tissue, diameter of Graafian follicles was measured using Vernier Calipers. On the basis of their size, follicles were

classified into three groups viz. small (5-9 mm) and large (10-20 mm). Fluid from each follicle was aspirated aseptically and stored at –20°C. Animals having ovaries with any pathological lesions, or those with cystic follicles (>20 mm in diameter; Tibary and Anouassi, 1996) were not included in the study. Before slaughter, about 15 ml peripheral blood was collected from each animal, serum was separated and stored at –20°C for hormonal analysis.

Histological and immunohistochemsitry:
Ovaries of non-pregnant she camel were collected and fixed in 4% neutral buffered formalin for 18-20 hours at 4°C, and then washed in phosphate-buffered saline and processed for paraffin embedding. Histological slides of 5 µm in thickness from each ovary were stained with haematoxylin and eosin for histological examination.

An indirect immunohistochemistry method (as described by Salvetti, (2004)) was used to detect estrogen receptor (ER α) protein and progesterone receptor (PR) protein using antibodies obtained from (Dako - Life Trade, Cairo, Egypt). Follicles were classified according to the criteria listed in the Nomina Histologica into the following groups: secondary, tertiary, atretic, and cystic follicles (Nomina Histologica, 1994).

Immunoassay for hormones
Blood serum and follicular fluid samples were analyzed for progesterone, estrogen, through EIA technique, using a Microstrip Elisa Reader (Stat-Fax-303, Awareness Technology, Inc.). Progesterone and estradiol concentrations were determined by using kits from Bremancos Diagnostic INC–GmbH, Germany (Cat. # BC-1113 & BC-1111, respectively). The lowest detectable level of progesterone during this test was 0.05 ng/ml, while the cross reactivity with other steroid hormones was <0.74%. For estradiol, the lowest detectable level was 5.9 pg/ml and cross reactivity with other steroids was <2.10%.

Statistical analysis:
Statistical analysis of the collected data was carried out according to procedures of completely random design, SAS (1995).

RESULTS

Morphologically, the ovary of the she-camel was flattened, lobulated measuring 3.17, 2.21 and 0.8 cm length, width and thickness respectively. Both right and left ovaries exhibited follicles in various stages of development, including primordial, primary, secondary and tertiary follicles, corpora albicantia, and late CL, as well as follicles with different degrees of atresia. The measurements of the different follicles and corpora lutea during different stage of the estrus cycle were summarized in (Table. 1) and the morphmetric measurements of the oocytes in different types of the ovarian follicles.

Immunohistochemically: In all examined she- camels, ERα was detected at low amounts in the follicular cells of the primordial and primary secondary follicles, moderate corpora lutea and corpora albicantia. Moreover, it was recorded at high amount in the vital and atretic tertiary follicles. Also, ERα was detected in cells of the deep and superficial stroma, tunica albuginea and surface epithelium but the reaction was weak in stroma cells surrounding the follicles. The reaction was strong in the cytoplasm of the ova of the primary and secondry follicles (Fig.1-3).

In mature ovarian follicles (Fig. 4); ERα was strongly expressed in the cellular nuclei and cytoplasm of the granulose, and moderate in that of theca interna, and theca externa layers. The reaction products in this cell type were granular in the nuclei and in the cytoplasm of the granulose cells but were homogeneous distribution in theca interna and theca externa. In corpora lutea, ERα was detected in low amount in the lutein cells and stroma cells. Moreover, it was higher in the capsular stroma than in the internal stroma.

On other hand, PR was detected as moderate reaction in cells of the stroma, corpora lutea and corpora albicantia, tunica albuginea and stroma cells surrounding the follicles surface epithelium but the reaction was strong in the blood vessels (Fig. 5). In addition, PR was detected in low amounts in secondary follicles and tertiary follicles. Furthermore in mature ovarian follicles, PR was expressed weak reaction in the cellular cytoplasm and nuclei of the granulose cells. The immunostaining was low or absent in the theca externa cells and strong in the cytoplasm and nuclei in the superficial layer of the mature follicles.

Serum and follicular fluid estrogen (Table. 2& 3 and Hist. 1& 2) was higher in follicles exceeding 15 mm more than the lesser follicular categories. Slight difference in the concetration of estrogen was found between follicles less than 10 mm diameter and those between 10 – 15 mm. A highly positive correlation (Table. 2 and Hist. 2) was detected between the size category of the corpus luteum and serum progesterone concentration ($r = 0.99$, $p<0.0001$).

Table 1: The morphmetric measurements of the oocytes in µm in the different ovarian follicles measured from histological sections of the she-camel ovaries stained with H&E

cell stage	cell diameter	cell surface	nucleus diameter	nucleus surface
Primordial follicle	12.9	143.5	7.9	45.7
Primary follicles	11.01	123.6	7.5	43.9
Secondary follicle	24.59	543.2	8.8	41.9
Growing follicle	22.2	499.5	8.2	33.9
Tertiary follicle	43.5	1229.3	10.1	35.7

Table 2: Estrogen (E_2) concentration in follicular fluid and serum of different size classes of ovarian follicles of she camels.

Size of the ovarian follicle (mm)	Follicular E_2 (pg/ml)	Serum E_2 (pg/ml)
> 10	67.18 ± 11.23	21.91 ± 2.06
10 to 15	69.33 ± 6.41	31.72 ± 3.65
>15	88.62 ± 7.46	44.98 ± 6.84

Table 3: Progesterone concentration in she camels relative to the size of the CL

Size of the ovarian CL (mm)	Serum P4 (ng/ml)
> 10	0.89 ± 0.01
10 to 20	2.50 ± 0.61
>20	4.67 ± 1.46

Hist. (1): Estrogen (E2) concentration in follicular fluid and serum of different size classes of ovarian follicles of she camels.

Hist. (2): progesterone concentration in she camels relative to the size of the CL

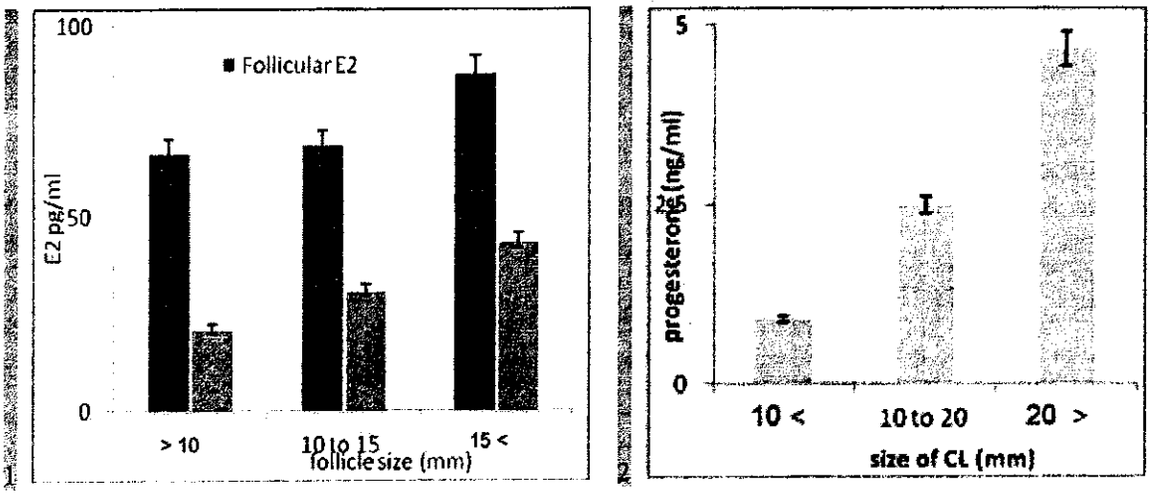


Fig. 1- 4: Micrographs of different she-camel ovarian cell types at various estrous stages showing immunostaining of ERα. By Streptavidin-Biotin method, Mayer's hematoxylin counterstain.

1- Outer region of the ovarian cortex during estrus with high immunoreactivity for ERα (SERα) in the surface epithelium, low immunoreactivty in the superficial stroma, and strong expression in the tunica albuginea, cytoplasm and nuclus of oocytes of the primordial follicles.X200.

2- Primary follicle of she-camel ovary showed ERα strong immunoreactions in the cytoplasm of oocyte and moderate reaction in the follicular cells. X100.

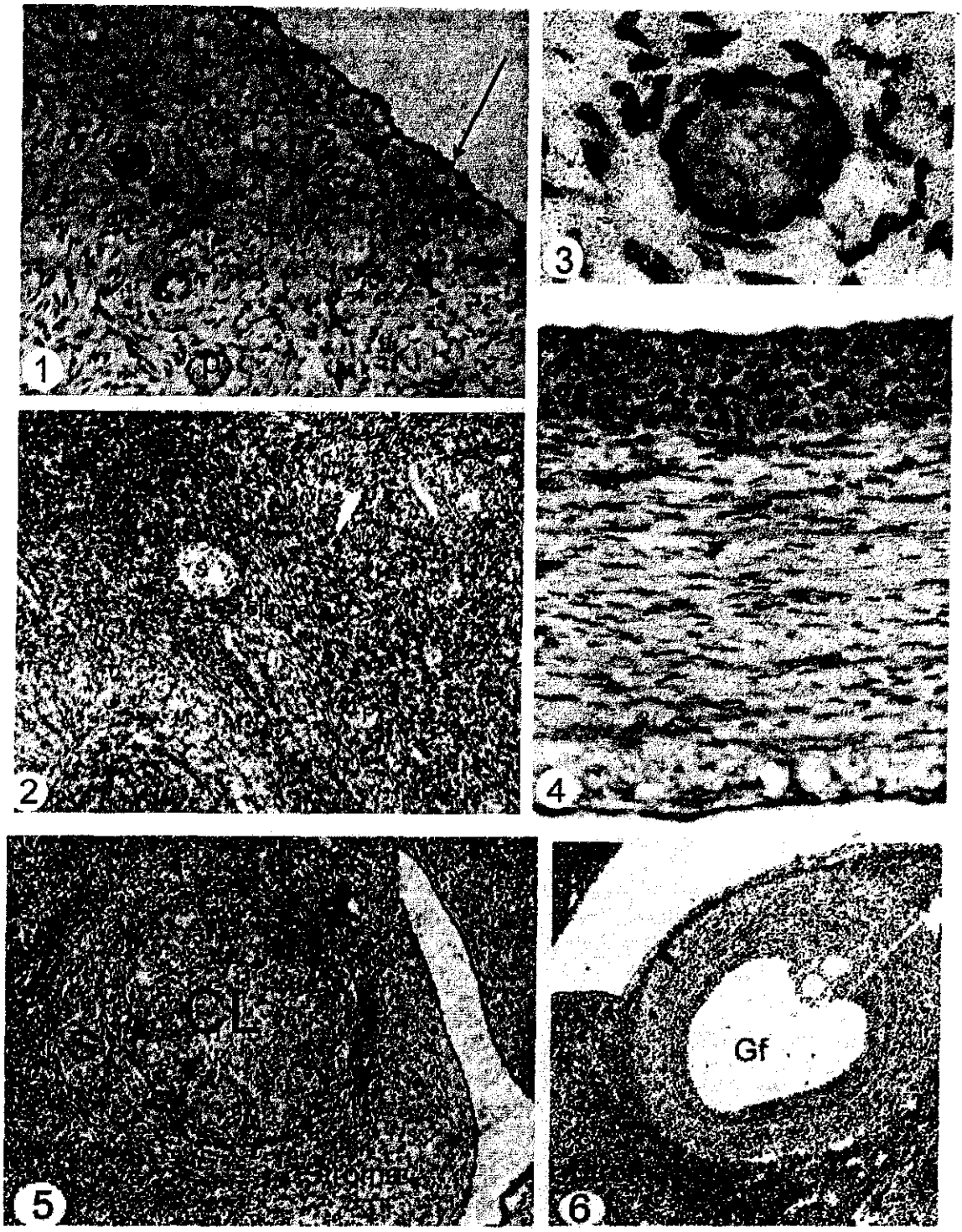
3- Low ERα immunostaining in the wall of a secondary follicle (s), stroma cells and atric follicles during proestrus. X100 .

4- Wall of the mature ovarian follicle presented strong immunoreactivity in the cytoplasm and nuclei granulosa, whereas theca interna and externa show moderate reaction but few nuclei show strong reaction. X200.

Fig. 5-6: Micrographs of different she-camel ovarian cell types showing immunostaining of PR. By Streptavidin-Biotin method, Mayer's hematoxylin counterstain.

5- PR immunoreactivity was moderated in corpus luteum, cells of the stroma and blood vessels. X40.

6- In the mature ovarian follicle, the PR immunoreactivity was low in the theca interna and externa but high in granulose layer X40



DISCUSSION

The obtained immunohistochemical observations indicate that the expression of ER and PR and the secretion of their specific hormones in the ovary of Arabian she-camel were well correlated with the reproductive cycle. But during ovarian activity, the expression of ER and PR is not always correlated with the presence of the hormones in the follicles and serum. The immunohistochemical expression of the ER was detected in the nuculei and cytoplasm of the ovarian cells whereas in the primate ovaries, the ER of the granulosa cells was nuclear only (Billiar *et al.*, 1992; Suzuki *et al.*, 1994 and Saunders *et al.*, 2000). In rat, hamster and pig, the ER is nuclear and cytosolic, and the cytosolic fraction is more important (Kawashima and Greenwald 1993). According to Guiochon-Mantel and Milgrom (1993), ER is essentially localized in the nucleus in the absence of estrogens. In addition, at the hypothalamic and hypophysal level, the estradiol injection induces the increase of the cytosolic fraction of the ER (Kawashima *et al.*, 1987). In she-camel ovary, the variation during the reproductive cycle would translate a functional action. Numerous studies show on the contrary that the theca cells are the major sites of ER expression in different species including rats, mice and primate (Chandrasekher

et al., 1994; Kuiper, *et al.*, 1996; Sanchez-Criado *et al.*, 2005) or total absence of the ER in the ovary (Saunders *et al.*, 1997).

As reported in the bovine ovaries (Van den Broeck *et al.*, 2002), in the present study, the PR localized in the different ovarian structure of the she-camel additionally shows that the various ovarian cell types exhibit different patterns of PR immunoreactivity during the ovarian activities. In the follicle cells of primordial, primary and secondary follicles the scores for PR were high and increased from primordial to secondary follicles. These data are in accordance with findings in primates (Hild-Petito *et al.*, 1988) and dogs (Vermeirsch *et al.*, 2001), and they indicate that progesterone may regulate follicular growth during the early stages of follicular development.

In she-camel follicular structures examined, the follicle/granulosa cells showed the high PR immunostaining during oestrus, when ovulation occurs. These results are concomitant with earlier observations in the dog (Vermeirsch *et al.*, 2001) and with a study on PR mRNA in the bovine ovary (Cassar *et al.*, 2002). The crucial role of PR in the ovulatory process has been demonstrated in PR-deficient mice, since such mice develop large follicles but fail to ovulate (Lydon *et al.*, 1995). All these findings emphasize the important role of progesterone and its receptor in the ovulation process. The expression of PR in granulosa cells in tertiary follicles is induced by the LH surge (Hild-Petito *et al.*, 1988). The induction of PR mRNA has also been observed in monkey granulosa cells during periovulatory stages (Chandrasekher *et al.*, 1994) and in porcine granulosa cells cultured *in vitro* after LH stimulation (Iwai *et al.*, 1991). The progesterone receptors are reported to mediate the protective effects of progesterone against apoptosis in the granulosa cells of preovulatory follicles (Quirk *et al.*, 2004).

The presence of PR in corpora lutea reflects the role of progesterone in corpus luteum activity (Revelli *et al.*, 1996; Rueda *et al.*, 2000). Progesterone regulates the proliferation and development of luteinized granulosa and theca cells in an autocrine and paracrine way (Sasano and Suzuki, 1997). The presence of PR in all lutein cells of the corpora lutea suggests the influence of progesterone in the luteinization process (Revelli *et al.*, 1996; Duffy *et al.*, 1997). However, in the present study, the PR immunostaining in the corpus luteum was lower than in most other ovarian structures, which can be due to a negative effect of the locally produced high levels of progesterone to the PR production. In contrast to all other ovarian cells, the lutein cells of the corpora lutea showed PR immunostaining not only in the nuclei, but in the cytoplasm as well.

A low but manifest PR immunoreactivity was observed in cells of the tunica albuginea and the surface epithelium. This corresponds with a study on ovine ovaries in which it has been suggested that cells of the ovarian surface epithelium are enzymatically involved in the ovulation process by the influence of progesterone and its receptors (Murdoch, 1998). Further investigations in cattle are necessary to verify the role of PR and progesterone in the ovarian surface epithelium.

The present study indicated that estrogen concentration in both the follicular fluid ($r = 0.06$) and serum ($r = 0.15$) correlated negatively (non-significantly) with the size of the follicle while a positive non significant correlation was found between serum progesterone and the size of the corpus luteum. However, positive non significant correlation was detected between serum progesterone and the size of the corpus luteum in the studied animals throughout the days of the cycle ($r = 0.25$). On other hand, present immunohistochemical observations indicate that the expression of ER and PR and the secretion of their specific hormones in the ovary of camel was not always correlated with the presence of the hormones.

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دراسة كيميائية هستومناعيه على مستقبلات هرمون الاستروجين والبروجستيرون في جريبات المبيض المختلفة في الجمال العربية

عبد المهيمن مصطفى صالح ، ايناس احمد عبدالحافظ ، ضرار رفعت ضرار

تهدف هذه الدراسة إلى دراسة توزيع مستقبلات هرمون الاستروجين والبروجستيرون في مختلفة جريبات المبيض من الناقة وعلاقتها مع مستوى هرمون الاستروجين والبروجسترون فى السائل الجريبى والدم. تم دراسة مستقبلات الاستروجين والبروجستيرون بواسطة طريقة تفاعل كيمياء النسيج المناعى الغير مباشرة. وتم قياس مستويات الهرمونات في الدم بواسطة المقايسة المناعية الاشعاعية. وجدت مستقبلات الاستروجين في كميات قليلة في الخلايا الجريبية لجريبات المبيضية الأساسية والاولية وكذلك فى الجسم الاصفر في حين وجدت متوسطة في الخلايا الجريبية للجريبات الثانوية وعالية فى البويضات والخلايا الجريبية للجريبات المبيضية الناضجة. في المقابل وجدت مستقبلات البروجستيرون فى كميات منخفضة في الجريبات المبيضية الثانوية والناضجة، ومعتدلة التفاعل فى الأجسام الصفراء وعالية التفاعل في الأوعية الدموية. وجد ان العلاقة بين تركيز هرمون الاستروجين في كل من السائل الجريبي ومصل الدم مع حجم الجريبات كانت عكسية، ولكن ليست بشكل كبير، في حين وجد ارتباط إيجابي كبير بين تركيز البروجسترون في مصل الدم وحجم الجسم الأصفر. وكما وجد ان تركيز الاستروجين فى مصل الدم والسائل الجريبي كان عاليا في الجريبات الاكبر من ١٥ مم أكثر من الجريبات الأقل حجما. لكن الاختلاف كان طفيفا بالجريبات المبيضية الأقل من القطر ١٠مم وتلك ما بين ١٠ - ١٥ مم. كما وجد ان توزيع مستقبلات هرمون الاستروجين والبروجستيرون المبيضى غير مرتبط بمستوى الهرمونين فى الدم والسائل الجريبى.