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SOME STUDIES ON THE HISTO-MORPHOLOGICAL CHARACTERISTICS AND BIOCHEMICAL CHANGES OF CORPORA LUTEA IN BUFFALOES (BOS BUBALIS) DURING ESTROUS CYCLE AND EARLY PREGNANCY

(With 5 Tables and 9 Figures)

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بعض الدراسات على الخصائص الهستومورفولوجية والتغيرات البيوكميائية للجسم الأصفر في الجاموس أثناء دورة الشبق و فترة الحمل المبكر

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هدفت هذه الدراسة إلى بحث الشكل الهستومورفولوجى وبعض التغيرات البيوكميائية للجسم الأصفر وعلاقتها بنشاطه الوظيفي أثناء دورة الشبق وفترة الحمل المبكر في الجاموس. لهذا الغرض فقد جمع عدد (٥٢) جسماً أصفراً من إناث الجاموس، منها عدد (٤٤) أثناء دورة الشبق وعدد (٨) أثناء فترة الحمل المبكر. وقد أعدت هذه الأجسام للفحص بالميكروسكوب الضوئى وبالميكروسكوب الالكترونى النافذ وكذلك للتحليل البيوكميائى؛ وقد أخذت عينات من الدم قبل ذبح الحيوانات مباشرة لقياس مستوى البروجيسترون (P_4) في البلازما. وقد أوضحت نتائج هذه الدراسة أن هناك نوعين من الخلايا الصفراء وأن الخصائص الوظيفية الواضحة كانت مرتبطة بدرجة كبيرة بالخلايا الصفراء الكبيرة عن الخلايا الصفراء الصغيرة ، وقد وجدت هناك علاقة بين متوسط مساحة السطح للمكونات الصفراء والتركيب الدقيق للخلايا الصفراء ومستوى البروجيستيرون في الدم عند الأطوار المختلفة للجسم الأصفر في أثناء دورة الشبق وفترة الحمل المبكر. وقد وصلت نسبة المساحة الكلية التى تشغلها الخلايا الصفراء والعناصر الوعائية الدموية إلى أعلى معدل لها عند بداية الفترة الوسطى من دورة الشبق وفترة الحمل المبكر، وتناسب مستوى البروجيستيرون إيجابيا في البلازما مع مساحة السطح للعناصر الوعائية الدموية والخلايا الصفراء أثناء الفترات المختلفة لدورة الشبق، وكذلك أثناء الفترة المبكرة من

الحمل، وقد لوحظت العلاقة المعنوية بين مستوى البروجيستيرون مع العناصر الوعائية أثناء الفترة الأخيرة من دورة الشبق، كما تناسب أيضا مستوى البروجيستيرون مع الخلايا الصفراء أثناء الفترة الوسطى من دورة الشبق، وكذلك الفترة المبكرة من الحمل. وسجلت هذه الدراسة ولأول مرة وجود أجسام داخل الميتوكوندريا (IMBs) في الجسم الأصفر في الجاموس، كما سجلت هذه الدراسة أيضا أن تفكك أو انحلال الجسم الأصفر يتميز بظهور بعض التغيرات التى تشمل: تكسير في الخلايا المبطنة للأوعية والشعيرات الدموية - تثبيط إنتاج البروجيستيرون - وتراكم حبيبات الدهون في الخلايا الصفراء. وقد أدى تراكم حبيبات الدهون في الخلايا الصفراء إلى زيادة تصاعدية في المحتوى الكلى للدهون في المخلوط المتجانس للجسم الأصفر، ووصل أعلى معدل له أثناء الفترة الأخيرة من دورة الشبق ، وفي مقابل ذلك أوضحت القياسات أن المحتوى الكلى للدهون أثناء فترة الحمل كان متقارباً جداً من المستوى الذي وُجد أثناء الفترة المبكرة للطور الأوسط من دورة الشبق. وأوضحت الدراسة أن التغيرات التى تحدث في إنتاج بيروكسيد الدهون (LPO) وأكسيد النيتريك (NO) ونشاط إنزيم سوبر أكسيد الدسميوتيز (SOD) تلعب دوراً هاماً ومعنوياً في تنظيم وظيفة الجسم الأصفر؛ حيث إن إنتاج أكسيد النيتريك أثبت وجود علاقة معنوية مع مساحة السطح الخاصة بالأوعية الدموية في الجسم الأصفر أثناء المرحلة الأخيرة من دورة الشبق، وكذلك فترة الحمل المبكر، ولكن العلاقة المعنوية القوية قد سُجلت أثناء الفترة الأخيرة من دورة الشبق. وقد إحتوى المخلوط المتجانس للجسم الأصفر على زيادة تدريجية من بيروكسيد الدهون (LPO) أثنا نمو الجسم الأصفر من بداية دورة الشبق إلى نهايتها ، وقد كانت هذه الزيادة المعنوية قوية أثناء الطور الأخير من دورة الشبق . وكان نشاط إنزيم سوبر أكسيد الدسميوتيز(SOD) عاليا ومعنويا أثناء الطور الأوسط من دورة الشبق وسُجّل أعلى معدل لنشاط هذا الإنزيم أثناء فترة الحمل المبكر، وكان شبيها للمعدل الذي وُجد أثناء بداية الطور الأوسط لدورة الشبق، ولوحظ أن أقل مستوى نشاط لهذا الإنزيم قد سُجل أثناء الطور الأخير من دورة الشبق. وقد خلصت هذه الدراسة إلى أن هناك عدداً من التغيرات المورفولوجية والكميائية التى تحدث أثناء كل من: (نمو الجسم الأصفر – حياته الوظيفيه – اضمحلاله) في الجاموس، ولهذه التغيرات أهمية كبيرة لإدراك الطريقة التى تنظم الوظيفة الطبيعية للجسم الأصفر، وقد يساعد هذا بدوره على تحسين الكفاءة الإنتاجية للجاموس.

SUMMARY

The present study aimed to investigate the histo-morphological appearance and some biochemical changes of corpus luteum (CL) in relation to its functional activity during the estrous cycle and early pregnancy in buffalo-cows. Fifty two corpora lutea were collected from cyclic (44) and early-pregnant (8) buffalo-cows. The corpora lutea were prepared for light microscopy, transmission electron microscopy and for biochemical analysis. Blood samples were collected directly before slaughtering for estimation of serum progesterone (P_4) level. The obtained results indicated the presence of two morphologically distinct steroidogenic cells. Distinct functional properties were associated with the large luteal cells (LLC) than the small (SLC) ones. There was a relationship between the average surface area of luteal component, the

fine structure of luteal cells and serum (P_4) concentration at different luteal phases and at early pregnancy. The total % of surface area of the streroidogenic cells and the vascular elements reached the maximum at early mid-luteal phase and early pregnancy. The serum (P_4) level was positively correlated with the surface area of the vascular components and steroidogenic cells during different luteal phases and early pregnancy, but the significant correlations ($p< 0.05$) were observed at late-mid luteal phase for vascular elements and at mid-luteal phases and early pregnancy for steroidogenic cells. The present study demonstrated, for the first time, the presense of intramitochondrial bodies (IMBs) in buffalo (CL). Luteal degeneration was characterized by destructive changes in the endothelial lining of the blood vessels and capillaries, inhibition of (P_4) production and accumulation of lipid droplets in the luteal cells. The latter was reflected by progressive increase in the total lipid content of corpus luteum periodicum (CLp) homogenate that reached higher level at late luteal phase. On the contrary, the average total lipid content of corpus luteum graviditatis (CLg) remained very close to that found at early mid-luteal phase. Changes in lipid peroxide (LPO) and nitric oxide (NO) production, and superoxide dismutase (SOD) activity, played a significant role in regulation of (CL) function. Whereas, (NO) production showed significant correlation with the surface area of vascular components of (CLp) at late luteal phase and (CLg) at early pregnancy, but a highly significant correlation was noticed at late mid luteal phase. The (LPO) content was gradually increased in (CLp) homogenate during development and it was significantly ($p< 0.05$) higher at late luteal phase. In the (CLg) the (LPO) content was generally lower than in the (CLp) at mid and late luteal phases. The (SOD) activity was significantly ($p< 0.05$) higher during mid luteal phase and the lowest (SOD) activity was observed at early and late luteal phases. However, highest (SOD) activity was noticed at early pregnancy and was similar to that observed during early mid luteal phase.

Key words:

ABREVIATIONS: CL: Corpus luteum, P_4: Progesteron, LLC: Large luteal cell, SLC: Small luteal cell, IMBs: Intramitochondrial bodies, CLp: Corpus luteum periodicum, CLg : Corpus luteum, graviditatis, LPO: Lipid peroxide, NO: Nitric oxide, SOD: Superoxide dismutase.

INTRODUCTION

The role of corpus luteum (CL) in providing progesterone (P_4) during luteal phase is a transient requirement ending within few days in bovine species, unless pregnancy ensues (Niswender *et al.*, 1994). The events of this brief time are complex and closely spaced. The wall of the ovarian follicle consists of granulosa and theca cells, which undergo vascularization and luteinization after ovulation to form corpus luteum (Hansel *et al.*, 1991 and Wiltbank and Niswender, 1992). At luteolysis, these processes become reversed, beginning with disconstruction of the vascular system (Modlich *et al.*, 1996), and degeneration of the luteal cells (Dhiarmarajan *et al.*, 1994).

This temporary steroid-producing gland undergoes marked structural, biochemical and functional changes during development, functional life and regression, and has been the subject of extensive morphological studies (Wiltbank, 1994; Fields and Fields, 1996; Liebermann *et al.*, 1996 and Singh *et al.*, 1997). Most of the recent investigations on the corpus luteum have been focused separately on: 1) Cell types, ultrastructure, and luteal changes during development and regression (Hansel *et al.*, 1991 and Garcia-Iglesias *et al.*1992). 2) Effects of local growth factors on the secretary function (Liebermann et al., 1996). 3) The role of oxygen free radicals (Superoxide, Nitric oxide and hydroxyl radicals) in the processes of luteal regression (Sawada and Carlson, 1994 and Shimamura *et al.*, 1995). 4) The changes in the levels of antioxidants in the ovary in relation to regulation of the luteal functions (Aten *et al.*, 1992 and EL-Din Zain and Omar, 1998). little is known about the aforementioned intricate relationships, therefore, the present study was conducted to clearify the relationship between histo-morphological appearance, morphometry, some biochemical changes, and serum (P_4) levels in buffalo corpus luteum during estrous cycle and early pregnancy.

MATERIAL and METHODS

Animals and specimens collection:

A total of 52 buffaloes (5-8 years old) of unknown reproductive history were used in this study. Just before slaughtering at a local abattoirs in Assiut, peripheral blood samples for determination of (P_4) level were collected via the jugular vein puncture and transfered directly to the laboratory. Immediately, after slaughtering and evisceration, the genital tract of each animal was excised and the reproductive state was

recorded [non-pregnant (n = 44) and early-pregnant about 45 days (n = 8)], then each pair of ovaries was collected and examined macroscopically.

The stage of the estrous cycle was defined from the gross observations of the follicles and corpora lutea according to Ireland *et al.* (1980) into: 1) Early luteal phase (n = 10), from the 1st to 4th day after ovulation and in which the CLp appeared red externally and filled with cloted blood internally, its diameter ranged from 0.5-1.5 cm. The follicles were absent or with diameter less than 10 mm. 2) Early mid-luteal phase (n = 12)), from the 5th to 10th day after ovulation, in which CLp appeared pale red and only the point of ovulation was red or brown externally, but internally it was orange in colour. The average diameter of the CLp at this phase was about 1.6-2 cm, and the folicles were larger than 10 mm in diameter. 3) Late mid-luteal phase (n = 11), from day 11th to day 17th after ovulation, in which CLp appeared tan to orange in colour either externally or internally, with an average diameter of 1.6-2 cm. Follicles with diameter less than 10 mm may be present or absent but growing follicles (4-8) mm in diameter were existed. 4) Late luteal phase (n = 11), after day 17 of ovulation. In this phase CLp appeared light yellow externally and orange to yellow internally with diameter less than 1 cm. The follicles were present with diameter more than 10 mm. The corpora lutea were excised from the ovaries (non-pregnant or pregnant), cleaned from the adhering tissues and bisected along the basal-apical axis into two parts. The first parts were transported within 1-2 h to the laboratory in a container containing ice bags (4°C) for biochemical analysis. The second parts of the corpora lutea were fixed for histological examination.

Preparation of tissues for histological examination:-

For light and transmission electron microscopy, immersion-fixation was performed by using 3 % glutaraldehyde in 0.1M phosphate buffer (pH 7.3). After osmication in OsO_4 for 2 h, the specimens were washed several times in phosphate buffer, then dehydrated in graded ethanol and embedded in Araldit 502 (Luft, 1961). Semithin sections were made at about 1µm thick by using a Reichert OmU2 microtome and stained with toluidine blue. The sections were examined by using a Zeiss Axiophot Microscope.

Corpora lutea from 5 animals were selected for each stage to estimate the cell number, diameter and surface area of the steroidogenic component (small and large luteal cells) and non-steroidogenic component (vascular and connective tissue elements). The measurments

were done using the image analysis program (Analysis 2.0, SIS, Munster, Germany). These measurements were taken in the parenchyma of (CL) and completely away from the connective tissue capsule. Tissue specimens for transmission electron microscopy were selected from the well fixed and prepared Araldit-embedded materials as described above. These specimens were sectioned by a Reichert Ultracut microtome (Leica) at 70 μ about using a diamond knife. The sections were contrast-stained with uranyl acetate and lead citrate, examined and photographed by a Zeiss transmission electron microscope (EM 109).

Preparation of samples for biochemical analysis :-

A suitable portion of the first bisected surface (10 % w/v) was homogenized in ice cold 0.1 M phosphate buffer solution adjusted to pH 7.4 in a glass homogenizer. A portion of homogenate was divided into two aliquots, the first aliquot used for measuring lipid peroxide (LPO) and the second used for determination of total lipid and cholesterol. The another portion of homogenate was centrifuged at 8000 r.p.m for 20 min and the supernatant (cytosol) was collected and used for determination of nitric oxide (NO) and superoxide dismutase (SOD).

Protein concentrations in the homogenate and cytosol were determined by the method of Lowry *et al.* (1951) using a commercial chemicals (Sigma Chemical Co.). Lipid peroxide in corpora lutea was assessed by the thiobarbituric reaction, which measures the secondary product of peroxidation, malondialdehyde (MDA) (Ohkawa *et al.*, 1979). The amount of (LPO) was expressed as nmol MDA/mg protein. Nitric oxide activity was measured using gires reagent with sodium nitrite as a standard according to Ding et al. (1988) and the activity was expressed as nmol/mg protein. Total (SOD) activity in the (CL) was measured by using the method described by Misra and Fridovich (1972) and total activity was expressed as ng/mg protein. Lipid was extracted according to the method described by Bligh and Dyer (1939). The total lipids was determined according to the method of Knight *et al.* (1972) and cholesterol content was measured using Cholesterol Kit (Diamond Diagnostic, Egypt). The amount of total lipids and cholesterol were expressed as mg/g wet tissue weight.

Hormonal assay:-

The blood samples kept overnight and centrifuged at 3000 r.p.m for 20 minutes. The serum was removed and stored at - 20°C untill assay. Serum P_4 concentration was determined by RIA method (Coat A-Count progesterone, Diagnostic Products Co. Los Angeles, U.S.A).

Statistical Analysis:

Morphometric data, biochemical constituents of corpora lutea and serum P_4 concentration were expressed as means ± S.E. The data were analyzed statistically by analysis of variance using general linear model (GLM) of statistical analysis system (SAS, 1985). Comparison between mean of variables at different luteal phases and during early pregnancy were carried out by using the new Duncan's multiple range test. Differences were considered to be significant if $p< 0.05$. Pearson correlation coefficient was used to determined the relationship between morphometric characteristics, some biochemical constituents and functional activity of corpora lutea periodicum and graviditatis.

RESULTS

Histo-morphological appearance of corpora lutea:

The corpus luteum periodicum or graviditatis of buffaloes was enclosed in a connective tissue capsule which seperated it from the adjoining ovarian tissues. Connective tissue septa were extended from the capsule to divide the gland into lobes. These septa carried large vessels to vascularize the parenchyma. A central cavity in the (CL) could be seen in most of the studied specimens but the size and content were variable. By light microscopy, there were two morphologically distinct steroidogenic cells (small and large). The two cell types showed an increase in the average diameter as the cycle progresses. Lipid droplets were gradually increased in the cyclic (CL) from early to late phase as well as the color of these droplets was greatly changed from light yellow at early luteal phase into dark yellow or even brown at late luteal one.

I) *Corpus luteum periodicum ((CLp)*

a) Early luteal phase (1^{st} –4^{th} day after ovulation) :-

The small luteal cells (SLC) appeared round to oval in shape with relatively small diameter 11.8 ± 4.1 μm (average). Each cell occupied an average surface area of 147.2 ± 68.1 μm^2 (Table 1). These cells were characterized by relatively large spherical nuclei with centrally located nucleoli and dense heterochromatin pattern against the nuclear membrane (Fig. 1). The cytoplasm of (SLC) was thin and contained few round mitochondria, some strands of rough endoplasmic reticulum, few secretary granules and few lipid droplets (Fig. 1). Moreover, these cells

represented 18.8 % from the total surface area of corpus luteum (Table 1).

The large luteal cells (LLC) were polygonal in shape with large vesicular centrally located nuclei. These cells were characterized by a relatively large size, 28.8 ± 5.3 μm in diameter and each cell occupied an average surface area of 407.9 ± 68.1 μm^2. These cells contained enormous amount of mitochondria, with tubular or/and lamellar cristae, considerable amount of smooth endoplasmic reticulum and secretory granules. However, lipid droplets were found in a little amount (Fig. 2). The blood capillaries were typically appeared having relatively thick endothelial lining with numerous cytoplasmic processes which extended into narrow capillary lumen (Fig. 3). Apoptotic cells were observed but in few number.

b) Early mid-luteal phase (5^{th} –10^{th} day after ovulation):

At this phase the number of (SLC) was reduced and this reflected by decrease in the average surface area occupied by these cells (12.6 %) (Table 1). The average diameter of (SLC) was non-significantly increased reaching 12.9 ± 3.90 μm. However, the general fine structure of these cells was not changed. The (LLC) appeared larger in size than before and their cytoplasm contained abundant mitochondria, free ribosomes, developed Golgi, smooth endoplasmic reticulum and tremendous amount of secretory granules. Moreover, intra-mitochondrial bodies (IMBs) with irregular outlines could be observed in the mitochondria of these cells (Fig. 4, 5). Generally, there was a considerable increase in the average diameter and surface area of the large luteal cells (Table 1). Blood capillaries with typical structure were expressed in large number than in the early luteal phase. Pre-capillary vessels with typical intact walls (endothelium and smooth muscle cells) and surrounded by pericytes were also demonstrated (Fig. 6).

c) Late mid-luteal phase (11^{th} –17^{th} day after ovulation):

The (SLC) appeared as described before and were found in a ratio (13.05 %) similar to that observed at early mid-luteal phase. The average diameter of these cells showed a little increase reaching 14.2 ± 4.1 μm. The average surface area of (SLC) was also increased to 150.9 ± 49.2 μm^2 (Table 1). The (LLC) were structurally similar to those of the early mid-luteal phase except for increase number of mitochondria which contained large IMBs (Fig. 5), as well as the amount of endoplasmic reticulum, secretory granules and lipid droplets. Most of the mitochondria which contained IMBs were dilated and showed some destrective changes in the mitochondrial cristae (Fig. 5). There was an

extensive folding of the plasma membranes of both small and large luteal cells as well as increase the intercellular distances (Fig. 7).

d) Late luteal phase (after day 17 of ovulation):-

At this phase, the changes were confined mainly on the structure of the (LLC) whereas their cytoplasm was totally packed with lipid droplets of different sizes (Fig. 8). These cells contained also numerous mitochondria, but few smooth endoplasmic reticulum, few secretory granules and few short strands of rough endoplasmic reticulum (Fig. 8). The average diameter of (LLC) and (SLC) was 28.49 ± 7.28 and 10.24 ± 2.50 μm, and the average surface area was about 492.61 ± 143.33 and 130.67 ± 40.76 μm^2, respectively (Table 1). However, the total surface area occupied by the (SLC) and (LLC) was greately reduced to 4.79 % and 55.52 % respectively. The vascular elements represented 3.10 % and the connective tissue matrix and other cells occupied 33.6 % from the total surface area of (CL) (Table 1). At this stage apoptotic cells were seen in large number, and the walls of most small blood vessels (arterioles, venules and capillaries) showed destructive changes (Fig. 9).

II) *Corpus luteum graviditatis (CLg)*:

At early pregnancy (about 45 days) stage, the (SLC) were few in number, representing only 6.6% from total surface area of (CL). These cells were similar in their structure and average diameter (12.9 ± 4.0 μm) to those of the early mid-luteal phase (Table 1). The (LLC) appeared also similar in their structure to the (LLC) of the early mid-luteal phase, but at this stage they contained less amount of lipid droplets and more secretory granules (Fig. 10). However, these cells were relatively large in size, having an average diameter of 40.3 ± 11.0 μm and occupied a large surface area (74.5 %) of (CL) (Table 1). The blood vessels and capillaries were similar to that described during mid-luteal phase, except for absence of the pericytes. The outlines of blood capillaries were clearly demarcated and separated by narrow distances from the neighboring luteal cells (Fig. 11).

Morphometric analysis of corpora lutea:

The mean diameter and surface area of (SLC) and (LLC) during different luteal phases and early pregnancy are presented in table 1. The mean % of the surface area occupied by (SLC) was gradually decreased ($p< 0.05$) during its development and functional life, reaching lowest level at late luteal phase. The average % of surface area (6.60 ± 0.95) occupied by these cells during early pregnancy was lower ($p< 0.05$) than during early and mid luteal phases (Table 1). However, the mean % of

surface area occupied by (LLC) was progressively increased ($p< 0.05$) reaching the maximum at mid luteal phase, then decreased again during late luteal phase (Table 1). The maximum % of surface area of the (LLC) reached the highest point ($p< 0.05$) at early pregnancy stage (Table 1).

The total % of the surface area of different components of the corpora lutea (either (CLp) or (CLg) are shown in table 2. The total % of the surface area occupied by streroidogenic cells (SLC) & (LLC) as well as that of the vascular elements reaching the maximum at early mid-luteal phase and early pregnancy, when compared with other luteal phases. These differences were statistically significant ($p< 0.05$).

Biochemical constituents of corpora lutea:

The mean levels of some biochemical constituents of (CL) homogenate (CLp) & (CLg) are presented in table 3. The total lipid content of (CL) homogenate increased ($p< 0.05$) progressively during its development and functional life, reaching its higher level (23.86 ± 0.53 mg/g wet tissue) at late luteal phase. However, the average total lipid content of (CLg) (14.87 ± 0.63 mg/g wet tissue) remained very close to that found at early mid-luteal phase. This was significantly ($p< 0.05$) lower than that found at late mid-luteal and late luteal phases. Moreover, this average was still higher ($p< 0.05$) than the average at early luteal and similar to that observed at mid-luteal phase (Table 3). The cholesterol content in the (CL) homogenate (CLp) was high ($p< 0.05$) during mid-luteal phase (both early and late) than during early and late luteal phase. The cholesterol content of (CLg) was generally higher (4.38 ± 0.42 mg/g wet tissue) than those found at different luteal phases of (CLp) (Table 3).

The (LPO) content was gradually increased in (CLp) homogenate during its development and functional life. This increament was significantly ($p< 0.05$) higher at late luteal phase than other phases (Table 3). However, the (LPO) content of (CLg) was generally lower (2.90 ± 0.44 nmol/mg) than those found at mid and late luteal phases. There was a gradual decrease in (NO) production in (CLp) as the cycle progress. This decreament was significantly ($p< 0.05$) lower (1.29 ± 0.23 nmol/mg) at late luteal phase than other luteal phases. In addition, the (NO) production in (CLg) was similar to that found during mid luteal phase of (CLp) (Table 3). The (SOD) activity in (CL) homogenate was significantly ($p< 0.05$) higher during mid luteal phase than in other luteal phases. The lowest (SOD) activities were found during early and late

luteal phases. Moreover, the (SOD) activity was highest (4.71 ± 0.27 ng/ml) during early pregnancy and was similar to that observed (4.60 ± o.21 ng/ml) during early mid luteal phase (Table 3).

Correlation between functional activity and other characteristics of corpora lutea:

The (NO) production showed positive correlation with the surface area of vascular components of (CLp) or (CLg). These correlations (r = 0.97; 0.91 and 0.93) were highly significant ($p< 0.01$) at late mid-luteal phase and significant ($p< 0.05$) at late luteal phase and during early pregnancy respectively (Table 4). Although, (NO) production showed positive correlation with serum (P_4) level at different luteal phases and at early pregnancy, it was only significant ($p< 0.05$, r = 0.97)) at late luteal phase (Table 5).

The serum (P_4) level was positively correlated with the surface area of vascular components and steroidogenic cells during different luteal phases and at early pregnancy (Tables 4 & 5). The significant correlations ($p< 0.05$) were observed at late-mid luteal phase for vascular elements (r = 0.87) and for steroidogenic cells (r = 0.91; 0.93 and 0.88) at mid-luteal phases and at early pregnancy, respectively.

DISCUSSION

The (CL) is known for its responsibility for (P_4) production and maintenance of early pregnancy in mammals. Ovulation of ovarian follicle leads to development of (CL) and secretion of (P_4). The secretion rate of (P_4) is dependent on the efficient and active production of such hormone within the steroidogenic cells of (CL) (Fields and Fields, 1996). Several investigations, therefore, have been done to characterize the cellular components of (CL) in different animal species and human.

The growth of (LLC) is associated with a marked increase in the metabolic functions which are characterized by increased activity of the mitochondrial enzymes (Richards and Almond, 1994) to meet the energy requirements for (P_4) production (Niswender *et al.* 1994). This support the current study, whereas the population of the mitochondria in the (LLC) was increased during mid-luteal phase of buffalo (CL). This could be correlated with the expected requirements for greater amount of cytochrome P450scc to accompany the increased (P_4) production at this phase. Regarding to the functional activity of (SOD) and (NO) production, the present data confirmed the previous results of El-Din Zain and Omar (1998). This is because there was a dependent

relationship between (SOD) activity, (LPO) production and serum (P_4) level at different luteal phases. The significant decline in (SOD) activity was matched by fall in serum (P_4) level and (NO) production as well as increase in (LPO) production at late luteal phase. Anthony *et al.* (1997) and Boiti *et al.* (2000) found that catalytic activity of nitric oxide synthase (NOS) was increased during regression stage of (CL) and they are suggested that NO/NOS system exert a regulatory role on steroidogenesis. This support the finding of the present study because (NO) production was greatly reduced at late luteal phases.

Oxygen free radicals (e.g. superoxide radical) created during mitochondrial electron-transported system and cytochrome P450scc oxidation, may be cause damage and decrease steroidogenesis if not detoxified by (SOD) and other scavenges (Weiss, 1980). Previous studies by Yamada *et al.* (1994) in cattle and Luthman (1971) in sheep reported the presence of small dense, uniform and non-structural bodies in the mitochondria of (LLC) named (IMBs). The present study demonstrated, for the first time, presence of (IMBs) in buffalo (CL). These bodies had different shapes and sizes and their outer surfaces were mostly course, they caused enlargement or dilatation of the mitochondria and destruction of the mitochondrial cristae. The (IMBs) are proteinaceous substance and may be represent pooling of enzymatic material resulting from dysfunction of the mitochondria (Walters *et al.*, 1984a; Fields and Fields, 1996). This occurred as a result of chemically cross linked of cytochrome P450scc to its electron donor adrenodoxin (Rodgers *et al.*, 1995). In the present study the (IMBs) demonstrated only in the (LLC) and at the late luteal phase of (CLp), where the (P_4) level was gradually decreased (minimum level). This agree with the previous study of Fields and Fields (1996) because they observed these bodies in the (LLC) on day 14 of estrous cycle, and after day 200 of pregnancy in the cow. During these phases (LH) level was very low (Little *et al.*, 1982 and Walters *et al.*, 1984b).

Steroidogenesis requires coordination of the anabolic and catabolic pathways of lipid metabolism (Viergutz *et al.*, 2000). The present study revealed that the cholesterol content and (P_4) level decreased towards the end of luteal phase. The main factors that reduces the (P_4) synthesis were determined by Yoshida *et al.* (1999). The latter authors, reported a marked increase in the level of $P450_{C26}$ (enzyme that catalyze 26 hydroxylation of cholesterol) between day 6 and 16 of pseudo-pregnant rat. The 26 hydroxy cholesterol blocks the endogenous cholesterol synthesis (Rennert *et al.*, 1990), leading to accumulation of

fat inside luteal cells and reduces the releases of (P_4) into the circulation as observed in the present study.

There is evidence that (LLC) produce over 80% of (P_4) secreted by (CL) during mid-luteal phase independent on LH stimulation (O'shea *et al.*, 1989 and Niswender *et al.*, 1994). Thi is in accordance with our findings because serum (P_4) level was positively correlated with the surface area of steroidogenic cells and the (LLC) were expressed in large ratio than the small ones during mid-luteal phase. Moreover, Mclean *et al.* (1992) mentioned that the (LLC) contains more growth hormone receptors, cytochrome P450scc and sterol carrier protein-2 suggesting a higher rate of cholesterol transport and conversion to pregnanolone than in (SLC). This may be reflect the importance of the (LLC) in (P_4) secretion in buffalo (CL) and the minimum role of (SLC) to produce the same hormone. In accordance with EL-Din Zain and Omar (1998) the present study demonstrated an increase in total lipid content in the regressing buffalo (CL) than in the developing or fully developed ones. The (LLC) have a higher capacity to convert cholesterol ester to steroid hormone (Wiltbank, 1994) and presence of lipid droplets in the (LLC) is a sign of luteal regression (Fields and Fields, 1996). This is support our findings because lipid droplets were seen in a moderate extent in the (LLC) of buffalo (CL) during developing stages and early pregnancy. These droplets were greatly increased in the amount and size during late luteal phase. On the contrary, lipid droplets in the (SLC) were very few and did not changed greatly at different luteal phases of the present study, such finding is in accordance with Weber *et al.* (1987) who mentioned that lipid droplets in the (SLC) are constant with lesser capacity to secrete (P_4) except in response to (LH).

Angiogenesis in the ovary is cycle-regulated process and this is necessary requirement for proper ovarian function (Fraser *et al.*, 2000). The present study found a great variation in the average surface area of the vascular elements which reached the maximum at mid-luteal phase and was positively correlated with (P_4) level at this phase. This is agree with Singh *et al.* (1997) and Fraser et al. (2000) who reported that the microvascular tree and functional activity of (CL) are fully established at mid-luteal phase. Snyder and Bredt, (1992) mentioned that (NO) stimulate angiogenesis in the (CL) and this is in accordance with the results of the present study because (NO) production was at higher levels in the developing, fully developed cyclic corpora lutea and those of early pregnancy than in the regressed ones. The current study demonstrated also the presence of large number of pericytes surrounding the small

blood vessels (arterioles, venules and capillaries) at early and mid-luteal phases. This can be considered as an indicator for the process of angiogenesis in the developing (CL) according to the report of Nehls *et al.* (1992) who mentioned that pericytes are believed to be involved in the earlier stages of capillary sprouting.

Nitric oxide (NO) can react with superoxide to form nitrate (non-radical product) and therefore, variation in production of (NO) and superoxide by the endothelial cells provide a mechanism that regulate the vascular tone of corpus luteum (Ward and Peters, 1995). In contrast to the previous suggestion and depending on the level of (LPO) production, the present study indicated but in indirect way that the level of superoxide production was minimum during early and mid-luteal phases and at early pregnancy, then reached the maximum at late luteal phase, this was due to low (SOD) activity. Moreover, the present study showed that there was no difference between the surface area of the vascular elements during mid-luteal phase and early pregnancy in buffalo (CL). This is in accordance with the findings of Fraser *et al.* (2000) who indicated that luteal rescue in early pregnancy is not associated with increased angiogenesis, and presumably the extended life span of (CLg) is associated with stabilization of blood vessels.

Secretory granules in the (LLC) of the present study were expressed in tremendous amount during early-mid luteal phase and early pregnancy. These granules were decreased gradually with the progress of the cycle, reaching minimum at late luteal phase. Secretory granules in the ruminants (CL) are believed to contain oxytocin and neurophysin (Walters *et al.*, 1984a and Theodosis *et al.*, 1986). The population of the secretory granules in ruminants fluctuates with the day of estrous cycle, reaching its maximum at day 7, and at early pregnancy (Fields *et al.*, 1992). However, granules depletion occurs at time preceding the luteolytic pulses of ($PGF_2\ \alpha$) from the uterus which would be expected to be further drive for the release of luteal oxytocin (Betteridge *et al.*, 1984). Both (LPO) and superoxide radical stimulate ($PGF_2\ \alpha$) biosynthesis (Aten *et al.*, 1992) and inhibit the LH-dependent component of steriodogenesis (Behrman and Aten, 1991).

Luteolysis is a complex process, involving massive vascular regression (Augustin *et al.*, 1995 and Singh *et al.*, 1997) during apoptosis of the luteal cells (Guo *et al.*, 1998). This may be due to oxidative stress which affect the endothelial cells, causing loss of function (Warren *et al.*, 2000 and Slater, 1984). The present study showed that large number of luteal cells with apoptotic features and

damaged endothelial cells were particularly seen at late luteal phase. In addition, a minimum (NO) level as well as a high (LPO) level were observed at the same phase in accordance with Kerr *et al.* (1972). The damaged luteal and endothelial cells were replaced by connective tissue which was reflected in the present study by increasing the surface area of the connective tissue matrix at late luteal phase.

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Table (1): Results of histo-morphometric characteristics of steroidogenic cells of CLp and CLg in buffalo- cows

Steroidogenic cells	Histo-morphometric characteristics	Corpus luteum periodicum (CLp)				Corpus luteum graviditatis (CLg)
		Early luteal phase	Early mid-luteal phase	Late mid-luteal phase	Late luteal phase	
Small luteal cells (SLC)	Diameter (µm)	11.80 ± 4.10	12.90 ± 3.90	14.23 ± 4.10	10.24± 2.50	12.90 ± 4.00
	Surface area (μm^2)	147.20 ± 68.10	128.60 ± 60.10	150.91 ± 49.20	130.67±40.76	147.30 ± 36.60
	% Surface area*	18.80±1.60 [a]	12.60±1.30 [bd]	13.0 ±1.64 [bd]	4.79±0.85 [c]	6.60±0.95 [c]
Large luteal cells (LLC)	Diameter (µm)	28.80 ± 5.30	32.20 ± 5.20	29.51 ± 6.12	28.49±7.28	40.30 ± 11.00
	Surface area (μm^2)	407.90 ± 108.90	582.90 ± 156.51	551.80± 161.60	492.61±143.33	821.50 ± 201.50
	% Surface area	27.80±1.54 [a]	65.10±1.61 [bd]	60.91±2.26 [bd]	55.52±1.37 [c]	74.50±2.11 [c]

*: In relation to the total area of luteal tissue examined microscopically.
[a,bd,c]: Values with different superscripts within the same row (different luteal phase, CLp) are significantly different (P< 0.05).
[a,c] : Values with different superscripts within the same row (mid-luteal phase of CLp and CLg)) are significantly different (P< 0.05).

Table (2): Percentage of surface area[1] of steroidogenic cells, vascular elements and connective tissue matrix in relation to the total area of luteal tissue in buffalo- cows

Luteal tissue	Corpus luteum periodicum (Cl.p)				Corpus luteum gravidtatis (CL.g)
	Early luteal phase	Early mid-luteal phase	Late mid-luteal phase	Late luteal phase	
Steroidogenic cells	46.60 ± 2.80 [a]	77.70 ± 1.70 [bc]	73.90 ± 2.60 [bd]	60.30 ± 2.70 [e]	81.00 ± 2.10 [c]
Vascular elements	4.80 ± 0.62 [a]	8.00 ± 0.65 [bc]	6.40 ± 1.02 [bc]	3.10 ± 0.68 [ac]	7.70 ± 1.10 [c]
C.T. Matrix	48.60 ± 2.30 [a]	14.30 ± 1.30 [bc]	19.70 ± 1.60 [cf]	33.60 ± 2.00 [d]	11.30 ± 1.45 [e]

[1]: In relation to the total area of luteal tissue examined microscopically.
[a,b,c,d]: Values with different superscripts within the same row (different luteal phase, CL.p) are significantly different (P< 0.05).
[e,f]: Values with different superscripts within the same row (mid-luteal phase of CL.p and CL.g)) are significantly different (P< 0.05).

Table (3): Results of some biochemical constituents of corpora lutea in buffalo-cows[1]

biochemical constituents	Corpus luteum periodicum (CLP)				Corpus luteum graviditatis (CLg)
	Early luteal (n = 10)	Early mid-luteal (n = 12)	Late mid-Luteal (n = 11)	Late Luteal (n = 11)	(n= 8)
Total Lipids (mg/g tissue)	12.53 ± 0.55[a]	15.22±0.48[b]	18.16±0.53[c]	23.86±0.53[d]	14.87±0.63[b]
Cholesterol (mg/g tissue)	2.29±0.21[a]	3.84±0.19[bd]	3.25±0.20[b]	2.61±0.20[a]	4.38±0.42[ac]
LPO (nmol/mg)	1.55±0.36[a]	3.36±0.31[b]	4.25±0.33[c]	7.86±0.34[d]	2.90±0.44[b]
NO (nmol/mg)	5.33±0.24[a]	4.06±0.21[b]	3.53±0.23[b]	1.29±0.23[c]	3.77±0.28[b]
SOD (ng/mg)	2.64±0.24[ac]	4.60±0.21[bd]	3.33±0.23[c]	2.33±0.23[a]	4.71±0.27[d]

[a,b,c,d]: Values with different superscripts within the same row [different luteal phases (CLp) and CLg] are
significantly different (P< 0.05).

Table (4):Correlation Coefficient between percentage of surface area of vascular elements, NO activity and P_4 concentration of CLp and CLg in buffalo-cows

	percentage of surface area of vascular elements	Correlation coefficient	
		P_4 Conc. (ng/ml)	NO activity (nmol/mg protein)
a) CLp			
- Early luteal	4.80 ± 0.62[a]	0.7178	0.8617
- Early mid-luteal.	8.00 ± 0.65[bc]	0.7584	0.7527
- Late mid-luteal.	6.40 ± 1.02[bc]	0.8726*	0.9699**
- Late luteal	3.10 ± 0.68[ac]	0.7830	0.9113*
b) CLg	7.70 ± 1.10[c]	0.9211*	0.9267*

*: P< 0.05. **: P< 0.01.
[a,b,c,d]: Values with different superscripts within the same row (different luteal phase, CLp) are significantly different (P< 0.05).
[c,f]: Values with different superscripts within the same row (mid-luteal phase of CLp and CLg)) are significantly different (P< 0.05).

Table 5: Correlation Coefficient between serum P_4 concentration, percentage of surface area of steroidogenic cells and nitric oxide activity of CLp and CLg in buffalo cows[1]

	Serum P_4 concentration (ng/ml)	Correlation coefficient	
		Surface area of steroidogenic cells (%)	NO activity (nmol/mg protein)
a) CLp			
- Early luteal phase.	0.47 ± 0.18^{a}	0.7326	0.7549
- Early mid-luteal phase.	3.49 ± 0.61^{b}	0.9112*	0.6928
- Late mid-luteal phase.	3.14 ± 0.44^{b}	0.9345*	0.3464
- Late luteal phase.	0.39 ± 0.20^{a}	0.4871	0.9695*
b) CLg	4.82 ± 0.89^{b}	0.8842*	0.7280

* : $p < 0.05$

[a,b]: Values with different superscripts within the same column are significantly different ($P < 0.05$)

LEGENDS

Fig. 1: Transmission electron micrograph of buffalo corpus luteum at early luteal phase showing small (SLC) and large (LLC) luteal cells. The small luteal cell contains relatively large nucleus (N) with clumps of dense heterochromatin and thin cytoplasm with few cytoplasmic structures. In the large luteal the nucleus (N) contains small heterochromatin clumps and the cytoplasm contains tremendous amount of cytoplasmic structures. X 6140.

Fig. 2A: Transmission electron micrograph of large luteal cell at early luteal phase showing spherical nucleus (N) with small heterochromatin clumps and cytoplasm containing tremendous amount of mitochondria (M) and secretory granules (S). X 11750

Fig. 2B: Magnification from figure 2A showing mitochondria (M) with lamellar (↙) and tubular (↙↙) cristae, endoplasmic reticulum (E), poly ribosomes (arrowheads), intermediate filaments (F), Golgi complex (G) and secretory granules (S). X 20000.

Fig. 3: Transmission electron micrograph of buffalo corpus luteum at early luteal phase showing a blood capillary in cross section with thick endothelial lining (E) and narrow lumen that contains irregular-shaped erythrocyte (ER). Note the basal lamina of the endothelium (▶) continues around the adjacent pericyte (P). X 10000.

Fig. 4A: Transmission electron micrograph of two adjacent large luteal cells at early mid-luteal phase. Small parts of the nuclei (N) are visible at the bottom of the micrograph, the lateral plasma membrane is greatly folded (▶) and the two cells are joined through desmosomes. (▶◀). The cytoplasm contains large amount of mitochondria (M) with intramitochondrial bodies in some of them (→), well developed Golgi complex (G) and tremendous amount of smooth endoplasmic reticulum (E). X 11750.

Fig. 4B: Higher magnification from figure 4A showing the intramitochondrial bodies (←) causing enlargement of some mitochondria and destruction of mitochondrial cristae. X 21300.

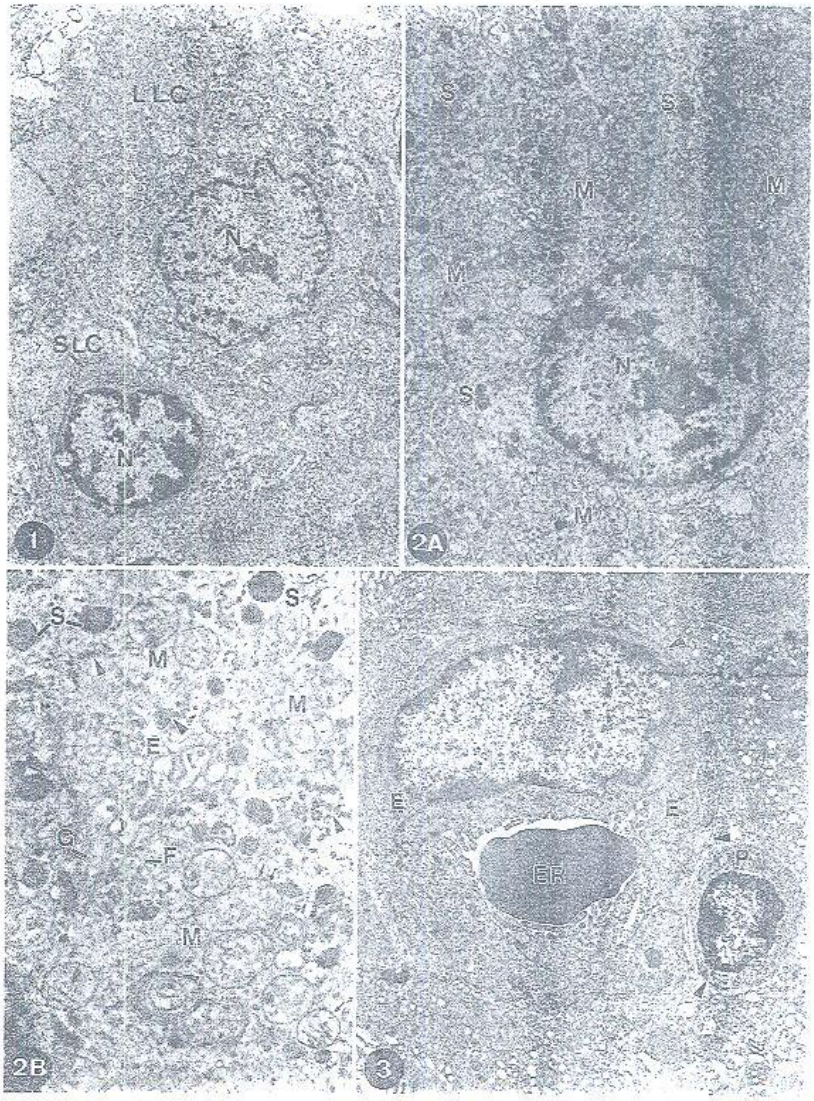
Fig. 5: Transmission electron micrograph of buffalo corpus luteum at mid-luteal phase showing a typical pre-capillary arteriole which continues into capillary (arrows direction from A to C). The arteriole has a relatively wide lumen that contains erythrocytes (ER), lined by thick endothelium (E) and surrounded by a single layer of smooth muscle cell (S). The lumen of the blood capillary appears narrow, the endothelial linning showing many cytoplasmic processes (↘) and the basal lamina of the endothelium (►) continues around the adjacent pericyte (P). X 7000.

Fig. 6: Transmission electron micrograph of buffalo corpus luteum at late-mid-luteal phase showing small luteal cell (SL) and the adjacent parts of large luteal cells (LL). The small luteal cell contains spherical nucleus (N) with condensed chromatin pattern, few mitochondria (M), few strands of rough endoplasmic reticulum (RE) and few lipid droplets (L). The large luteal cell showing abundant mitochondria (M), large amount of smooth endoplasmic reticulum (SE) and many secretory granules (S). Note wide intercellular spaces (✳) between the two cells. X 21300.

Fig. 7: Transmission electron micrograph of large luteal cell at late luteal phase showing large amount of lipid droplets (L), considerable amount of mitochondria (M), short strands of rough endoplasmic reticulum (RE) and very few secretory granules (S). X 21300.

Fig. 8: Transmission electron micrograph of buffalo corpus luteum at late-luteal phase showing degeneration of the endothelial lining (E) of a blood capillary which appears in oblique section. Note lysis of the endothelial basal lamina (▼) and destruction of the mitochondrial cristae (↓). X 7000.

Fig. 9: Transmission electron micrograph of large luteal cell at early-pregnancy showing vesicular nucleus (N) with light heterochromatin pattern and cytoplasm containing large amount of mitochondria (M), secretory granules (S), Golgi complex (G) and large vacuolated dense bodies (D) X 11000.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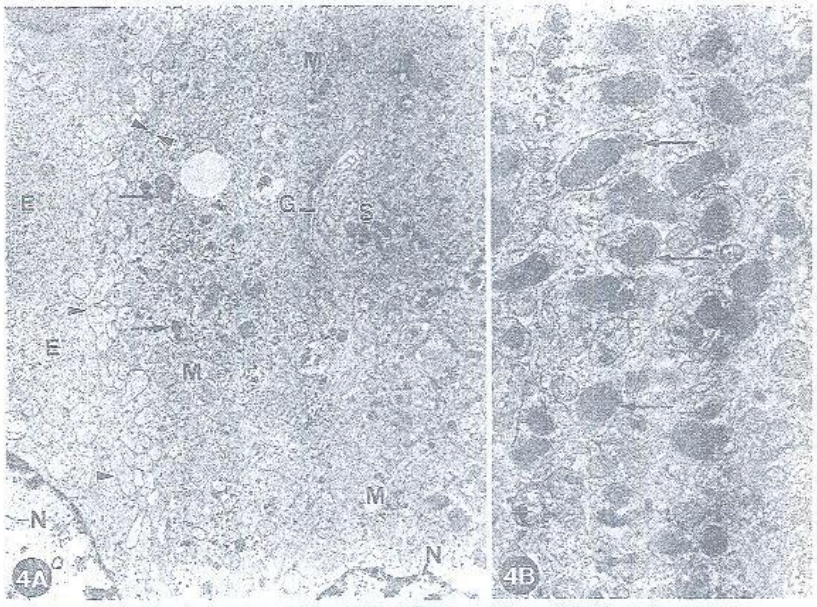
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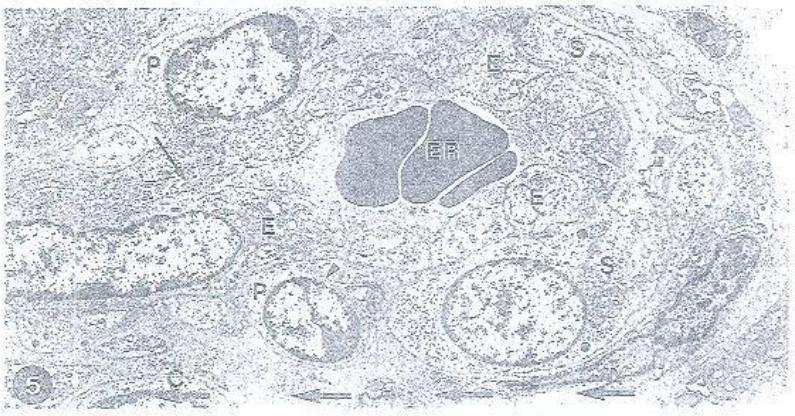
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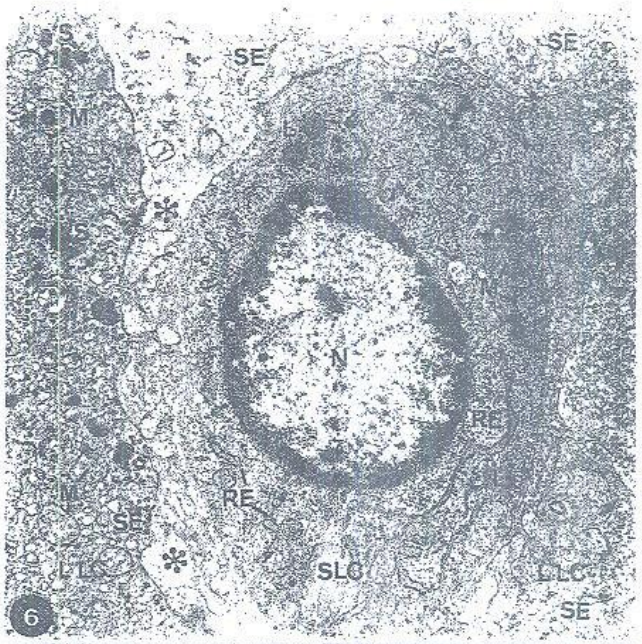

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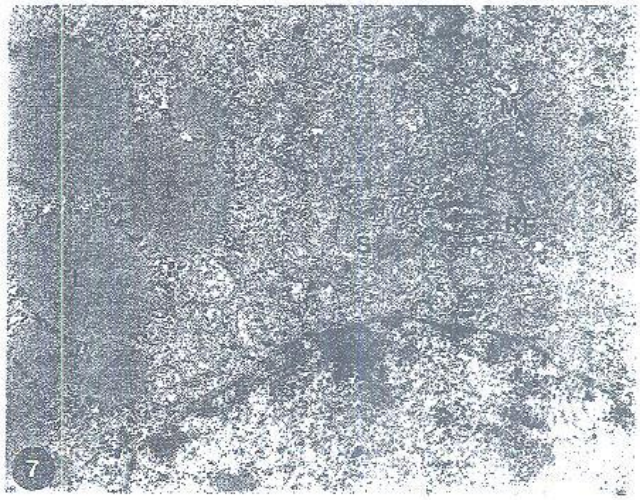

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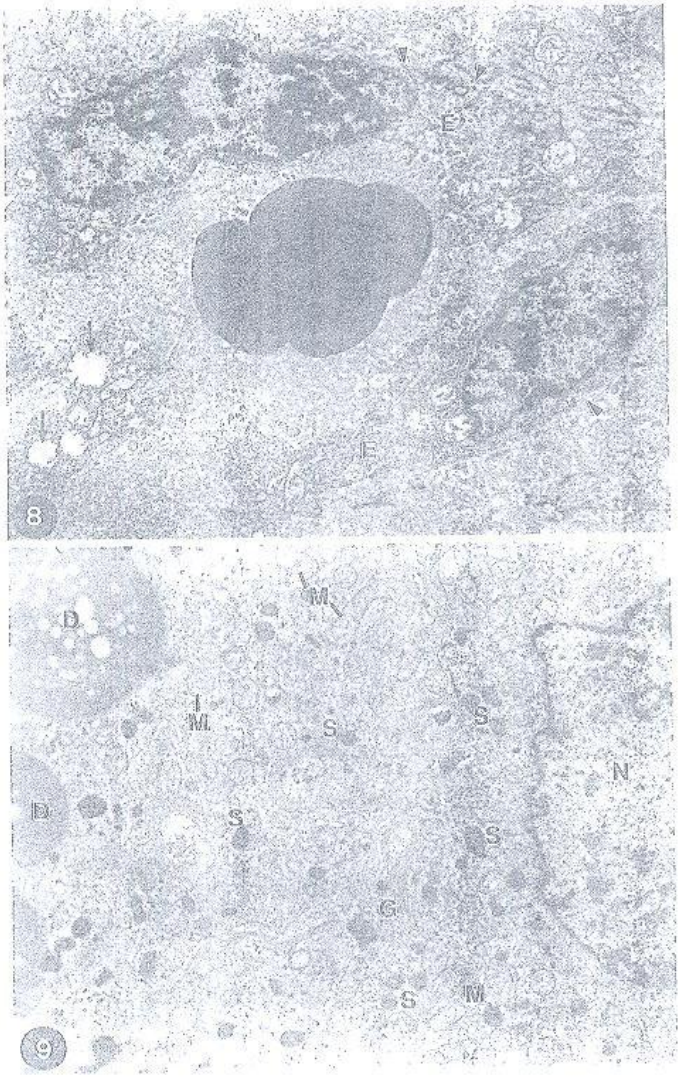
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