SUPEROXIDE DISMUTASE ACTIVITY, NITRIC OXIDE AND LIPID PEROXIDE PRODUCTIONS AND IT'S RELATION TO APOPTOTIC CHANGES AND SERUM PROGESTERONE HORMONE LEVEL DURING PHYSIOLOGICAL LIFESPAN OF BUFFALO'S CORPORA LUTEA

By

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SUMMARY

The activity of superoxide dismutase (SOD), nitric oxide (NO) and lipid peroxide (LPO), productions were focused in different stages of corpora lutea development, as well as their relation to serum progesterone level (P_4) and intracellular selenium (Se) and calcium (Ca^{2+}) with apoptotic changes in luteal cells. Seventy-eight corpora lutea were dissected from ovaries, which were obtained from slaughter house near Assiut City. Blood samples were collected from each buffalo-cow. The corpora lutea were classified into developing (n =22), fully developed (n = 30) or regressing (n = 26) corpora lutea.

During physiological lifespan of corpora lutea, there are changeable activity and production of SOD, NO and LPO. In regressing corpora lutea, there are a significant higher production of LPO and significant lower production and activity of NO and SOD than in developing and fully developed corpora lutea. LPO production had a significant negative correlation with serum P_4 concentration in regressing and fully developed corpora lutea, however, this correlation was not significant in developing corpora lutea. SOD activity and NO production were lower significantly in regressing corpora lutea than in other developmental stages of corpora lutea. SOD activity was significantly positive correlation with P_4 levels in developing and fully developed corpora lutea while, NO had a significantly positive correlation with serum P_4 levels in all developmental stages of corpora lutea.

Intracellular contents of Ca^{2+} and Se had a significantly negative and positive correlation respectively with serum P_4 concentrations in all developmental corpora lutea stages. Ca^{2+} and Se were significantly higher in regressing and fully developed corpora lutea respectively than in other developmental stages. Furthermore, the

ultrastructure of corpora lutea specially during the regressing process showed cell membrane integrity and fragmentation in luteal nucleus. This finding is related to the increasing of LPO production and decreasing in SOD activity, Se and NO production during regressing stage of corpora lutea.

These results suggest that SOD, LPO, NO and intracellular Ca^{2+} may be involved in regulating luteal function (through their relation with P_4 production) during physiological lifespan of the corpus luteum. Therefore, the inbalance between natural antioxidant (Selenium for example) and reactive oxygen species, which are considered oxidative stress, may lead to dysfunction of corpus luteum either during estrous cycle or during pregnancy as well as, it can be of possible importance in reproduction and fertility in buffalo-cows.

INTRODUCTION

In domestic ruminants, it is well known that, luteolysis occurs as a result of the release of luteolytic episodes of uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in response to the binding of oxytocin to the newly developed endometrial oxytocin receptors (Wathes and Lamming, 1995). The corpus luteum secretes progesterone and this steroid is an important regulator of estrous cycle length and is essential for maintenance of pregnancy (Baird, 1992 and Garverick, et al., 1992).

The role of progesterone during the luteal phase is to inhibit the development of the endometrial oxytocin receptor until the appropriate time, while at the same time inducing endometrial ability to synthesis and release $PGF_{2\alpha}$ in response to oxytocin (Lamming and Mann, 1995). Many investigators have focused on cell ultrastructure, cell types and their origin on one hand and luteal changes taking place during regression on the other hand (Garcia-Iglesias, et al., 1992 and Yamada, et al., 1994). There is a high correlation between plasma progesterone concentration and corpus luteum mass (Marcial, et al., 1992 and Young, et al., 1997) and histomorphology (Zayed and Megahed, 1999).

The steroidogenic interaction which occurs between the theca cells and granulosa cells during the development and growth of ovarian follicles has been well documented in many species. After ovulation, these cells differentiate into luteal cells and form the corpus luteum which increase in weight as it becomes vascularised largely owing to increase in size of the individual luteal cells (Baird, 1992). In general, granulosa cells form large luteal cells and theca cells form small luteal cells (Hansel, et al., 1991). The interactions among large and small luteal cells may play a significant role in the regulation of corpus luteum function (Del Vecchio, et al., 1995).

It is possible that, apoptosis, an intracellular genetic programme resulting in cell death (Bellamy, et al., 1995), is an important pathway and which is

demonstrated in the regressing corpus luteum of cows (Zheng, et al., 1994 and Rueda, et al., 1995), sheep (Kenny, et al., 1994), rabbits (Dharmarajan, et al., 1994) and rats (Orlicky, et al., 1992). Evidence of apoptosis can be sought by demonstration of the specific morphological appearance of apoptosis. The first visible features are cytoplasmic vacuoles and nuclear condensation accompanied by loss of microvilli and cell-cell junctions (Young, et al., 1997). Apoptosis commonly involves the activation of endonucleases which lead to DNA fragmentation (Oberhammer, et al., 1993). Calcium (Ca^{2+}) ion is one of the most important factor stimulating endonucleases activities and thereby the apoptosis (Gaido and Cidlowski, 1991).

It is well known that reactive oxygen species (ROS) and their product lipid peroxide (LPO) damage the cell membrane (Slater, 1984). The production of superoxide radical was increased in the plasma membrane of the corpus luteum during the regression (Sawada and Carlson, 1989). Furthermore, luteal contents of superoxide dismutase (SOD), a scavenger of the superoxide radical, change during the estrous cycle (Shiotani, et al., 1991) and increased by luteinizing hormone (Latoraya, et al., 1988). It is therefore likely that, superoxide radical and their scavenging (SOD) with LPO play important roles in generating and regulating the luteal function during pregnancy (Sugino, et al., 1993). The present study aimed to demonstrate the corpus luteum SOD activity and LPO production and it's correlation with progesterone production as well as Ca^{2+}, selenium % in luteal cells during physiological lifespan of the buffalo's corpus luteum. Moreover, attention to ultrastructure picture of corpus luteum, especially apoptotic picture during physiological lifespan was emphasized.

MATERIAL AND METHODS

This study was undertaken on 78 specimens collected from a local slaughter house near Assiut city. The age of the included specimens were procured by dentition (Kartha, 1975) and ranged between 3-6 years old. Seventy-eight blood samples were also collected from the animals through jugular veinipuncture before slaughter in clean sterile centrifuge tubes without anti-coagulant . Serum was separated by centrifugating the blood samples at 3000 rpm for 20 min, then stored at –20°C until hormone assaying.

Immediately after slaughter, the genitalia were removed and the stage of the estrous cycle was determined according to Eissa (1996). The ovaries were collected and transferred to the laboratory within 1-2 h in an ice-box. The corpora lutea were divided according to Okuda et al. (1988) into developing (n = 22), fully developed (n = 30) and regressing (n = 26). These corpora lutea were immediately dissected, free from the stroma of the ovary for each animal, then divided into 3 portions. The first portion was stored at –20°C until used for biochemical assay. The second portion was prepared for X-rays analysis by using scanning technique and Link ISIS programme for intracellular selenium (Se) and calcium (Ca^{2+}) in Assiut University EM unit. The third portion was fixed in 5% glutaraldehyde for 24 h. The specimens were then washed in Cacodylate buffer (pH 7.2) and post-fixed in cold 1% Osmium tetraoxide for 24 h, then they were washed in Cacodylate buffer. After dehydration,

the samples were embedded in Epon 812 using gelatin capsules (Gupta, 1983). Semithin sections were prepared by using LKB ultramicrotome. The sections were stained with toluidine blue, then examined by light microscope. Ultrathin sections, from the selected areas of the trimmed block, were made and collected on copper grids. These ultrathin sections were contrasted in uranyle acetate and lead citrate, then examined by transmission electron microscope (JEOL-EM 100 CX II) at an accelerating voltage of 80 Kv, in Assiut University EM unit.

The first portion of the corpora lutea was prepared for biochemical assays. A suitable portion of corpus luteum (10% w/v) was homogenized in ice-cold phosphate buffer (0.1 mol/ml and pH 7.4) in a special glass homogenizer then stored at –20°C until used. A part of homogenated sample was centrifuged at 8000 rpm for 10 min. The supernatant (cytosol) was collected and used for estimation of superoxide dismutase (SOD) and nitric oxide (NO). The other part of homogenate was used for determination of lipid peroxide (LPO) and protein concentration.

Total SOD activity was measured by the nitrite method reported by Oyanagi (1984). The data were expressed as ng/mg protein. NO activity was measured using Gries reagent with sodium nitrite as a standard according to Ding et al. (1988). Its activity was expressed as nmol/mg protein. The concentrations of LPO in the corpus luteum were measured by the thiobarbituric acid method according to Ohkawa et al. (1979). The results of LPO were expressed as nmol of malondialdehyde (MDA) per g wet weight of tissue (nmol/mg protein). Protein concentration was measured by the method according to Lowry et al. (1951) using commercial kit. Serum progesterone (P_4) concentration was determined by using commerical ELISA kits (BIO SOURCE, EUROPS, S.A., Belgium). The sensitivity of the assay, defined as the smallest concentration distinguishable from zero, was 0.1 ng/ml. The intra and interassay coefficient of variation were 6.8% and 8.2% respectively. Serum estradiol-17β (E_2) levels were determined by using commerical ELISA kits (BIO SOURCE, EUROPS, S.A., Belgium). The sensitivity of the assay was 5 pg/ml. The intra and interassay coefficient of variation were 4.6% and 7.5% respectively.

Data were expressed as means + SE for all parameter in this study, then analysed statistically using analysis of variance (ANOVA). The means + SE were tested at least significant difference (LSD). All tests were done by using PC-stat computer programme. Results were considered significant at $P< 0.05$ or less.

RESULTS

The obtained results in this study are presented in Tables 1 and 2 and Figures 1 and 2. The mean concentrations of progesterone (P_4) changes in different stages of developmental cyclic corpora lutea. P_4 concentration increased significantly ($P< 0.01$) for fully developed corpora lutea (3.27+0.97 ng/ml) than that for developing (0.93+0.27 ng/ml) and regressing (0.63+0.29 ng/ml) corpora lutea (Table 1). However, this hormone had non-significant difference during developing and regressing stages of corpora lutea. Concerning estradiol-17β (E_2), the highest significantly ($P< 0.01$) mean levels was noticeable during regressing stage (25.03 +

3.79 pg/ml) and the lowest level (9.88+2.07 pg/ml) was detected during fully developed corpora lutea.

There are variable changes in the mean levels of lipid peroxide (LPO), superoxide dismutase (SOD) and nitric oxide (NO) activities of homogenated corpora lutea during physiological lifespan of corpus luteum (Table 1). The LPO contents increased gradually in different stages of corpora lutea as well as, its levels were significantly ($P< 0.01$) lower (1.78+0.58 nmol/mg protein) in developing corpora lutea and significantly ($P< 0.01$) higher (7.19+1.05 nmol/mg protein) in regressing corpora lutea. In contrast NO contents in homogenated corpora lutea decreased significantly ($P< 0.01$) in regressed corpora lutea (1.77+0.73 nmol/mg protein) as well as, increased (5.29+0.71 nmol/mg protein) significantly ($P< 001$) in developing corpora lutea. The increasing activity of SOD was recorded in developing (3.55 + 0.57 ng/mg protein) and fully developed (4.52 + 1.17 ng/mg protein) than in regressed corpora lutea (2.66 + 0.67 ng/mg protein).

X-ray analysis of different developmental stages of corpora lutea (Fig. 1) revealed that, the intracellular selenium (Se), which is considered as a natural antioxidant, showed as significantly ($P< 0.05$) lower % (17.07 + 2.73%) in regressing corpora lutea than in fully developed (22.86 + 2.59%) and in developing corpora lutea (19.37 + 1.82%). Moreover, the intracellular calcium (Ca^{2+}) increased significantly ($P< 0.01$) in regressing corpora lutea (4.34 + 1.01 %) than in developing and fully developed corpora lutea (1.71 + 1.04 and 2.69 + 0.85 % respectively).

The ultrastructure changes during regression of corpora lutea are illustrated in Fig. 2. This figure revealed that, the regression of corpora lutea was completed through apoptosis of its luteal cells. The apoptotic process started during regressing stage of corpora lutea by condensation and clumping of the nuclear chromatin under intact nuclear membrane and formation of nuclear projections which, later on, lead to nuclear fragmentation. There is condensation of the cytoplasmic organella (Fig. 2a). In other cells, there was condensation of the chromatin (Fig. 2b) or clumping of the nuclear chromatin under intact nuclear membrane with formation of nuclear crescent (Fig. 2c). This process is proceeded by fragmentation and lysis of the nucleus (Fig. 2d). Finally, the cell fragments were phagocytosed in an apoptotic vacuole which lead to complete disappearance of the apoptotic cells (Fig. 2e)

DISCUSSION

This study indicated that the marked significant decrease in serum progesterone concentration during the regressing phase of corpora lutea coincided with the rapid increase in lipid peroxide activity and decreased superoxide dismutase in the corpus luteum. These data are consistent with the other obtained findings that superoxide dismutase activity decreased during the regressing phase and increased luteal cell integrity with plasma membrane damage (via ultrastructure finding). The results of this study would be supported by the well established observation that the reason (s) for degeneration of luteal cells and capillary endothelial cells during regressing phase may be due to alterations in blood flow

(Wiltbank and Niswender, 1992) or the action of some secondary factor produced by the degenerating luteal cells (Bagavandoss, et al., 1988).

From these secondary factor, reactive oxygen species (ROS) may induce tissue injury through several mechanisms. Reduction of blood flow which occurs during regression of the corpus luteum stimulates the conversion of xanthine dehydrogenase to xanthine oxidase which stimulates the production of superoxide radical and cell damage (McCord, 1985). Although ROS is charged directly permeate defined lipid bilayers and it can cross plasma membranes of luteal cells via anion channels (Weiss, 1986 and Hesla, et al., 1992). An oxidant attack on membrane, lipoprotein or albumin associated polyunsaturated fatty acids can initiate a complex cascade of events leading to the formation of more ROS or biologically active inflammatory mediators that have the potential to propagate cell damage.

Furthermore, superoxides may directly alter protein and nucleic acid structure or function (Freeman and Crapo, 1982). Superoxide radicals and lipid peroxidation increase during the regressing phase of corporus luteum (Sawada and Carlson, 1989) and inhibit luteal function (Gatzuli, et al., 1991). In addition, the ovary contains SOD which enzymatically inactivate these highly toxic oxygen metabolities and is considered the specific inhibitor of superoxide anion radical. The ROS created, during the process of steroidogenesis, may cause local damage and decrease steroidogenesis if not detoxified by SOD and other scavengers. Moreover, the presence of more superoxide anions may inhibit the activity of SOD (Sinet and Garber, 1981). The toxic hydroxyl radical may be produced when H_2O_2 (the end-product of SOD) reacts with O_2 in the presence of iron (Fridovich, 1986). Without the presence of sufficient activity of cytosolic enzyme (e.g SOD), H_2O_2 generated in the dismutation reaction could itself inhibit progesterone production (Riely and Behrman, 1991).

Moreover, Behrman and Aten (1991) suggested that H_2O_2 may affect progesterone synthesis by blocking the transfer of intracellular cholesterol from cytosol to mitochondria as well as, it caused rapid inhibition of luteinizing hormone-stimulated cAMP accumulation in the membrane of the corpus luteum that resulted in the decrease of progesterone production (Behrman and Preston, 1989 and Sugino, et al., 1993). On the other hand, during full development of corpora lutea, when the concentration of luteinizing hormone rises during the pulse (Sawada and Carlson, 1994), there is a temporary increase in progestrone secretion due to increase concentration of intracellular cAMP. Eventually, the secretion of $PGF_{2\alpha}$ is sufficient to uncouple the LH receptor activation system and luteal regression occurs (Baird, 1992).

This association between SOD and corpus luteum function is consistent with the hypothesis that very high concentrations of oxygen free radicals may be present at the end of the lifespan of the corpus luteum which inactivate the cellular defenses provided by SOD, promote tissue damage and further reduce the availability of protective enzymes such as SOD (Hesla, et al., 1992). The obtained results generally coincided with data reported by Aten et al. (1992), Sugino et al. (1993) and Shimamura et al. (1995) in experiments animals.

The present study revealed that, nitric oxide (NO) production was significantly higher in developing and full developed corpora lutea as well as, NO production was decreased significantly in regressing phase of corpora lutea. In addition, the increase in production of NO and P_4 may be attributed to large increase in blood flow to the corpus luteum allowing utilization of serum-derived lipoprotein as a source of cholesterol for steroidogenesis (Grummer and Carroll, 1988 and Wiltbank and Niswender, 1992). The luteal cells (especially lagre luteal cells) also have the ability to produce peptides and proteins for secretion. Two growth factors (basic fibroblast growth factor and insulin-like growth factor-1) are produced in the corpus luteum with the greatest concentrations of mRNA for these proteins expressed in the luteal phase (Stirling, et al., 1991). These growth factors may stimulate luteal angiogenesis (Gospodarawicz, et al., 1985) or regulate luteal steroidogenesis (Einspanier, et al., 1990).

In addition, the NO increases in developing and fully developed corpora lutea may be attributed to increases in blood flow where, the blood capillary endothelial cells produced NO (van der Zee, et al., 1997) which is an important mediator of vasodilator responses induced by pharmacological agents (Ward and Peters, 1995). However, regressing phase of corpora lutea is accompanied by degeneration of luteal capillaries endothelial cells (Braden, et al., 1988) and decrease blood flow within the subsequent hypoxia within the luteal tissue (Wiltbank, et al., 1990) and dropped in NO production.

Natural antioxidant in the cells protects plasma cell membrane against oxidative damage and prolonging the biologic life of polyunsaturated fatty acids which are important membrane constituents (Ward and Peters, 1995). A decline in antioxidant (free-radical scavengers) capability of the regressing corpus luteum may result in increased local concentrations of oxygen free radicals, which stimulate functional and structural regression (Hesla, et al., 1992). This hypothesis support the results of the present study which indicated that the intracellular Se was significantly lower in regressing corpora lutea than in developing and full developed corpora lutea as well as, a positive significantly correlation between Se and P_4 production. Concerning the intracellular calcium in luteal cells, the present data also showed that, intracellular calcium was higher significantly during the regression process of corpora lutea than in developing and fully developed corpora lutea. These results support the theory that during luteolysis of corpus luteum, the activation of phosphoinositide-specific phospholipase C and protein kinase C sustained the production of inositol trisphosphate (IP_3) and diacylglycerol (Jacobs, et al., 1991 and Wiltbank, et al., 1991). The increase concentrations of IP_3 leads to elevation in free intracellular calcium concentrations (Wiltbank, et al., 1989).

In conclusion, apoptosis occurs in regressing corpora lutea which is characterized by condensation and clumping of nuclear chromatin as well as fragmentation of the nucleus and cell membrane. These are associated with increase production of LPO and intracellular free calcium as well as, reduction of NO levels, SOD activate, and antioxidant (Se) with serum P_4 concentrations. Moreover, LPO, NO and SOD with antioxidant activation may play a very important role in regulating the function of corpus luteum during physiological lifespan in

buffalo-cows. Therefore, the imbalance between antioxidant and ROS (oxidative stress) may be lead to dysfunction of corpus luteum during estrous cycle or pregnancy which can be possible importance of reproduction and fertility in buffalo-cows.

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Table (1): Mean (+ SE) values of serum steroid hormones, intracellular % of selenium (Se) and calcium (Ca^{2+}) and some biochemical constituents of buffalo's corpora lutea.

Items	Developing CLs (n = 22)	Fully developed CLs (n = 30)	Regressing CLs (n = 26)
* Serum steroid hormone			
P_4 (ng/ml)	0.93±0.27^a	3.27±0.97^b	0.63±0.29^a
E_2 (pg/ml)	19.05±2.35^a	9.88±2.07^b	25.03±3.79^c
* Intracellular contents			
Se (%)	19.37±1.82^a	22.86±2.59^b	17.07±2.73^c
Ca^{2+} (%)	1.71±1.04^a	2.69±0.85^b	4.34±1.01^c
* Biochemical constituents			
LPO (nmol/mg protein)	1.78±0.58^a	3.64±0.64^b	7.19±1.05^c
NO (nmol/mg protein)	5.29±0.71^a	3.73±0.58^b	1.77±0.73^c
SOD (ng/mg protein)	3.55±0.57^a	4.52±1.17^b	2.66±0.67^c

P_4 = Progesterone E_2 = Estradiol-17β
LPO = Lipid peroxide NO = Nitric oxide
SOD = Superoxide dismutase.
Values with the same superscript letter within the same row are non-significant.
abc ($P< 0.01$) within each of E_2, LPO, NO, SOD.
ac ($P< 0.05$) and ab, bc ($P< 0.01$) within P_4, Se and Ca^{2+}.

Table (2): Correlation coefficient between superoxide dismutase (SOD) activity, lipid peroxide (LPO) production, nitric oxide (NO), intracellular selenium (Se), calcium (Ca^{2+}) and serum progesteron (P_4) production in different stage of buffalo's corpora lutea.

	Developing CLs	Fully developed CLs	Regressing CLs
SOD/P_4	0.5812*	0.6442*	0.2665ns
LPO/P_4	-0.3746ns	-0.4439*	-0.7539**
NO/P_4	0.7524**	0.4762*	0.5581*
Se/P_4	0.6931*	0.4312*	0.7787**
Ca^{2+}/P_4	-0.5256*	-0.5292*	-0.6139**

- ** P < 0.01 * P< 0.05 ns = non-significant.

LEGENDS

Figure 2 A:TEM showing Apototic luteal cell (L), notice clumping and condensation of nuclear chromatin (N), Start of nuclear fragmentation through formation of nuclear projections (arrow) and condensation of the intact cytoplasmic organelles(O). (Uranyl acetate and lead citrate X 16,000).

B: TEM showing condensation of the nuclear chromatin(N) in apoptotic luteal cell. (Uranyl acetate and lead citrate X 28,000).

C: TEM showing clumping of the nuclear chromatin (N) under intact nuclear membrane (arrow) (Uranyl acetate and lead citrate X 28,000).

D: TEM showing fragmentation and lysis of the nuclear chromatin (LY), the remaining nuclear chromatin forming the typical crescent shape (C) in an apoptotic luteal cell, (Uranyl acetate and lead citrate X 16,000).

E: TEM showing the end stage of the apoptotic luteal cells. This stage is characterized by phagocytosis of the cell organelles in an apoptotic vacuole (V) (Uranyl acetate and lead citrate X 8,000).

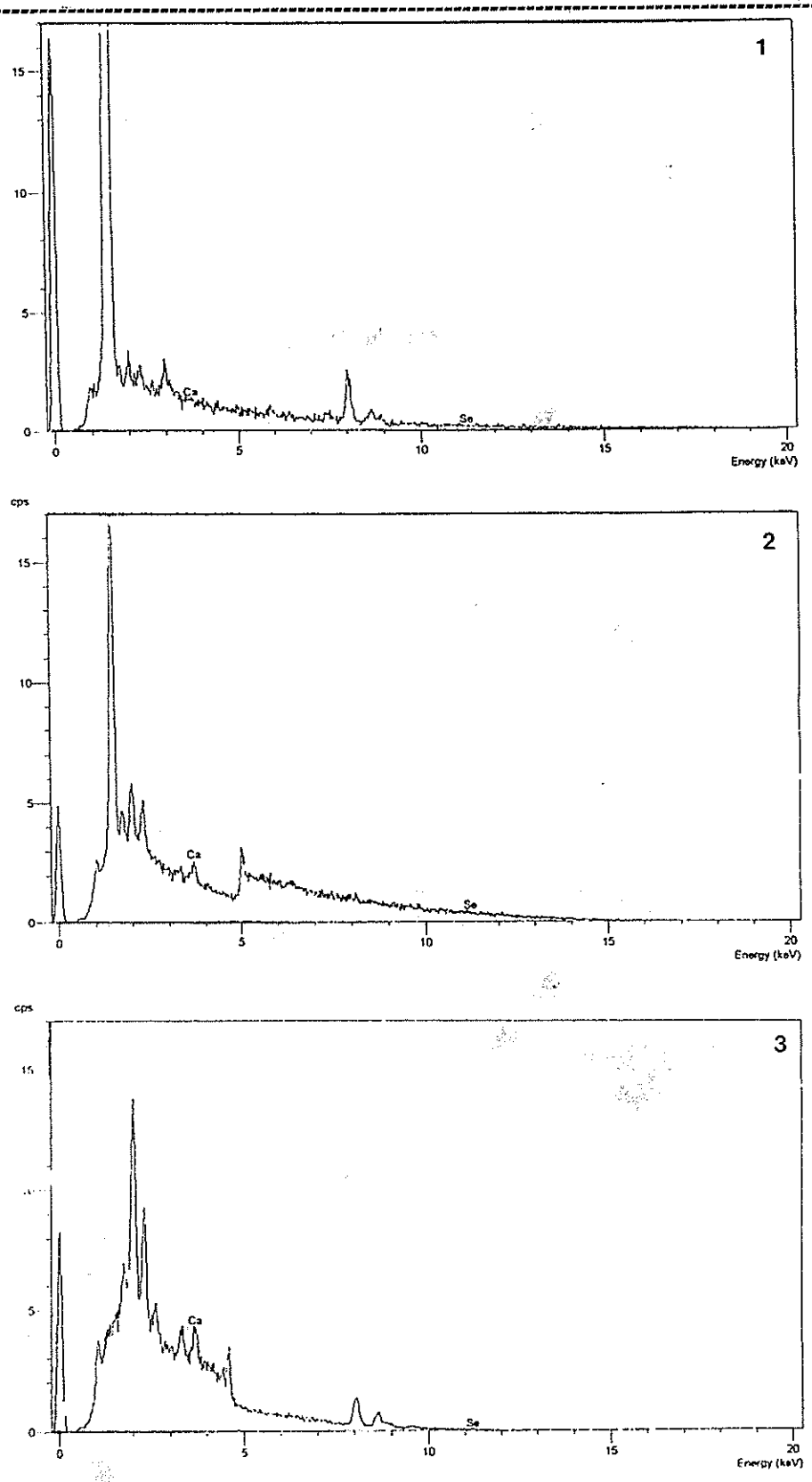

Figure (1): The percentages of intracellular free calcium Ca^{2+} and selenium (Se) (by using scanning electron microscope and Linke ISIS programme) in buffalo-cow corpora lutea stages: (1) Developing, (2) Fully developed and (3) Regressing.

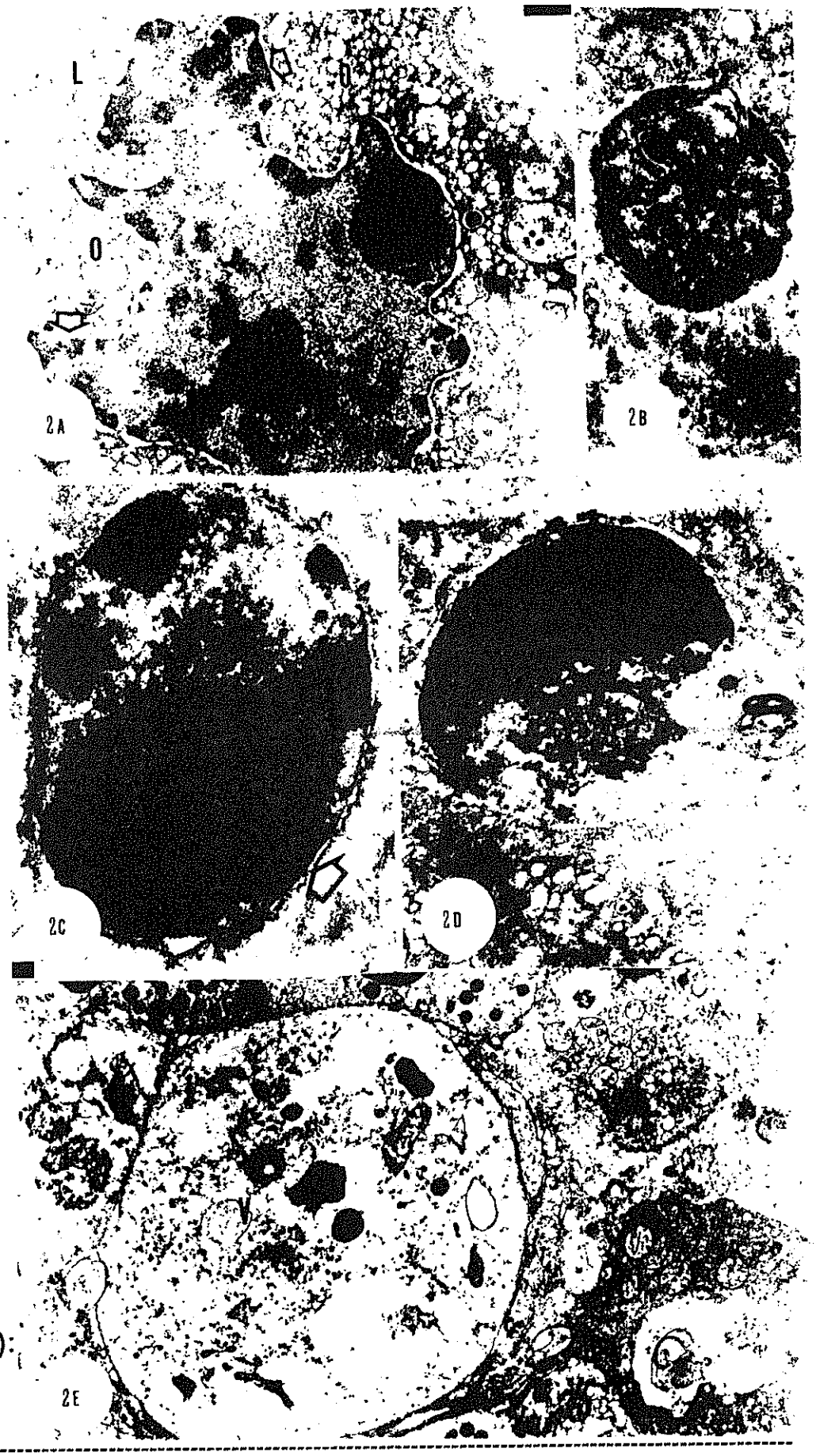

Figure (2):