

Effect of crude and steroid free bovine follicular fluid on some hematological and biochemical parameters and ovarian development in rats

Hayder Abdulkadem AL-Zamely Mohammad Abdulazeez AL-llozy*

Dept. of Physiology, College of Vet. Med. Univ. Of AL-Qadisiya

*College of Agriculture, Univ. of AL-Mothanna

Abstract:

Ovarian growth and function is under the control of pituitary gonadotropins and various local intraovarian factors. The present study has been undertaken to assess the efficacy of crude follicular fluid and steroid free FF taken from bovine ovaries on some hematological aspect as well as ovarian growth and function of mature female Wistar rats. Follicular fluid aspirated from large size Graffian follicles of abattoir – derived bovine ovaries was made cell free by centrifugation and steroid free by charcoal treatment. follicular fluid has been fractioned using ammonium sulphate precipitation at various saturation levels and then subjected to get filtration chromatography. The 20 – 40% fraction yielded the detectable peak and the average yield of peptide was 0.659 mg/ml of FF . The crude and steroid free FF were tested for effects on some hematological parameters and ovarian and uteri weights, numbers of different types of ovarian follicles and assessment of histophysiological appearance. Twenty four mature female Wistar rats were randomly assigned into three equal groups (control and two treatment groups), injected with normal saline (Control), SFFF (T1) and CFF (T2) for 4 consecutive days. At the fifth day, all female rats were anesthetized, dissected and blood samples were obtained for hematological study and ovaries and uteri were removed , weighted and fixed in formalin 10% for histophysiological study .

The results revealed no significant effects in Hb concentration , PCV , AST , concentrations, cholesterol , urea , and glucose concentrations .

On the other hand , the result revealed no effect on uteri and ovarian weights , an increment in the numbers of primary and secondary follicles and a decrement in the number of Graffian follicles . Within each group , the results showed that primary follicles recorded the highest number among the different types of ovarian follicles. While the highest total number of the different types of ovarian follicles were recorded in CFF and SFFF groups compared with control .

The present study demonstrated no side effect on blood constituents as well as the effect of a bovine intrafollicular factor/s regulating ovarian growth and function in rats , especially the development of primary and secondary follicles unlike Graffian follicles which underwent from the inhibition .

تأثير السائل الجريبي الخام ومنزوع الستيرويدات للأبقار في بعض المعايير الدمية والكيموحيوية والتطور المبيضي لدى الجرذان

حيدر عبد الكاظم الزاملي محمد عبد العزيز علي اللوزي*

فرع الفلسجة، كلية الطب البيطري، جامعة القادسية
*كلية الزراعة، جامعة المثنى

الخلاصة:

يكون نمو المبايض ووظائفها تحت سيطرة هرمونات الغدة النخامية المحرصة للقند وعدد من العوامل داخل المبيض ، أجريت الدراسة الحالية بهدف تقييم فعالية السائل الجريبي الخام والسائل الجريبي منزوع الستيرويدات المأخوذة من مبايض الأبقار في بعض المقادير الدمية والكيموحيوية صورة الدم علاوة على دراسة تأثيرها في نمو ووظائف مبايض جرذان الوستر الناضجة . تم جمع السائل الجريبي من جريبات كراف لمبايض الأبقار وأزيل منها بقايا الخلايا بطريقة التدوير بجهاز الطرد المركزي وأزيلت منها الستيرويدات باستخدام المعاملة بفحم الجار كول . تمت تجزئة السائل الجريبي باستخدام الترسيب بسلفات الامونيوم بمستويات مختلفة ثم تمت تنقيته بطريقة كروماتوغرافيا الهلام . وجد أن الجزء المترسب بالتركيز 20 – 40% يحتوي على المستوى المحسوس من الستيرويدات والتي قدرت قيمتها 0.659 ملغم/مل من السائل الجريبي بعدد تمت دراسة تأثير السائل الجريبي الخام ومنزوع الستيرويدات على بعض المصادر الدمية والكيموحيوية صورة الدم وأوزان المبايض والأرحام وأعداد جريبات المبايض بأنواعها الثلاث (الابتدائية والثانوية وكراف) علاوة على دراسة المظهر النسيجي – الفسيولوجي للمبايض . أجريت الدراسة على 24 من إناث جرذان الوستر الناضجة التي التي قسمت على ثلاث مجموعات متساوية ، حقنت الأولى (السيطرة) بالمحلول الملحي الفسلجي وحقنت الثانية (T1) بالسائل الجريبي منزوع الستيرويدات وحقنت الثالثة (T2) بالسائل الجريبي الخام لمدة أربعة أيام متتالية . في اليوم الخامس تم تخدير الحيوانات وأخذت منها نماذج دم لدراسة صورة الدم وأزيلت منها المبايض والأرحام لدراسة نموها وتطورها. أظهرت النتائج عدم وجود تغيرات ذات معنوية احصائياً بين مجموعات التجربة في كل من تركيز الهيموغلوبين وحجم الخلايا المضغوطة وعدد الخلايا البيض الكلي والإنزيمات الناقلة للأمين AST و ALT والكوليسترول واليوريا والكلور . من جانب آخر ، أظهرت النتائج عدم تأثير أوزان المبايض والأرحام بحصول زيادة معنوية في أعداد الجريبات الابتدائية والثانوية وانخفاض معنوي في أعداد جريبات كراف ، وعند إجراء المقارنة داخل كل مجموعة ، وجد أن أعداد الجريبات الابتدائية هي الأعلى معنوياً من بين الأنواع الأخرى . كما بينت الدراسة أن أعداد الجريبات الكلي كان الأعلى معنوياً في المجموعتين المعاملتين بالسائل الجريبي الخام ومنزوع الستيرويدات بالمقارنة مع السيطرة . يمكن الاستنتاج أن السائل الجريبي البقري يحتوي على عامل أو عوامل تؤثر في نمو المبايض ووظائفها وعلى وجه الخصوص نمو وزيادة أعداد الجريبات المبيضية الابتدائية والثانوية (الجريبات قبل تكوين التجويف) ، إلا أن تلك العوامل تكون مثبتة لنمو جريبات كراف (الجريبات الحاوية على التجويف) من دون أن تكون أية جوانب سلبية على صورة الدم التي تناولتها الدراسة .

Introduction:

It was originally proposed in 1948 that follicular fluid (FF) was merely

a simple transudate of blood that accumulated between the layers of the granulosa cells in growing follicles (1) . Then it is became

known that FF contains a large variety of components of serum along with an array of secretions which are synthesized by the specialized cells of the follicular microenvironment, as a result, the components of FF change during growth and expansion of each follicle (2).

Follicular fluid obtained from mammalian ovaries influences various reproductive performances and endocrinological attributes when injected into farm animals (3 & 4). Steroid – stripped FF is rich in inhibin bioactivity (5) and has been used extensively, as a crude preparation of inhibin, in various *in vivo* and *in vitro* studies (6). Inhibin acts as a negative feedback regulator of FSH, which is a key determinant of follicle growth and development (7). Furthermore, an inverse relationship between the release of pituitary FSH and follicular inhibin has recently been demonstrated in goats (8).

Rich source of the ovarian hormone inhibin (9), which with estradiol, controls the concentration of the FSH in peripheral circulation (10). It showed that treatment of ewes with steroid – free ovine FF during the late follicular phase of the estrous cycle resulted suppression in the ovarian secretion of estradiol, androstendione, testosterone and inhibin.

Aim of study:

The present study was undertaken to evaluate the efficacy of crude and steroid – free BFF from on ovarian growth and function as well as some of hematological parameters of mature female Wistar rats.

Materials and Methods:

1- Collection and preparation of follicular fluid (FF)

(A) Follicular fluid was obtained by aspirating bovine follicles (17 – 25 mm in diameter) collected from local abattoir of AL-Najaf governorate.

(B) The collected follicular fluid were kept on ice until centrifugation at 8000 rpm for 15 minute at 4°C to remove cellular debris and stored at – 20°C (collection period extended for 2 months).

(C) After a sufficient amount of FF were collected to conduct the experiment, the FF thawed and pooled.

(D) Activated charcoal (10 mg/ml) was added to the FF and mixed for 1 hour at 4°C.

(E) Charcoal was removed from the FF by centrifugation at 14000 rpm for 90 minute at 4°C.

(F) Charcoal treated FF were frozen at – 20°C until used in the present experiment (12).

It is reported that 99% of the original steroids were removed by this technique (3).

2- Detection of proteins in follicular fluid which is done by Biuret assay (13).

3- The estimation of cholesterol in charcoal treated follicular fluid , as described by 14 .

4- Experimental animals :

Twenty four mature Wister albino females rate with a mean weight of 180 ± 20 gm . These animals obtained from the animal house at the College of medicine , Kufa University , and animal house at the College of veterinary medicine , Al-Basra University.

All rats were kept in alternating light and dark (12 : 12) . Rats chow and water ad libitum , and the protein ratio in feed was 19% and the energy 3000 kilo calorie (15) .

The rats were housed 3/cage under controlled condition for 2 months until their weight became 160 – 250 , vaginal smear made for 4 consecutive days and only the rats that exhibited 2 consecutive estrus cycles were included in the experiment.

5- Vaginal Smear :-

The purpose of making vaginal smear to determine the rats that have different histological changes during 4 sequential days , and only these rats included in the experiments.

6- Injection of chorcoal treated follicular fluid :-

The rats were randomly assigned into three groups , number of animals in each group (8) assigned as following :

A- Control (C) : injected intraperitoneally by 100 μ of normal saline.

B- Steroid free follicular fluid treated group (SFFF) : injected intraperitoneally by 100 μ of SFFF .

C- Crude follicular fluid treated group (CFF) : injected intraperitoneally by 100 μ of CFF .

Injection process continued for four consecutive days .

7- hematological and biochemical parameters , were measured by Beckman counter which is directly give the result , these parameters are hemoglobin concentration , packed cell volume , cholesterol , alinine transfersae and asparatate transferase , urea , and glucose concentration .

8- Histopathological protocol for uteri and ovaries , done by routine histopathological procedure which include , fixation and sectioning and hematoxylin and eosin staining , (16) .

Results:

1- Steroid – free follicular fluid

Gel filtration chromatography of the 20 – 40 % fraction of FF resulted in an elution peak other than but slightly similar to the void volume peak (Fig. 1) . he average yield of peptide in this fraction was 0.659 mg/ml of Graffian FF .

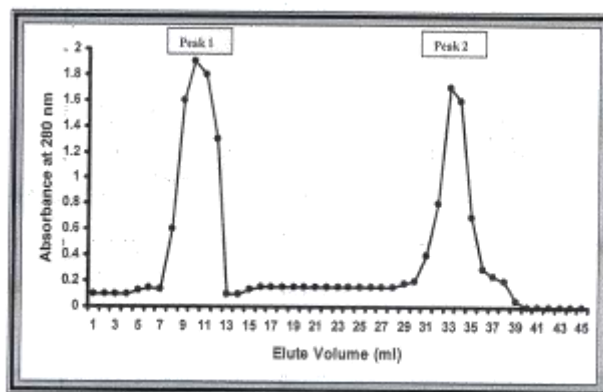


Figure 1 . Elution profile of Sephadex G-75 chromatographic separation of the fraction 20 – 40 % of FF from Graffian follicle . Peak 1 represents the void volume , and peak 2 represents the fraction of FF rich in ovarian peptide used in this study .

4.2. Uteri and ovarian weights

There was no effect of treatment with CFF and DFFF on the weight of ovaries and uteri (fig. 2 and 3) . However ovaries of CFF

treatment group tend to increase compared with other groups, but this increment didn't reach the significant level during the experimental period

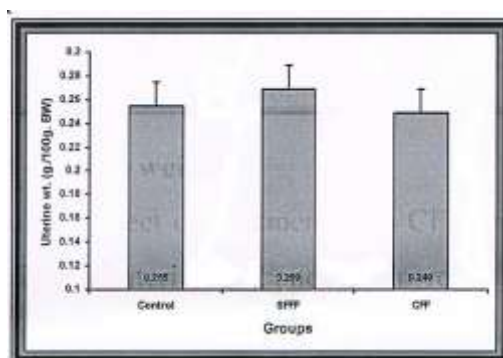


Figure 2. Effect of bovine CFF and SFFF on uterine weight (g./100g. BW) of mature female Wistar rats .

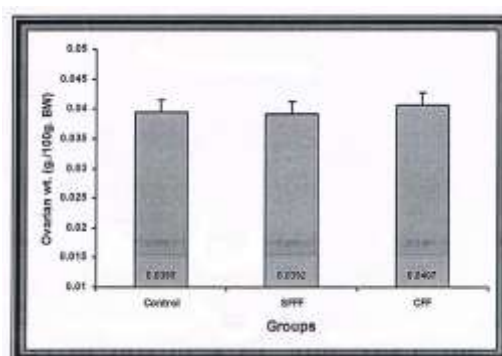


Figure 3. Effect of bovine CFF and SFFF on ovarian weight (g./100g. BW) of mature female Wistar rats .

4.3. Numbers of ovarian follicles

The data pertaining to mean values of the number of different types of ovarian follicles were depicted in table (1). A significant increase has been observed in primary and secondary follicles in CFF group compared with SFFF and control group . On the other hand SFFF group was significantly higher than control , while Graffian follicle number was higher in control followed by SFFF group compared

with CFF group . In comparison within each group , it was found that primary follicles recorded the highest number in the three groups followed by secondary follicles compared with the number of Graffian follicles .

Total number of the different types of ovarian follicles from CFF and SFFF groups recorded mean values significantly higher than that of control (figure 4).

Table 1. Effect of BCFF and SFFF on number of different types of ovarian follicles in mature female Wistar rats.

Type of follicle	Control	SFFF	CFF
Primary follicles	7.416 c A ± 0.273	14.334 b A ± 1.272	15.501 a A ± 1.522
Secondary follicles	5.418 c B ± 0.431	6.166 b B ± 0.260	6.625 a B ± 0.318
Graffian follicles	5.000 a C ± 0.167	3.709 b C ± 0.543	3.224 c C ± 0.246

- The numbers = mean ± standard error .
- The different capital letters = significant differences ($P \leq 0.05$) between groups .
- The different small letters = significant differences within the same group .

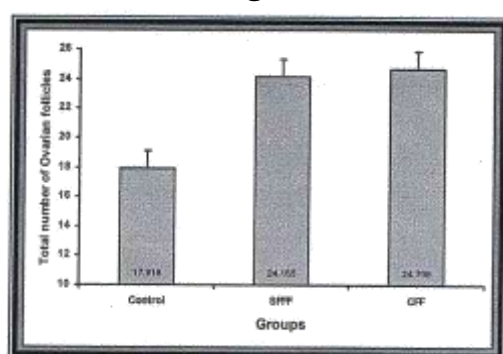


Figure 4. Effect of bovine CFF and SFFF on total number of ovarian follicles of mature female Wistar rats .

4.4. Histophysiological appearance of ovaries

Histological sections obtained from of CFF and SFFF groups showed higher number of primary

follicles and lower numbers of Graffian follicles compared with that of control , while secondary follicles were nearly similar among the experimental groups (figure 5.) .

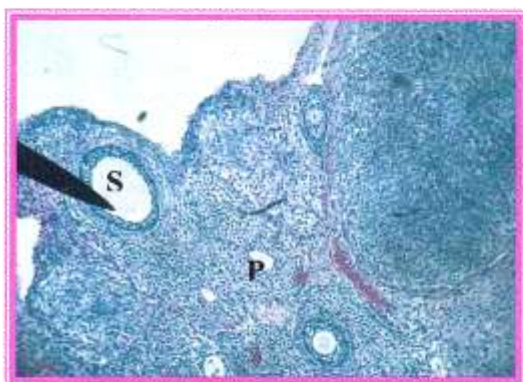
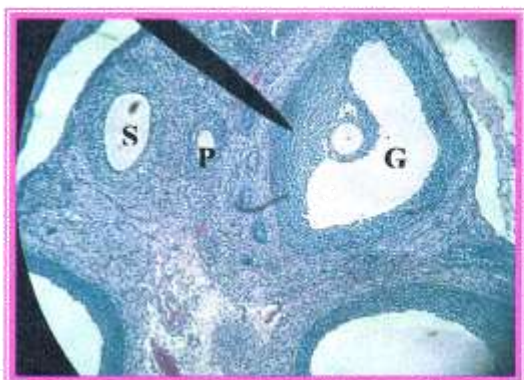


Figure 5. sections obtained from ovaries of C, SFFF and CFF groups shows the differences in the number of primary (P), secondary (S) and Graffian follicles (G). (H&E x100).



4.5. Hematological Parameters

Results demonstrated in figures (6 , 7 , 8) and table (2) revealed no significant differences in Packed cell volume (g/100 ml) , hemoglobin concentration (%) , total leukocytes count (N/ million) , glucose concentration (mg / 100 ml) , urea

concentration (mg / 100 ml) , cholesterol concentration (mg / 100 ml) as well as blood serum enzymes (aspartate aminotransferase ; AST and alann aminotransferase ; ALT) (mmol / 100 ml) among the three groups of the experiment .

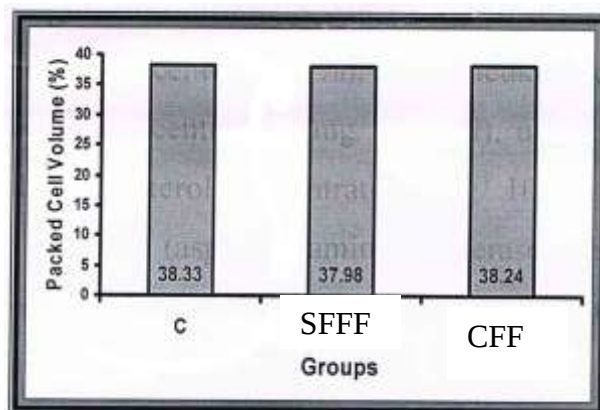


Figure 6. Effect of bovine CFF and SFFF on packed cell volume (%) of mature female Wistar rats .

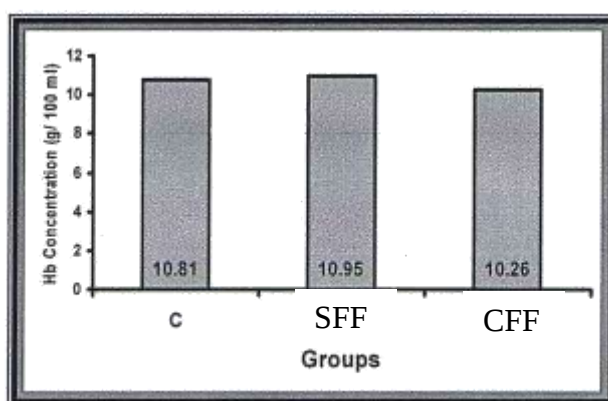


Figure 7. Effect of bovine CFF and SFFF on hemoglobin concentration (g/100 ml) of mature female Wistar rats.

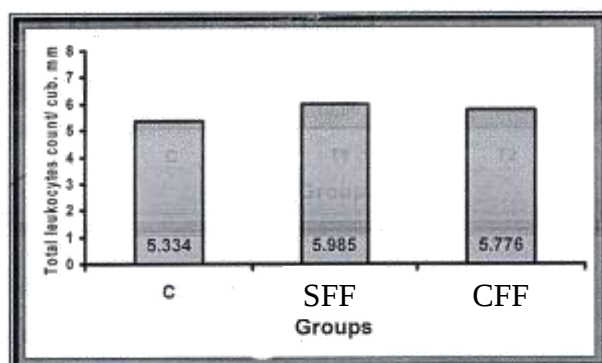


Figure 8. Effect of bovine CFF and SFFF on total leukocytes count (per cubic millimeter) of mature female Wistar rats .

Table (2) : Effect of bovine CFF and SFFF on some serum biochemical constituents of mature female Wistar rats .

Groups	C	SFFF	CFF
Glucose (mg / 100 ml)	122.45 ± 8.28	128.83 ± 10.45	127.22 ± 8.88
Urea (g/ 100 ml)	5.896 ± 0.345	6.162 ± 0.582	6.072 ± 0.484
Cholesterol (mg / 100 ml)	2.934 ± 0.022	2.383 ± 0.028	2.675 ± 0.031
AST (mmol / 100 ml)	78.66 ± 8.77	70.48 ± 6.34	75.52 ± 7.56
ALT (mmol / 100 ml)	45.73 ± 1.87	42.39 ± 1.67	43.88 ± 1.94

- There is no significant differences between all groups .

Discussion:

The present study demonstrated that CFF and SFFF did not affect follicular maturation which led to decrease of antral (Graffian) follicles numbers , but on the other hand , preantral (primary) follicles increased dramatically in SFFF treated group , and these opposite results reflected on ovaries which recorded no significant differences in their weight compared with other groups.

Inhibin , found in follicles of various sizes and physiological states (17) , has been shown to be a powerful inhibitor of FSH secretion (18) and is likely involved in FSH suppression (19) ; however , the isoform of inhibin responsible for the FSH suppression (20) has not

been fully elucidated and seems to be confounded with the effects of estradiol (21) in growing and regressing follicles (22) .

On the other hand , estradiol has been shown to be effective in suppressing FSH concentrations either alone or synergistically with inhibin (18) . The synergistic FSH – suppressive effects of estradiol and immunoreactive inhibin have also been shown to be associated with early follicular development , as both substances increased in the follicular fluid . There was a dramatic increase in the intrafollicular estradiol concentrations on the day after the beginning of follicle deviation , suggesting that estradiol from the

dominant follicle may not be produced in sufficient quantities to suppress systemic FSH until approximately the time of deviation (23). In uteri weights were also not affected by treatment with CFF and SFFF, which may be attributed to the decrement in estrogens secretion from ovarian follicles, particularly it has been shown that antral (large especially Graafian) follicles were decreased in number. These follicles were considered the main source of estrogens during the oestrus cycle. On the other hand, estrogens acts on the uterus to increase the mass of both endometrium and myometrium by increasing the rate of hyperplasia and hypertrophy (24).

The significant decrement of antral (Graafian) follicles in SFFF group may be attributed to the inhibitory effect of TGF – B (mainly inhibins) on FSH secretion from adenohypophysis (25 & 26), particularly FSH has shown to play a role in follicle cells proliferation along with the development of the basement membrane, however, FSH and LH together promote the formation of the antrum of each follicle, in addition to helping enable the theca interna cells to become vascularized during development (27).

Crude FF and SFFF have no inhibitory effect on the growth of preantral (primary and secondary) follicles. It has been reported that the growth of preantral follicles to be gonadotropin independent; however, FSH was shown to promote preantral

follicle growth *in vitro* in buffalo (28). The CFF and SFFF did show significant effect on preantral follicles growth, especially primary follicles, unlike the effect on Graafian follicles growth. Factors responsible for stimulation and inhibition of cumulus cell expansion and oocytes maturation *in vitro* have been isolated from FF (29). Follicle – enclosed oocytes did not resume meiosis either *in vivo* or *in vitro* in the absence of appropriate signals. This suggested that the presence of inhibitory agent(s) within the antral follicle is responsible for maintaining the oocyte in the germinal vesicle (GV) stage. It might be possible that the CFF and SFFF containing peptides inhibited binding of FSH to receptors on cumulus cells, thereby reducing the stimulatory action of FSH on cumulus – oocyte complex maturation.

On the other hand, the present study revealed no significant differences in some of hematological and biochemical constituents, which, in part, indicated that using follicular fluid in a crude or steroid free status was, safe and have no side effects on general health of the experimental animals, otherwise, blood picture reflect the health status of the animals.

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