Adrenal Suppression Induced by Megestrol Acetate and the possible protective role of selenium
A Histological , Histochemical and Ultrastructural Study

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Abstract

The anticancer drug megestrol acetate (MA) is suspected to cause adrenal insufficiency. Several clinical reports indicated that suppressed adrenal function might be possible in long term administration of MA . The present study was designed to evaluate this issue by using the histological, histochemical and ultrastructural techniques. The data obtained from the present study indicated that MA caused various cellular and subcellular damages in most of the cells of the three zones of adrenal cortex. Histochemical investigations indicated increased lipid content associated with increased storage of ascorbic acid and acid phosphatase, and all these data might reflect a state of suppressed steroidogenesis. The results also indicated that selenium might have a protective role against the cytotoxicity of megestrol acetate on adrenocortical cells. All the results were discussed and it is concluded that more followup of adrenal function should be done during the long term treatment with MA, and it is suggested that selenium can be used as an additional protective supplement .

Introduction

Cancer is a disease in which there is uncontrolled multiplication and spread within the body of abnormal forms of body's own cells. It is one of the major causes of death all around the world. Figures for the last century give the impression that the disease is increasing in spite of the advances made in the main treatment approaches; surgical excision, irradiation and chemotherapy (Rang *et al*., 2003). In chemotherapy, hormonal maneuveres have considerable use in the management of breast and uterine cancer, and progestins are hormonal agents with considerable antitumor activity (Aisner *et al*., 1987). The hormonal therapy of tumors is dependent on the presence of receptors for these hormones in the malignant cells (Rang *et al*., 2003).

Megestrol acetate (MA) is a synthetic progestin which is known commercially as megace and used in human medicine for the treatment of endometrial and breast carcinomas as well as for the treatment of acne, hirsutism, and sexual infantilism in females and metastatic prostate carcinoma in males (Naing *et al*., 1999). Some countries, use MA in combination oral contraceptive preparations and recently it became one of the most commonly used drugs for the treatment of AIDS and cancer related weight loss. However, despite this apparent benefit, available data suggest that weight gain which is caused as a consequence of treatment with MA is largely adipose tissue and not body muscles (Inui, 2002).

The major challenge facing chemotherapy is that cancer cells and normal cells are so similar in many respects that it is more difficult to find general, exploitable, biochemical differences between them. Hence, chemotherapy is usually associated with unavoidable side effects. Like all drugs, MA may develop several side effects among the patients to

whom it is prescribed as a long term medication , however, the people react to the drugs in different ways, specially if we consider the gender. So the animal model in the present study focused on the female subjects since they represent the major users of MA.

Glucocorticoid activity of MA has been reported, and some concern has been expressed about the use of MA to treat AIDS patients and patients with metastatic breast carcinoma due to the risk of secondary adrenal suppression that might develop in those patients (Naing *et al*., 1999 and Stockheim *et al.,* 2000). Also, the incidence of hyperglycemia and hypertension in patients with MA may suggest adrenal insufficiency as a side effect of that drug (Brockman, 2004). So, the present study was achieved to introduce more conformation and to evaluate the possible effect of MA on the adrenal gland by using the histological, histochemical and ultrastructural techniques with special reference to the possible role of selenium in inducing protection.

Materials and Methods

Forty five adult (about 35.0 g in weight) female CD-1 mice were used in the present study . They were kept in normal laboratory conditions and supplied with food and water ad libitum . The animals were divided into 3 main groups , 15 animals in each group . The first group served as control, and was given saline only, while the second group was given daily oral dose of 0.4 mg/day of megestrol acetate dissolved in water for the period of two months, and this dose represnt the equivalent of the human therapeutic dose as calculated according to the formula of Paget and Barnes (1964) . The third group of mice received daily dose of 1.4 mcg/kg of selenium one hour before the daily oral dose of 0.4 mg/day MA for two months . Adrenal gland was taken out after dissection and processed for paraffin procedure after fixation in 4% phosphate-buffered paraformaldehyde. Paraffin sections were stained with the silver

method of Gordon and Sweet (1936) for reticulin and with Hx and eosin for routine histology . Frozen sections of paraformaldehyde fixed tissues were examined with the fluorescent microscope for the examination of lipoidal and liofuscin materials. Other frozen sections were subjected to the metallic ion precipitation method of Gomori (1941) for acid phosphatase. Ascorbic acid was demonstrated by using the silver method mentioned by Gabe (1976). Glycol methacrylate embedding was used to obtain semithin sections which were stained with basic fuchsinmethylene blue according to the method of Bennett *et al.(*1976). For electron microscopy, tissues were fixed in phosphate buffered glutaraldehyde ,post fixed in 2% osmium tetroxide , dehydrated and embedded in Epon 812 . Semithin section were obtained using glass knives and stained with toluidine blue . Ultrathin sections were obtained using diamond knife, double stained with uranyl acetate and lead citrate, then examined with the Jeol 1200 EX electron microscope. Measurements of the dimentions of the adrenal cortex and its zones were taken by using a slide micrometer and micrometer eye piece . Statistical analysis was achieved by using the t-test .

Results

It should be recorded that the group of mice treated with megestrol acetate (MA) , showed apparent weight gain if compared with the control animals or with the animals treated with selenium prior to MA administration. Moreover, during dissection it was noticed that there was massive accumulation of adipose tissue inbetween the mesentries and fats were completely covering the kidney and the adrenal gland .

Histology, ultrastructure and lipid content :
The adrenal gland contains two structurally and functionally different tissues, the cortex and medulla, and it is surrounded by the connective tissue capsule that contains fibroelastic tissue, in which

smooth muscle cells are present (Fig. 1). The present study is concerned with the adrenal cortex which can be distinguished into; zona glomerulosa, zona intermedia, zona fasciculata and zona reticularis (Fig.1)

The connective tissue framework of the gland is derived from the thick capsule that surrounds the gland (Fig. 1). The group of cells in zona glomerulosa are found outlined with septa of connective tissue fibers that extend to form trabeculae extending at right angles to the region of medulla, demarcating the columns of cells on the zona fasciculata and form a dense meshwork in the zona reticularis that surrounds individual or grouped cells (Fig. 1).

Histological examination of semithin sections of the gland from animals administered MA revealed prominent signs of fibrosis specially in zona glomerulosa and zona fasciculata (Fig. 2). This was much more confirmed by applying the silver method for reticulin in paraffin sections (Fig. 3). Individuals given selenium prior to MA administration did not show prominent changes in increased fibrosis (Fig. 4). In MA-treated mice, large blood sinusoids were found filled with abnormal and fragmented blood corpuscles, besides the appearance of fibrocytes surrounding their walls (Fig.5) .

a) **The zona glomerulosa** (Figs. 1) is the most peripheral area about 62 um (mean value) in width in which the cells form irregular, arch-like struct-ures. The cells are polyhedral in shape, with microvilli and large spherical nuclei that possess prominent nucleoli and peripherally located heterochromatin. The cells possess many free ribosomes, tubular SER associated with lipid droplets that have the diameter range of 1000 nm – 1800 nm, elongated mitochondria with lamelliform or tubular cristae and small Golgi appara-tus (Fig. 6a,b). In electron micrographs with low magnification it is hard to distinguish between the mitochondria and the vesicular or tubular SER due to increased number of the latter elements (Fig. 6a). Between the cells there is intercellular space (about 1000 A° in diameter) and around the blood sinusoids there is periendothelial space

(about 3000A^{o} in diameter) similar to the space of Disse in liver (Fig. 6a,). Microvilli projecting from the cells extend into these spaces (Fig. 6a). In some profiles the liposomes (lipid droplets) appear protruding into the intercellular or periendothelial spaces through endopla-smocrine secretion and newly synth-esized small lipid droplets are found instead (Fig. 6a).

In animals treated with MA, some of the cells showed signs of necrosis as the nucleus became irregular, shrunken, more heterochromatic and surrounded by a halo which might be due to the separation of the two leaflets of the nuclear membrane. The liposomes appeared highly electron dense indicating increased unsaturated fats (Fig. 7) and the sinusoidal endothelium was damaged . In case of treatment with selenium prior to MA administ-ration, the cells showed the same profile like the control (Fig.8 and 13), while the periendot-helial areas showed great damage. The endothelium was almost completely damaged, the sinusoids were dilated and some of the cells lost their microvilli which were usually found projecting towards the sinusoids (Fig. 8). Moreover, abnormally shaped blood corpuscles were found inside the sinusoids in addition to fragments of blood corpuscles, the same feature that was observed in MA-treated animals (Figs 5 and 8). In MA-treated animals the cells were found more heavily loaded with liposomes than in control (Figs.9 and 10). For more conformation, light microscopical invest-ingations by using the fluorescence micro-scopy showed more yellow fluorescence in MA- treated animals than in control or MA + selenium – treated animals (Figs.11-13) ,thus indicating more lipid content while, the animals treated with selenium prior to MA displayed the same lipid profile in fluorescent micrographs and electron micrographs like the control (Figs. 8 and 13) .

b) **The zona intermedia** (Fig. 14) is small, one or two cells thick and relatively lipid free. The mitochondria of these cells are slightly elongated, spherical or cup shaped and possess electron dense matrix and slightly dilated cristae, while the cytoplasm is richly

filled with smooth ER and small Golgi apparatus (Fig. 14). No observable change could be detected between the cells of the animals treated with MA or with MA + selenium when compared with the control .

c) **The zona fasciculata** (Fig. 1) is the region of the largest cells of the four main zones about 215 um in thickness . The cells have large, spherical mitochondria with tubular cristae and very dense tubular smooth ER, numerous ribosomes and few polysomes (Figs. 15 and 16). They possess well developed microvilli that extend into the intercellular spaces and appear intermingled between each two adjacent cells (Fig. 15). Lipid droplets are larger in size (about 2000-2800 nm in diameter) and more numerous than those found in the cells of the other zones. They appear separate or fused with sometimes eccentric empty vacuoles which might be due to loss of some lipoidal content during the dehydr-ation process (Fig. 15). The nucleus is euchromatic with few peripherally located heterochromatin and one or sometimes two or three nucleoli (Figs. 15 and 16).

In MA-treated animals some of the cells appeared degenerated. The nucleus became shrunken, with a perinuclear halo similar to that noticed in zona glomerulosa and the heterochromatin remarkably increased while the contour of the nucleus became irregular (Fig. 17). The same features were also observed in light microscopical preparations and moreover pyknosis of nuclei was more frequently noticed (Fig. 18). Cytoplasmic areas between lipid droplets were degenerated and great number of mitochondria were lost while the lipid droplets became abnormally larger in size, more electron dense and sometimes were found as empty vacuoles containing myelin figures instead (Fig. 17). As a consequence of the presence of large number of damaged cells which suffered from dissolution or turnover of their lipid droplets into myelin figures, light microscopical preparations examined by the fluorescence microscopy showed decreased yellow fluorescence as indication of loss of some lipoidal materials in the MA-treated animals (Fig. 12). On the other hand in intact cells lipid droplets increased greatly

and the intercellular spaces became wider, while the periendothelial areas and the endothelial linings of the sinusoids were greatly damaged (Fig. 19) .

By comparing the group given selenium prior to MA with the control , no noticeable change in lipid content could be distinguished (Figs. 10 and 13). The cells displayed normal ultrastructural features except the frequent appearance of myelin figures and lipofuscin granules with irregularity of the nuclear contour(Fig.20)

d) **The zona reticularis** (Figs. 1) is the capillaries form a loose network (Fig.1). The capillaries are wider than those in the other zones and the macrophages, histio-cytes, are more obviously demonstrated innermost layer of adrenal cortex and it is about 116 um (mean value) in width . The cell cords and accompanying in semithin sections stained with basic fuchsin-methylene blue and in preparations examined by the fluorescence microscope (Fig. 21). The histiocytes take the orange fluorescent colour indicating the presence of lipofuscin granules in these cells while in methylene blue-basic fuchsin preparations they take red and blue colours (Fig. 21). The cells of zona reticularis appear as small polyhedral or cuboidal with relatively large more heterochromatic nuclei than those in the other zones . The nuclei have irregular shape with well developed nucleoli, which might exist as one or more in each nucleus (Fig. 22a). Most of the cells have clearly spherical mitochondria with cristae of the tubular type. Some mitochondria appear flattened or cup-shaped, often including a portion of the cytoplasm (Fig. 22b). Cellular particulate glycogen is abundant and there is a considerable number of smooth ER, lysosomes and lipofuscin granules, while Golgi apparatus is small (Fig. 22a). Microvilli are very few in contrast to the epithelial cells of zona glomerulosa and fasciculata.

In MA-treated animals the most remarkable feature was the spread of numerous vacuoles specially at the deeper areas of zona reticularis (Fig. 23). The second remarkable feature was the massive accumulation of lipofuscin granules which

are considered to contain the "fall out" products of the lipid metabolism in the cells that lie in the deeper zones near the medulla. The lipofuscin granules were characterized by having two components, an electron lucent homogeneous, lipid-like component and a very electron dense component (Fig. 24a,b). Also, prominent increase of lysosomes and glycogen was noticed, meanwhile, the contours of the cells and their nuclei became highly irregular and the Golgi apparatus showed atrophy (Fig. 24a,b). The periendothelial and intercellular spaces became wider and the cells displayed more numerous microvilli projecting to these spaces if compared with the control (Figs. 22 and 24a). Endothelial lining was massively damaged, so microvilli projecting from the cells became directly in contact with the lumen of sinusoids (Fig. 24a).

Treatment with selenium prior to MA administration was not associated with obvious ultrastructural changes except the damage of the endothelium of sinusoids, widening of intercellular spaces and periendothelial spaces in addition to microvillarization of the plasma membrane of the cells (Fig. 25).

Histochemistry :

Ascorbic acid, which is known to be necessary for corticosteroidogenesis is found as small dark brown granules after staining with the silver method and can be localized in all the cortical zones with relative abundance in zona fasciculata (Fig.26) . Examination of the cortical cells from animals administered MA revealed that there was marked increase of the ascorbic acid granules in groups fasciculata cells than those in the other groups . This increase was more prominent in zona fasciculata cells which lie near zona glomerulosa (Fig.27) . On the other hand, preparations from animals given selenium prior to MA didn't show considerable variation in their ascorbic acid content than the control (Fig.28) .

Acid phosphatase, the marker enzyme for lysosomes was demonstrated by the method of metallic ion precipitation of Gomori and the enzyme appeared in the form of black granules of variable sizes which appeared clearly in zona reticularis than the other zones . This might be attributed to the presence of more considerable amount of lysosomes in this zone (Fig.29) than the other zones. Treatment with MA invariably caused noticeable increase in acid phosphatase activity (Fig.30)as compared with the sections from control animals or with the examined sections from animals treated with selenium prior to MA administration (Fig.31) .

Dimensions of zones of the adrenal cortex :

The adrenal cortex of the mice used in the present study displayed significant changes in its thickness between the control and the different treatment groups . The thickness of the adrenal cortex in MA treated mice was found significantly decreased (320.8 um at $p < 0.05$) if compared with control (392.2 um) or with animals given selenium prior to MA (381.6 um) . Also the relative thickness of the major 2 layers ; zona fasciculata and zona reticularis showed significant changes if compared with control or with MA + selenium − treated animals (Table 1) . The thickness of zona glomerulosa in MA-treated animals didn't show significant changes ($p > 0.05$) if compared with the control or with MA+selenium treated animals ($p > 0.05$), while the thickness of zona fasciculata and reticularis decreased in a highly significant value ($p < 0.0001$) . On the other hand, the thickness of the whole adrenal cortex in animals treated with selenium prior to MA administration was found significantly ($p < 0.05$)larger (381.6 um) than that in the MA-treated (320.8 um) and was non significantly ($p > 0.05$) changed if compared with the control . Also, the thickness of zona fasciculata (210.8 um) and zona reticularis (113.2 um) in animals treated with selenium prior to MA was significantly ($p < 0.0001$) larger than in MA-treated (170 um and 86 um) respectively . In light micrographs , (Figs. 32-34) the difference

in the thickness of the adrenal cortex between the control and the MA-treated is so obvious, while it seems that there is no difference between the control and the MA+selenium - treated animals .

Table 1 : Mean diameter of the adrenal cortex and its major 3 zones ; zona glomerulosa, fasciculata and reticularis in µm in control and treated mice.

Region / Group	Adrenal Cortex	Zona Glomerulosa	Zona fasciculata	Zona Reticularis
Control Mean ±SD	392.2 ±37.83	61.8 ±1.30	215 ±5.38	116.4 ±1.14
MA- treated Mean ±SD P	320.8 ±19.11 $< 0.05^a$	64.2 ±2.16 $> 0.05^a$	170 ±2.74 $< 0.0001^a$	86 ±1.58 $< 0.0001^a$
Selenium + MA treated Mean ±SD P	381.6 ±45.11 $>0.05^a$ $< 0.05^b$	65.2 ±1.64 $< 0.05^a$ $> 0.05^b$	210.8 ±2.28 $> 0.05^a$ $< 0.0001^b$	113.2 ±2.38 $< 0.05^a$ $< 0.0001^b$

Non significant > 0.05
Significant < 0.05
Very highly significant < 0.0001
a ⟶ compared to control
b ⟶ compared to MA –treated

Explanation of figures

Figure 1.: Transverse section of the adrenal gland of normal female CD-1 mouse , showing the thick outer connective tissue capsule (CP), zona glomerulosa (zg) , zona fasciculata (zf) , zona reticularis (zr) , the mesh of fibres in zona reticularis (arrow head) and medulla (md) **.(Gordon and Sweet for reticulin X400)**

Figure 2: Semithin section of part of the adrenal cortex of a mouse treated with MA for 2 months . Signs of fibrosis in zona glomerulosa are quite clear and can be noticed as accumulations of fibrocytes forming thick sheaths (arrows) around the groups of cells. Cells are loaded with numerous lipid droplets (arrow heads). **(Toluidine blue X850)**

Figure 3: Paraffin section in adrenal cortex of a mouse treated with MA for 2 months stained with the silver method for reticulin showing the thick fibres formed surrounding the groups of zona glomerulosa cells. **(Gordon and Sweet for reticulin X850)**

figure 4: Semithin section in adrenal cortex of a mouse treated with selenium 1 hr prior to MA administration for 2 months . The cells of zona glomerulosa and fasciculata are still surrounded by sheaths of connective tissue. Arrows indicate fibrosis. **(Toluidine blue X850)**

Figure 5: Semithin section in adrenal cortex of a mouse treated with MA for 2 months, the profile is near a large vein showing abnormally formed and fragmented RBCs . Cells of zona fasciculata are massively loaded with lipid droplets (arrows). **(Toluidne blue X850)**

Figure 6:a - Electron micrograph of zona glomerulosa of control mouse showing ployhedral cells with microvilli projecting into the intercellular spaces (arrow) and periendothelial space (arrow head) , large nucleus with two nucleoli (asterisks) , and nume-rous darkly stained lipid droplets (thick arrow) . One of the cells shows apical vacuoles (V) due to endoplasmocrine secretion , while it contained small newly synthesized lipid droplets . **(X5000)**

b- Higher magnification of a part of the zona glomerulosa cell showing numerous vesicles of smooth endoplasmic reticulum, mitochondria with lamelliform cristae and Golgi apparatus (arrow) . **(X20000)**

Figure 7: Electron micrograph showing zona glomerulosa cell from a mouse treated with MA for 2 months . The cell possess shrunken nucleus with dense heterochromatin, and surruonded by a halo . Lipid droplets are more electron dense if compared with figures 6 and 13 **. (X7140)**

Figure 8: Electron micrograph of zona glomerulosa cells from a mouse treated with selenium before MA . The cells show the same profile like the control in figure 6, but the endothelial lining is damaged (arrow head) and one of the cells lost its microvilli (arrow)**.(X7500)**

Figure 9: Semithin section of adrenal cortex of a control mouse showing lipid droplets in the cells of zona glomerulosa (zg) and zona fasciculata(zf). **(Toluidine blue X850)**

Figure 10: Semithin section of adrenal cortex of a mouse treated with MA for 2 months , showing marked increase of lipid droplets in zona glomerulosa (zg) and in intact cells (arrow heads) of zona fasciculata (zf) . **(Toluidine blue X850)**

Figure 11: Fluorescence micrograph of frozen section of adrenal cortex of control mouse showing slight yellow fluorescence of lipid content in zona glomerulosa (zg) as compared with brighter fluorescence in zona fasciculata (zf) . **(X450)**

Figure 12: Fluorescence micrograph of frozen section of adrenal cortex of a mouse treated with MA showing increased yellow fluorescence due to the large amount of lipid droplets in zona glomerulosa (zg) while less fluorecence appear in zona fasci-culata (zf) in addition to empty spaces indicating loss of lipid droplets while intact cells showed brighter yellow fluorescence (arrow head) . **(X650)**

Figure 13: Fluorescence micrograph of frozen section of adrenal gland from a mouse treated with selenium prior to MA , showing the same yellow fluorescence like that in Fig. 9 , indicating no change in lipoidal content of zona glomerulosa (zg) or zona fasciculata (zf) . **(X450)**

Figure14: Electron micrograph of a zona intermedia cell from a control mouse, characterized by its dense-matrix mitochondria and plenty of smooth ER (arrow heads). **(X65000)**

Figure 15: Electron micrograph of zona fasciculata cells from a control mouse . The cells are large in size and contain large nuclei . Cytoplasm is filled with numerous smooth endoplasmic reticulum , spherical mirochondria (m) and large lipid droplets. Numerous microvilli project into the intercellular space (arrow heads) . **(X7500)**

Figure 16: A higher magnification of zona fasciculata cells from a control mouse, showing the spherical mitochondria with tubular cristae (m), numerous ribosomes (r) and large lipid droplets**. (X15300)**

Figure 17: Zona fasciculata cell from adrenal cortex of a mouse treated with MA for 2 months showing obvious necrosis . The nucleus is shrunken with a clear halo surro-unding it, large lipid droplets are degenerated and myelin figures (arrow heads) are found instead . Large areas of cytoplasm are degenerated **.(X6825)**

Figure18: Glycol methacrylate semithin section of adrenal cortex from MA-treated mouse, showing three pyknotic nuclei at the top (arrows). One nucleus shows shrinkage and a perinuclear halo (arrow head) . The lipid droplets take the dark green colour. **(Basic fuchsin-methylene blue . X2000)**

Figure 19: Electron micrograph of zona fasciculata cells from a mouse treated with MA showing, widen-ing of the intercellular spaces (arrow heads) and destruction of the endothelial linings of sinus-oids (arrows)**. (X4300)**

Figure 20: Electron micrograph of zona fasciculata cells from a mouse treated with selenium before MA. Most of the nuclei are irregular and several myelin figures are noticed (arrow heads) in addition to lipofuscin granules (arrows). **(X5200)**

Figure 21: Light micrographs of adrenal of a control mouse showing the appear-ance of histiocytes in fluorescent preparations (a) as orange fluorescent (arrows) and in semithin sections stained with basic fuchsin – methylene blue (b) differentiated by their red and blue granules. (Basic fuchsin-methyene blue) **(a) X 850 (b)X 2000**

Figure 22: Electron micrograph showing a zona reticularis cell. (a) From a control mouse with relatively large heteroch-romatic nucleus with 2 prominent nucleoli, lipofuscin granules (arrow heads), few microvilli and small Golgi apparatus (arrow). (b) higher magnifyication showing the cup shaped mitochondria in cross section surrounded by numerous smooth ER. **(a)X 10000 (b)X 20000**

Figure 23: Transverse section of adrenal gland from a mouse treated with MA for 2 months stained with silver method for reticulin showing prominent vacuolization in zona reticularis. **(Godron and sweet for reticulin X 250)**

Figure 24: Electron micrograph of zona reticularis from a mouse treated with MA for 2 months. (a)The cells lie adjacent to the sinusonid which has lost its lining (arrow), and they are heavily loaded with lipofuscins with their two char-acteristic components and large amount of lysosmes (arrow heads). Nuclei became irregular (asterisks).(b) Part of the cytoplasm showing atrophied Golgi apparatus (arrow heads) .**(a)X 10000 (b) X 12000**

Figure 25: Electron micrograph of zona reticularis cells from a mouse treated with selenium prior to MA. The intercellular spaces became wider (arrows), endot-helial linings of sinusoids are damaged (arrow heads), and more microvilli appeared (thick arrows) . **(X 7800)**

Figure 26: Transverse section of adrenal gland from a control mouse stained with the silver method for accorbic acid, showing dark brown granules of ascorbic acid **. (Silver method for ascorbic acid X 250)**

Figure 27: Transverse section of adrenal gland from a mouse treated with MA for 2 months and stained with silver method for ascorbic acid. The ascorbic acid granules are more numerous in this region of zona fasciculata (compare with the previous figure). The section is counterstained with Hx. **(Silver method for ascorbic acid X 250)**

Figure 28: Transverse section of adrenal gland from a mouse treated with selenium prior to MA. The distribution of ascorbic acid granules in zona fasciculata is nearly similar to the control in figure 26. **(Silver method for reticulin X 250)**

Figure 29: Transverse frozen section of adrenal gland in zona reticularis from a control mouse showing distribution of acid phosphatase which appear as black granules which are sometimes accumulated. **(Gomori method for acid phosphatase X 450)**

Figure 30: Tansverse frozen section of adrenal gland in zona reticularis from a mouse treated with MA for 2 months. The acid phosphatase activity inceased greatly in zona reticularis. **(Gomori method for acid phosphatase X 450)**

Figure 31: Transverse frozen section of adrenal gland in zona reticularis from a mouse treated with selenium prior to MA. The distribution of acid phosphatase is slightly less than that in control or that in MA-treated. **(Gomori method for acid phosphatase X 450)**

Figures 32-34 : Transverse sections of adrenal glands from control mouse (32) , MA-treated mouse (33) and MA+ selenium-treated mouse showing the difference in the thickness of the cortex between the 3 cases . **(Hx&eosin X250)**
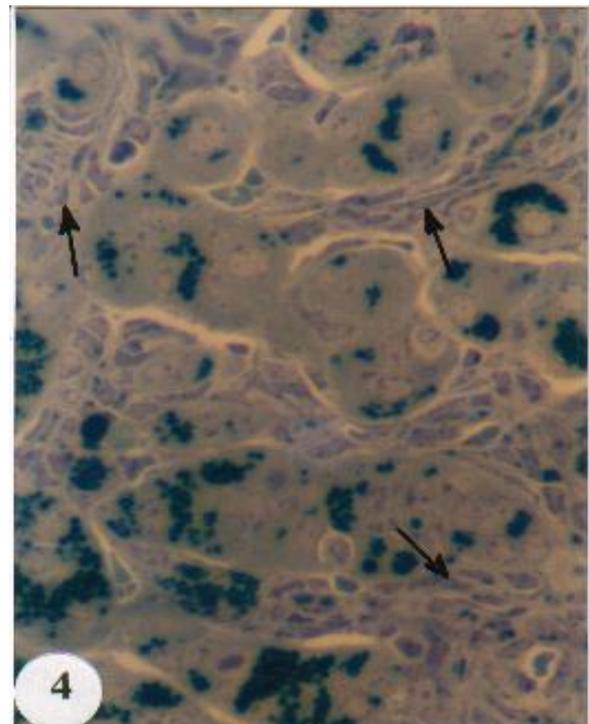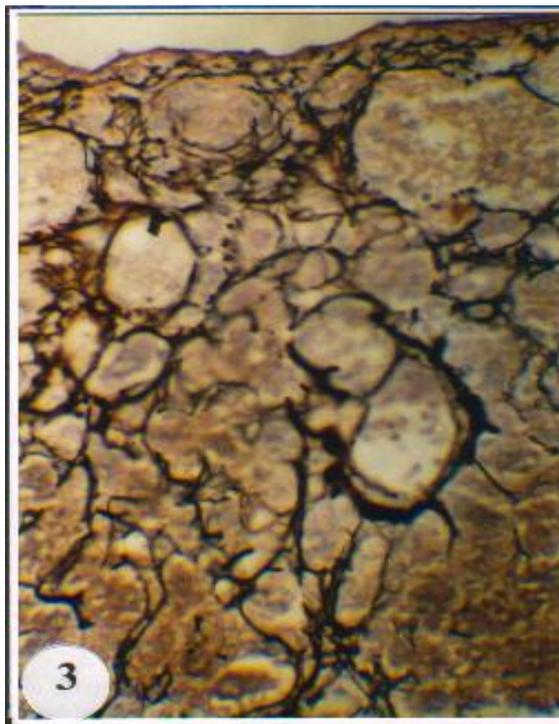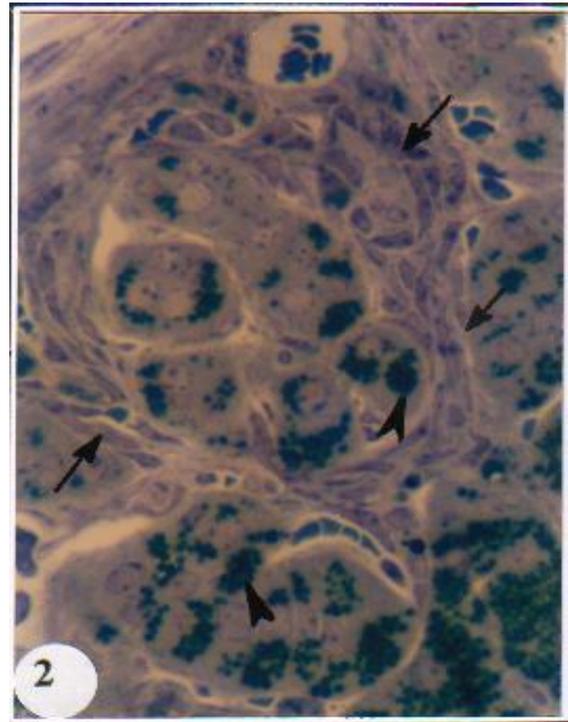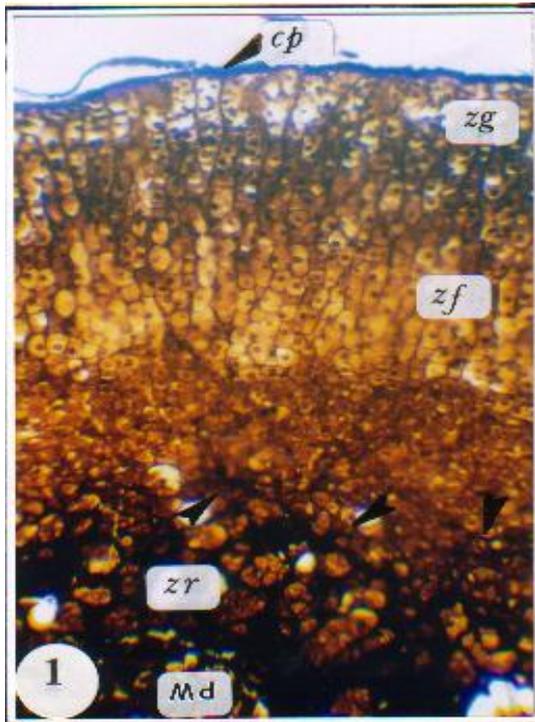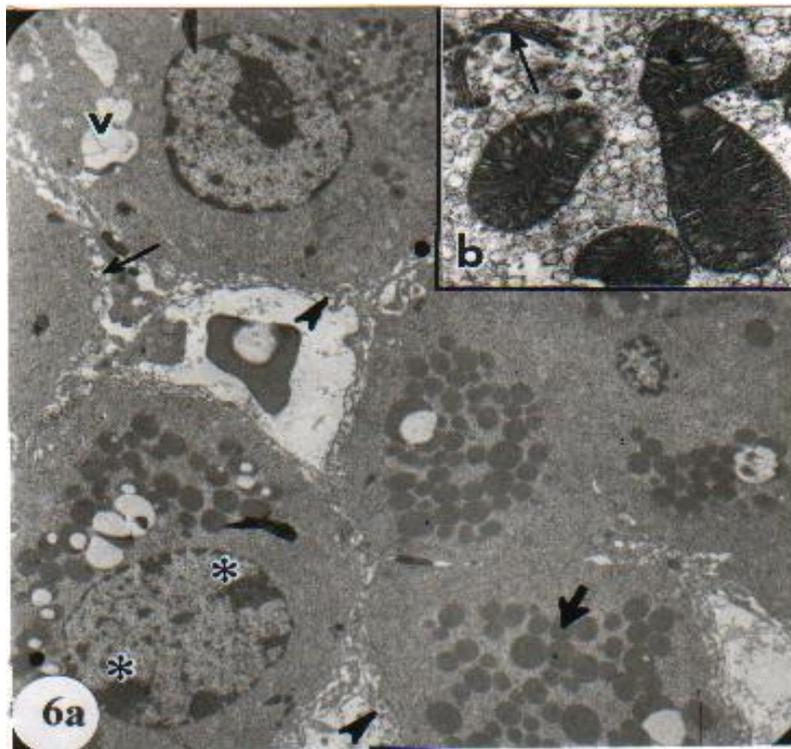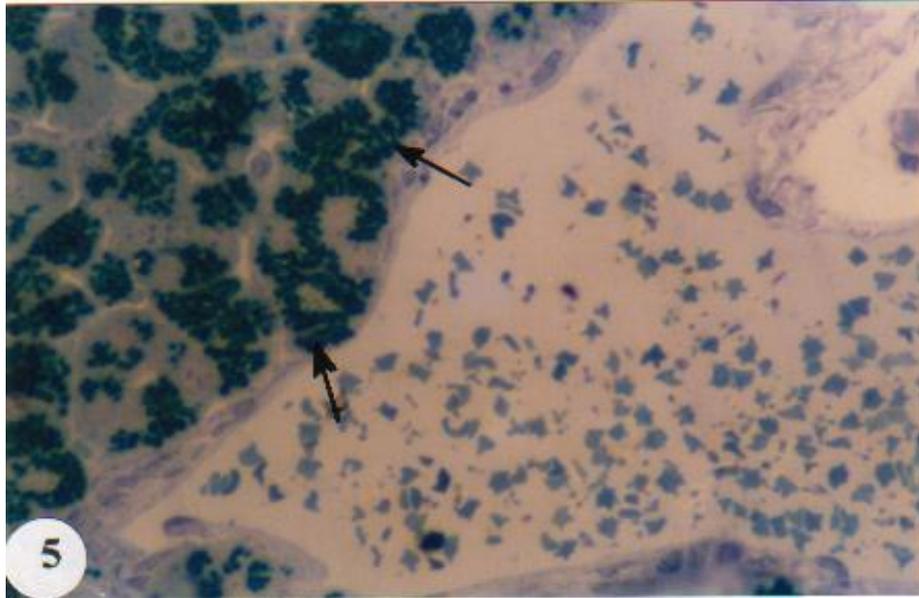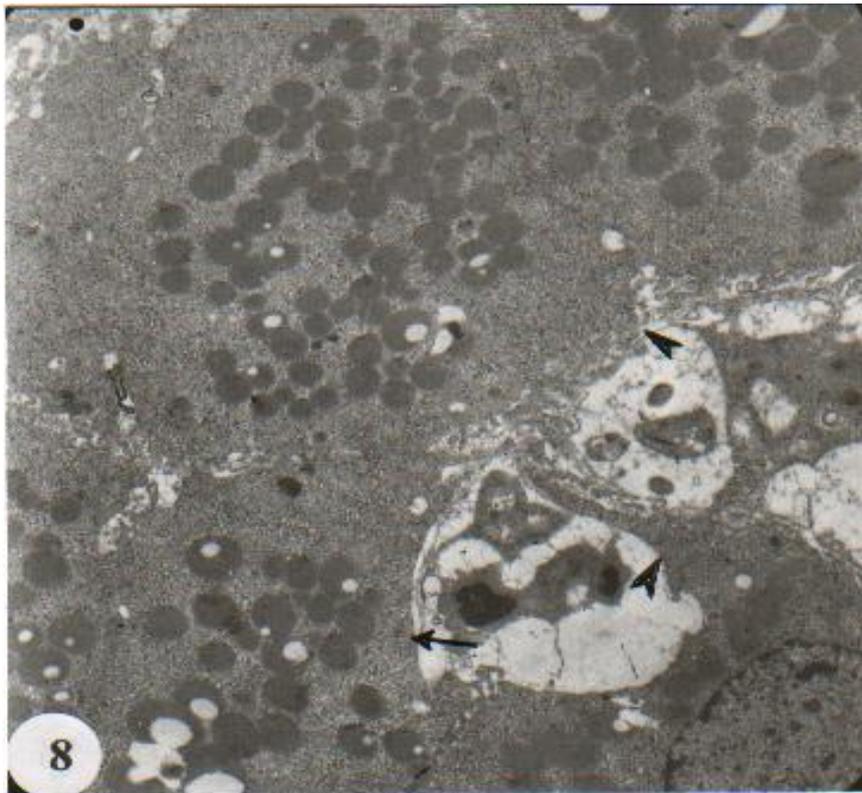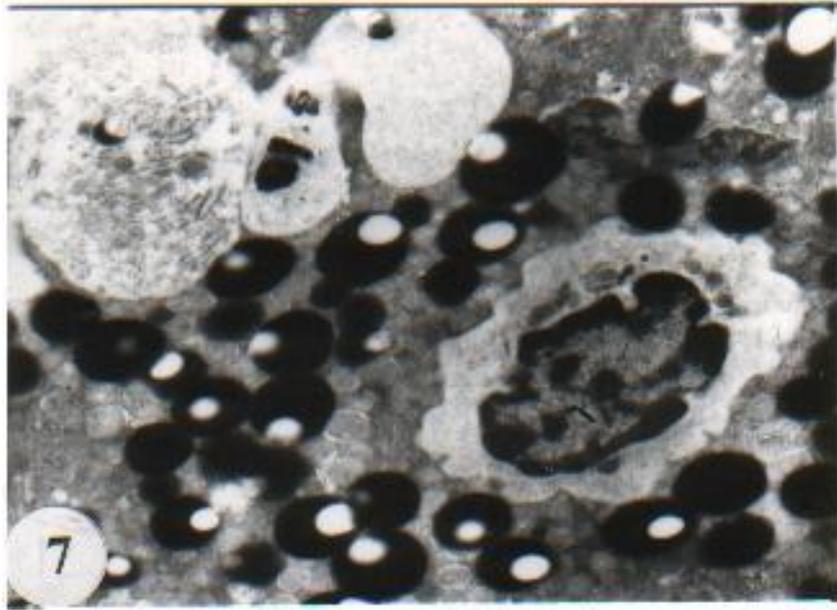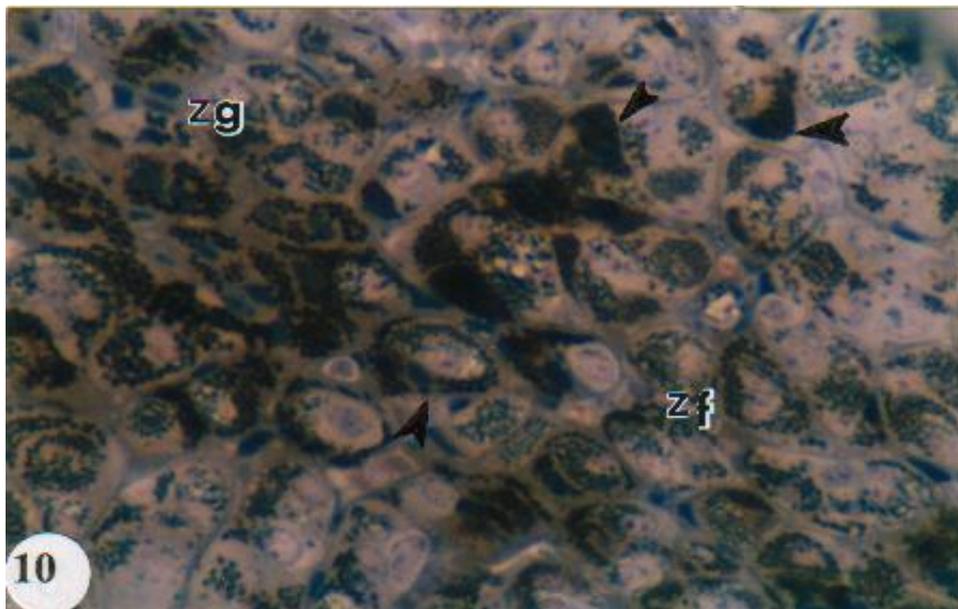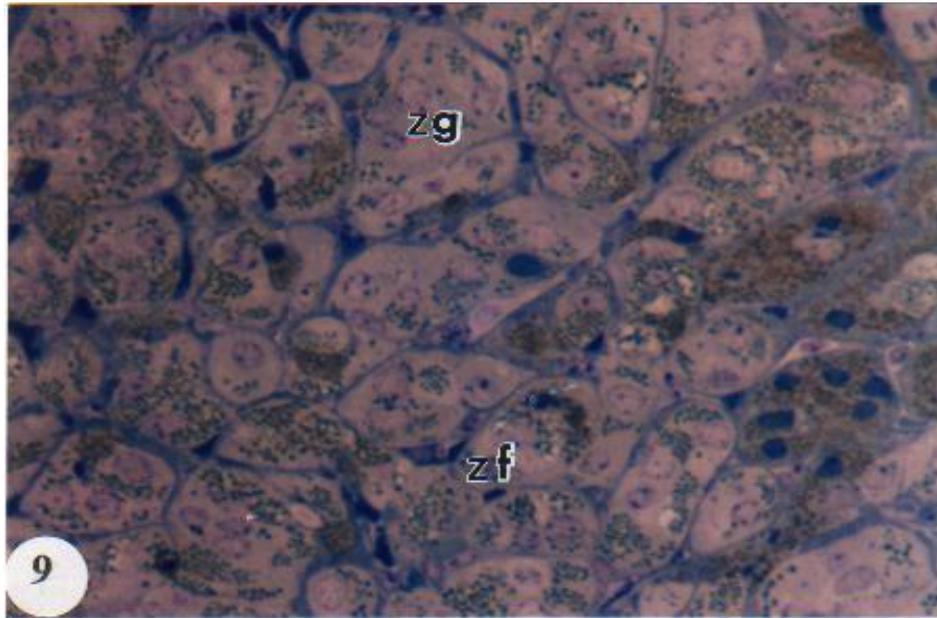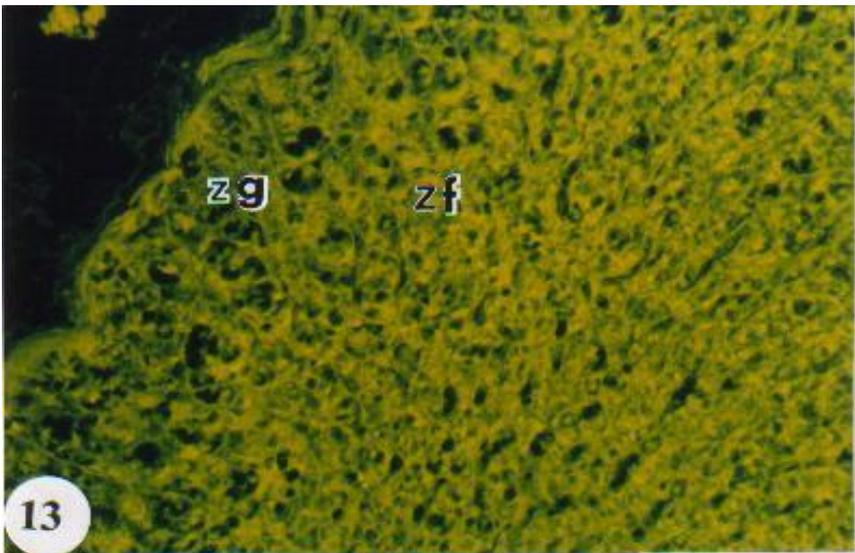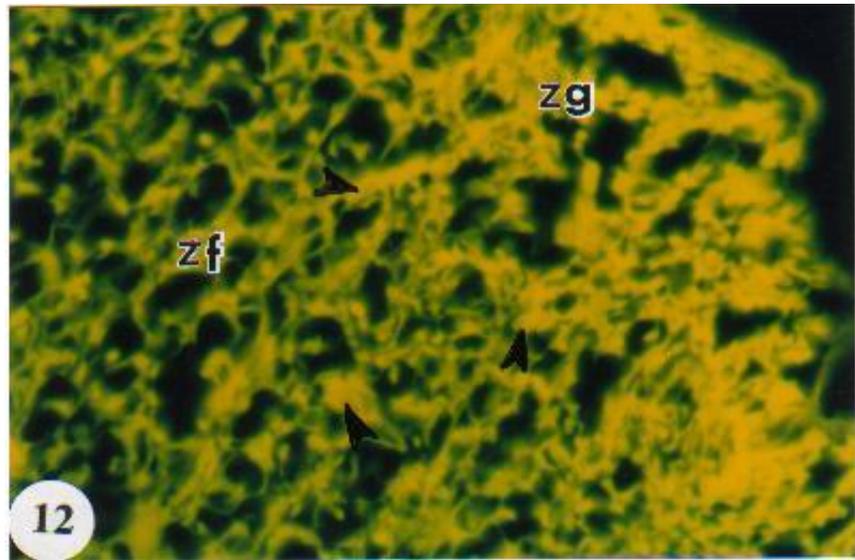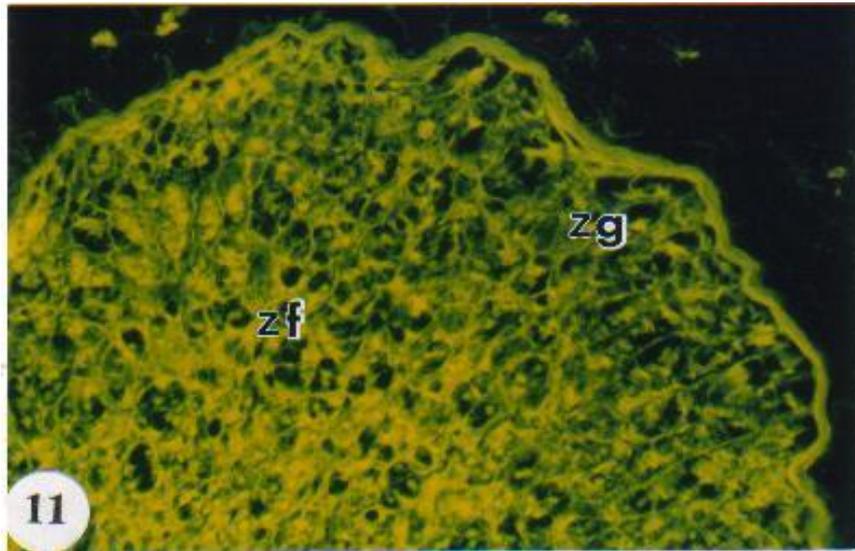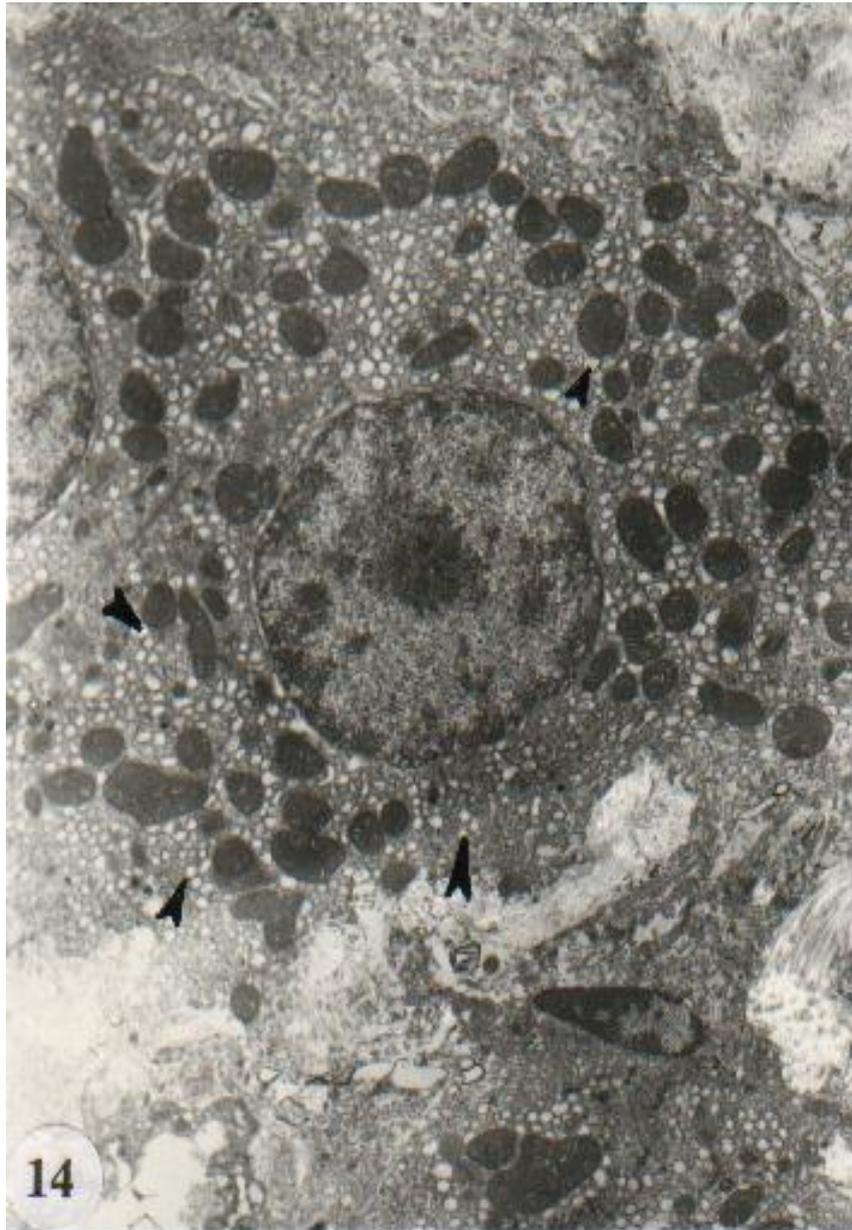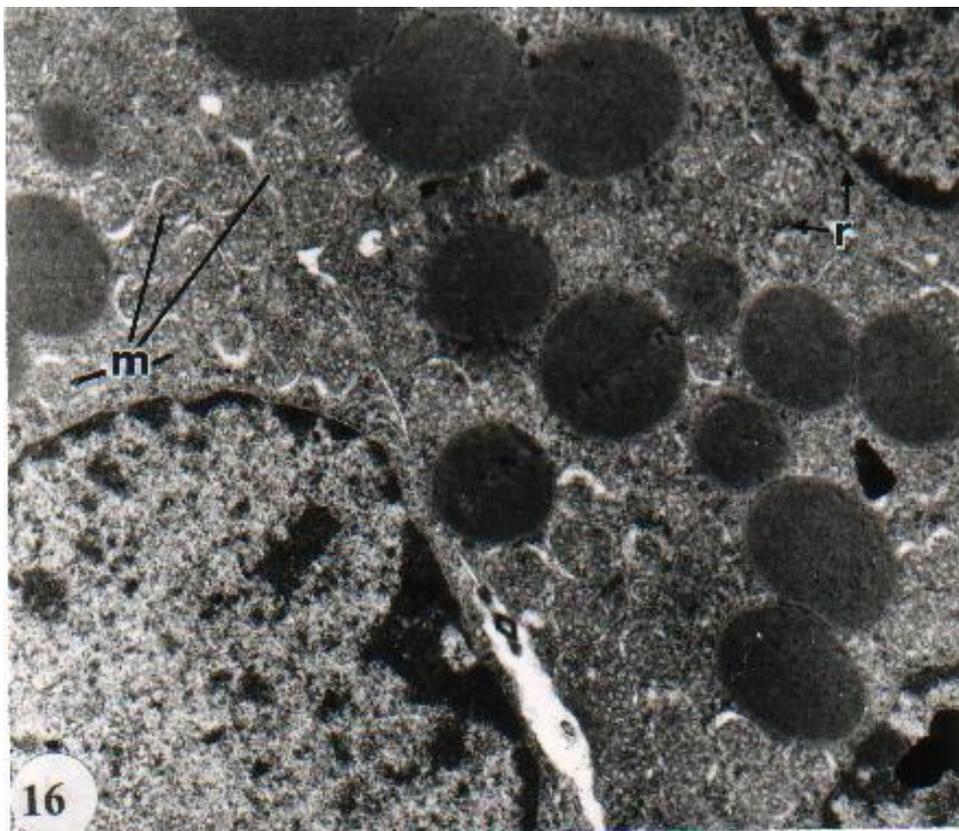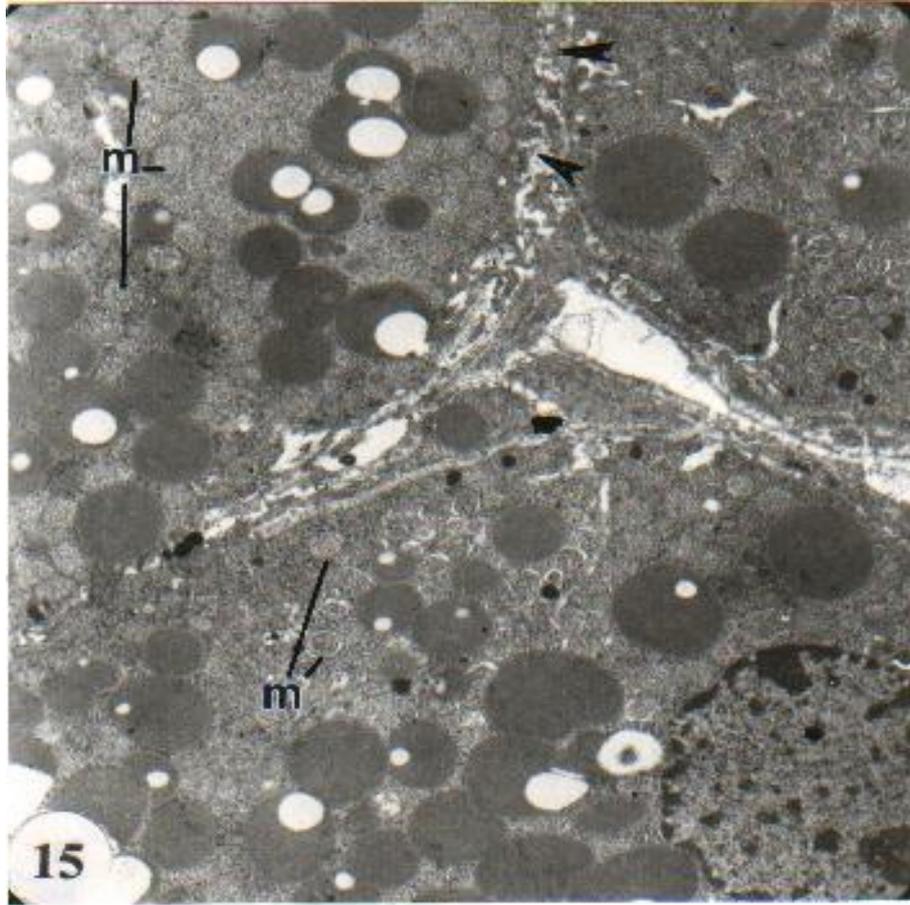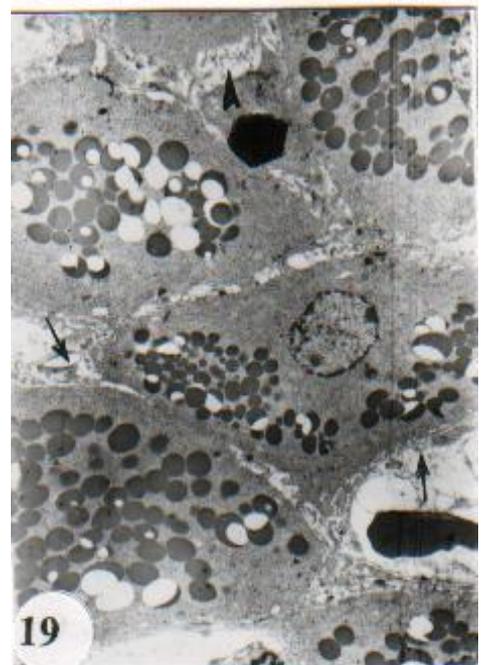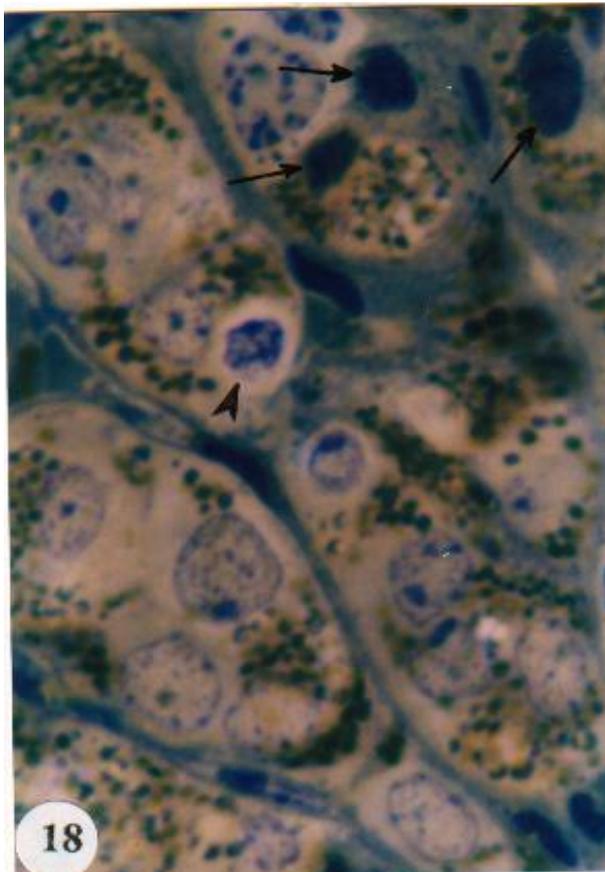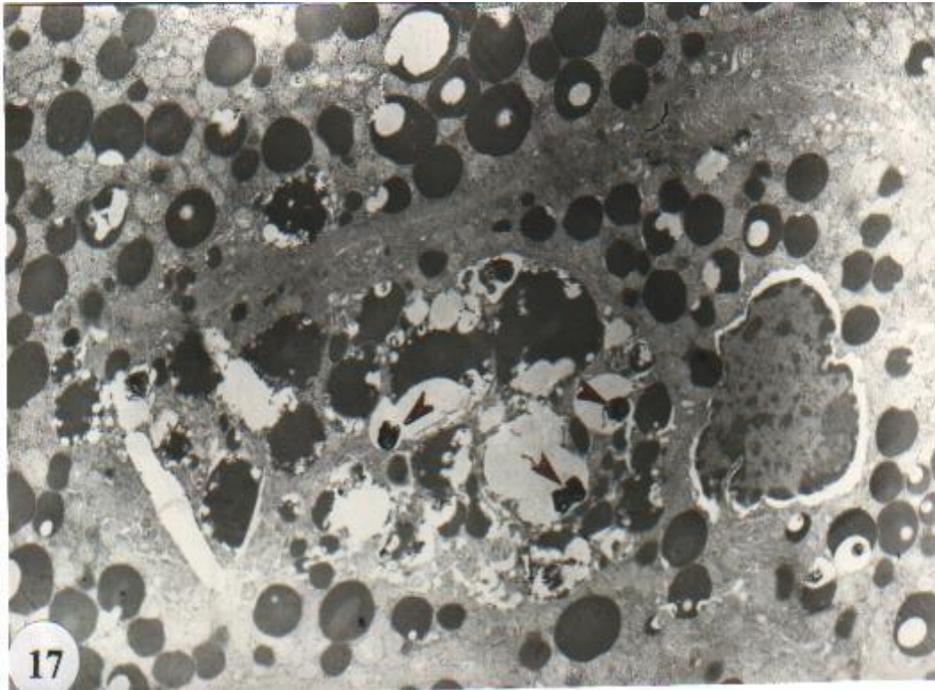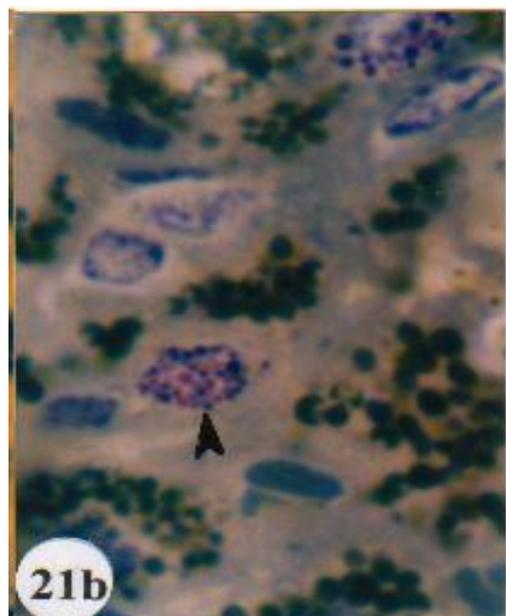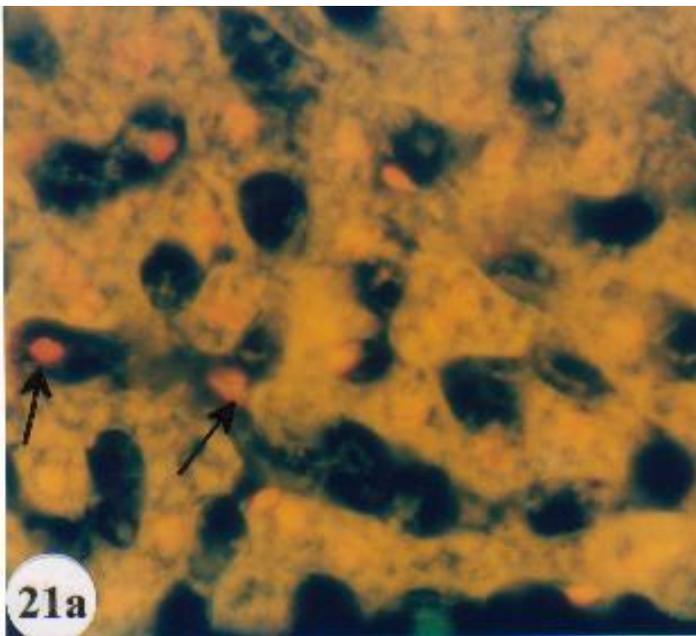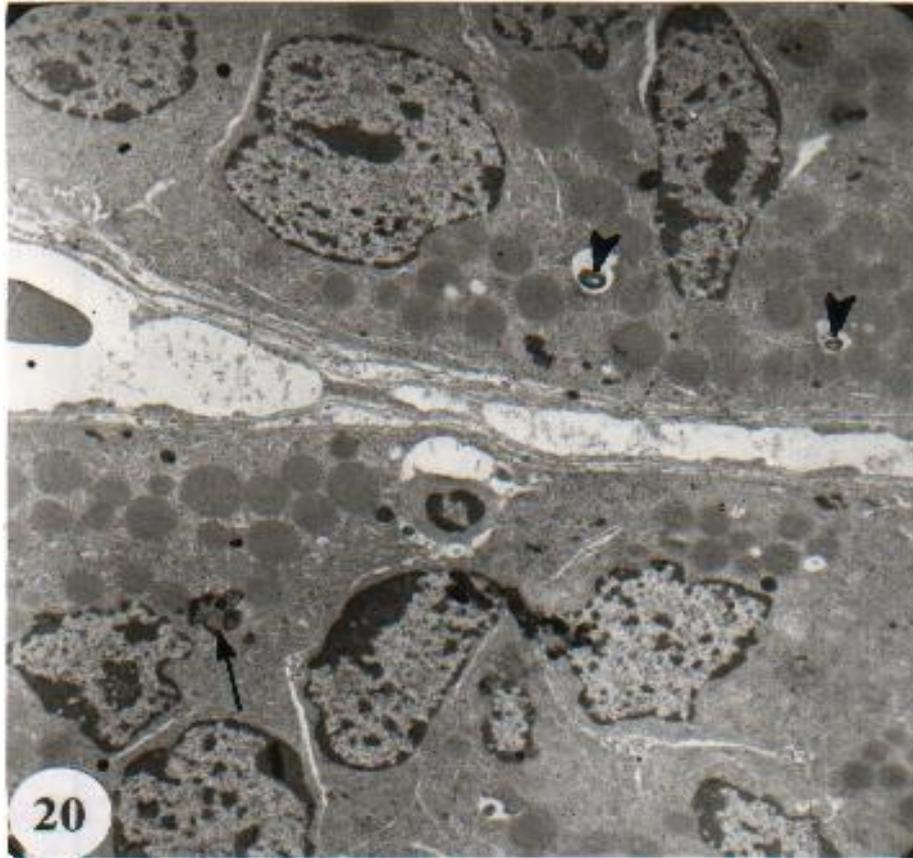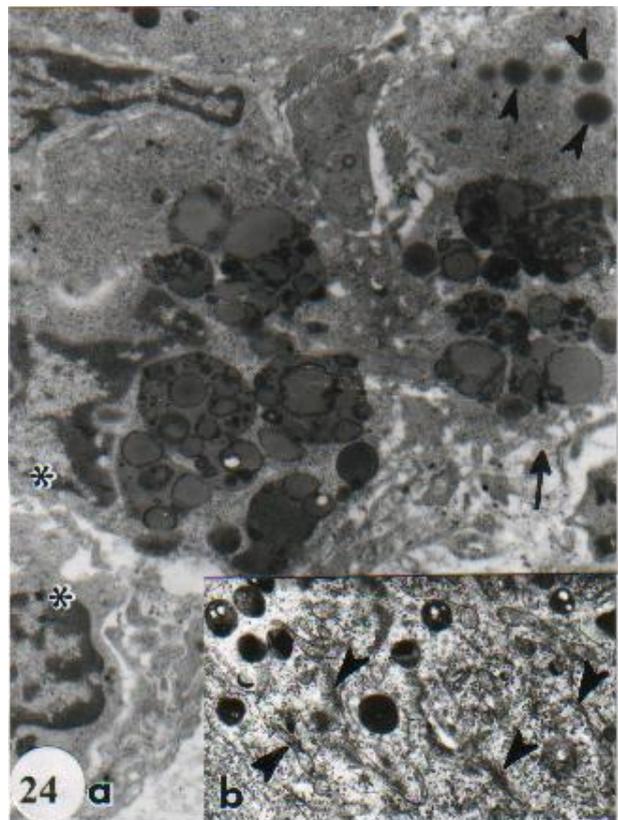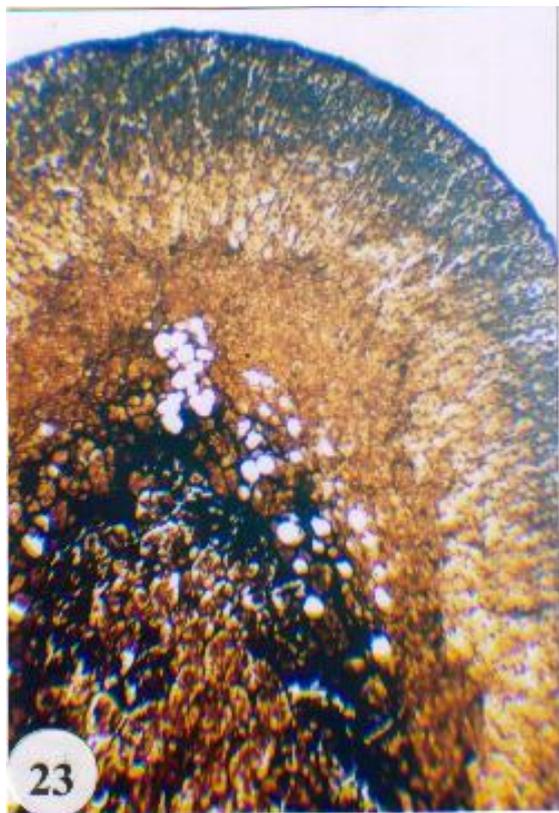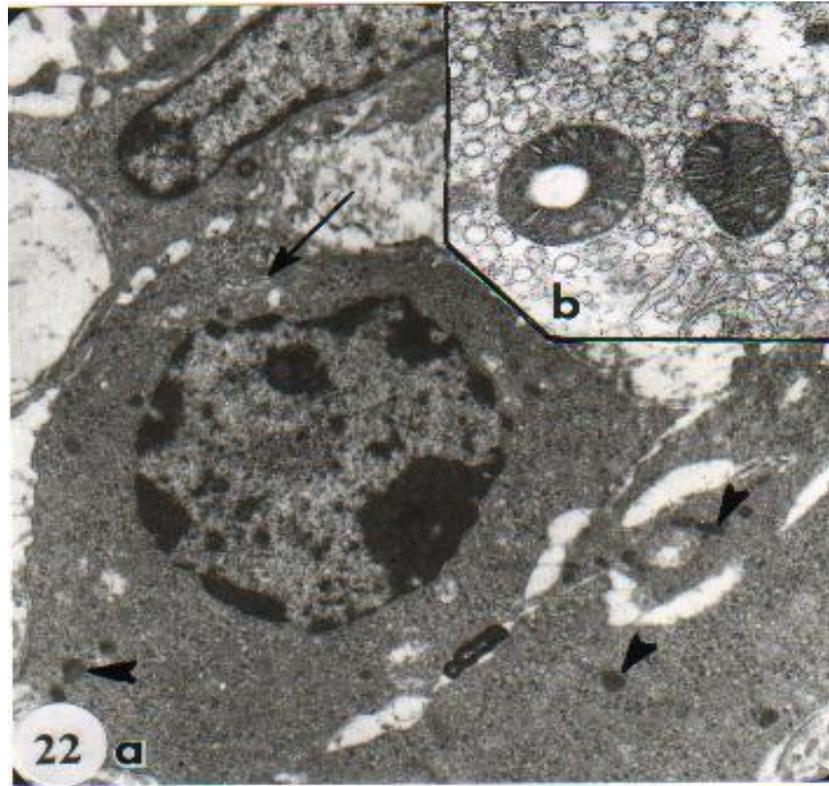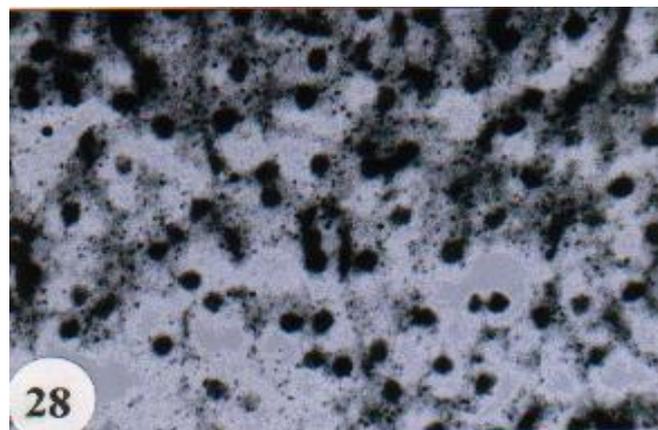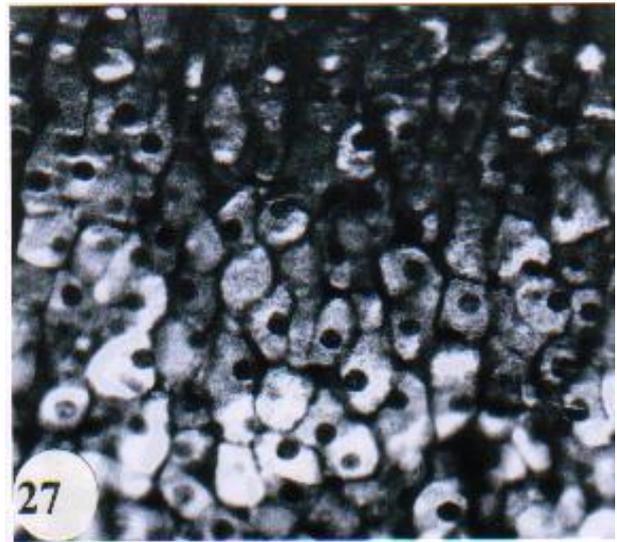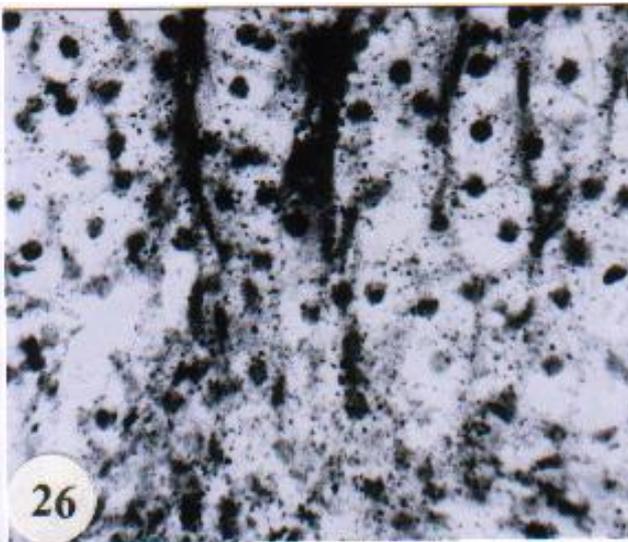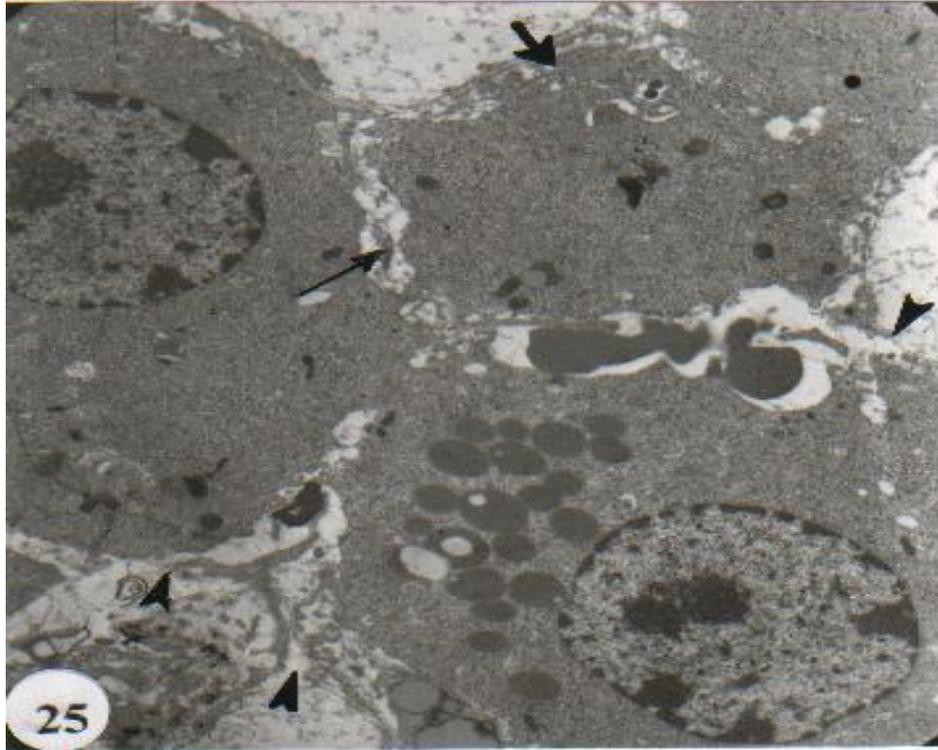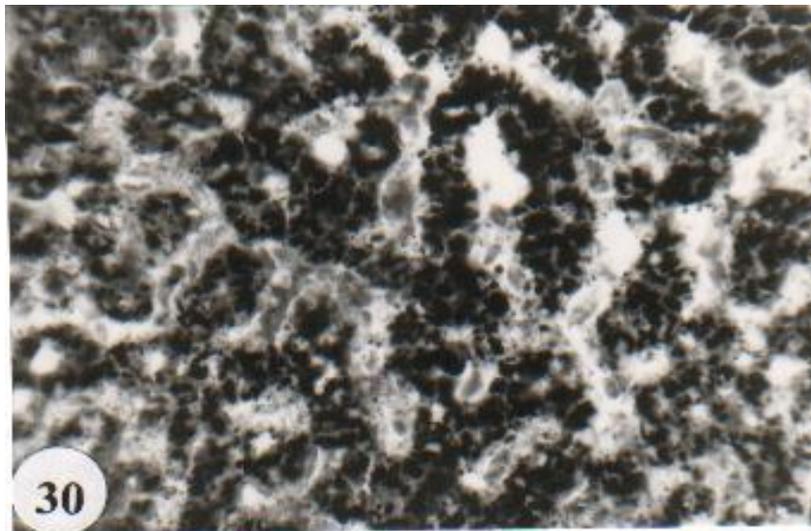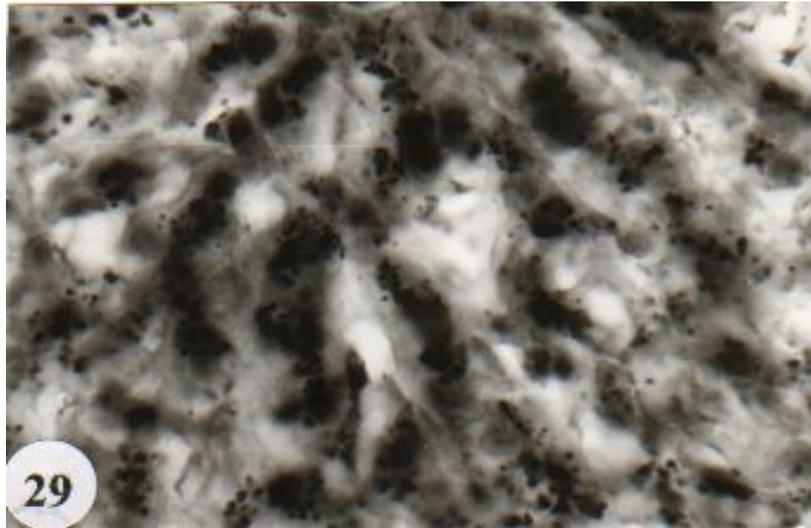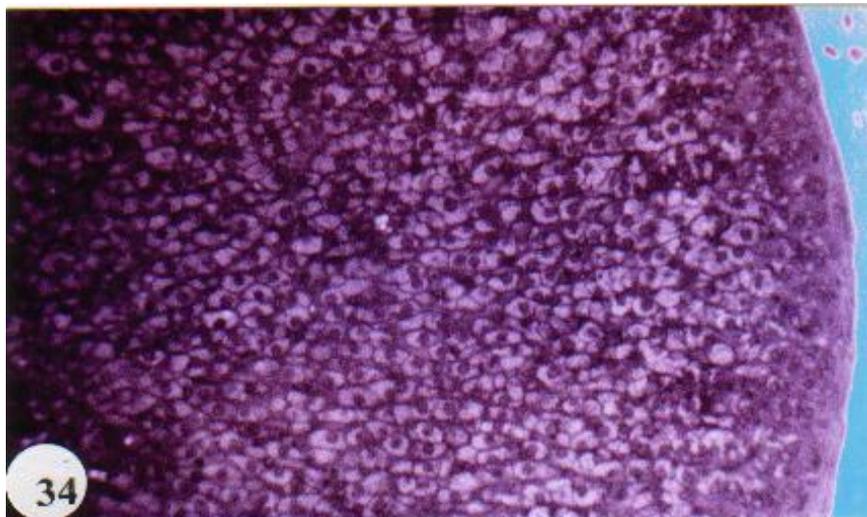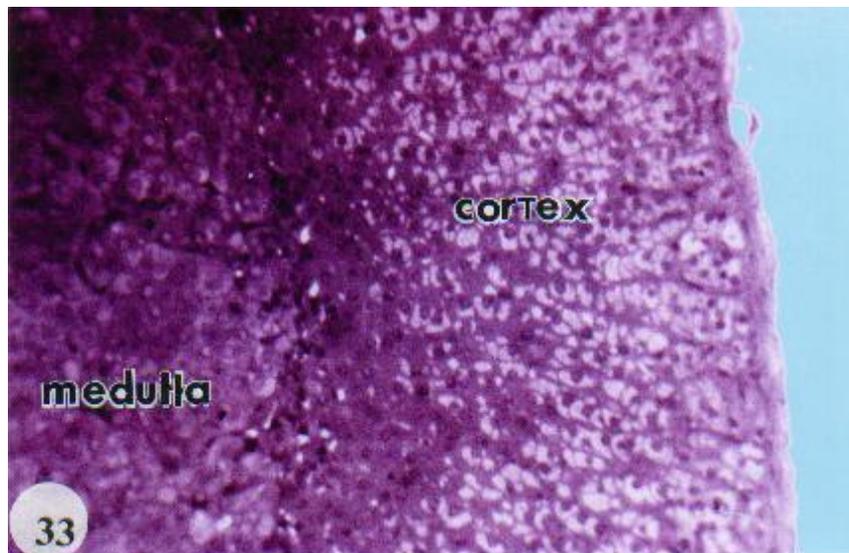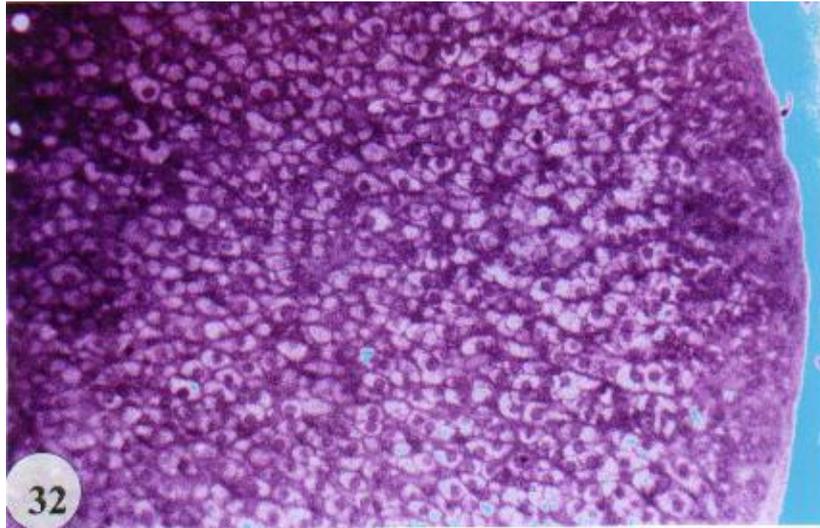
Discussion

The synthetic progestin MA used in the present study caused significant weight gain. This weight gain in primarily fat as it was found in the present study that excess adipose tissue accumulated between the mesentries, around the kidneys and covering the whole adrenals. Progesterone and other gestagens are well known to have an effect on the appetite and the energy metabolism. Gestagens induce increased food intake by direct stimulation of the appetite centers in the brain (Wade *et al*., 1979). Moreover, through interactions with cortisol receptors in the body, specific metabolites of MA were found to promote water retension (Harte *et al*., 1995). The megestrol acetate-associated weight gain made it invariably as one of the most commonly used drugs for treatment of AIDS-related weight loss as it mimic the actions of the naturally occurring female hormone progesterone (Brockman, 2004).

The increase of the connective tissue spaces containing connective tissue fibres in zona glomerulosa and fasciculata in the current study is to a great extent similar to the reported findings of Fujita (1972) in his study on the adrenal of rat after 90 days of hypophysectomy . Invasion of the adrenal tissue by fibrocytes was also recorded in chickens treated with the antiandrogenic drug cyproterone acetate (Wahba and Ezzat ,1988) .Generally fibrosis may result from the drugs that can have the capability to cause cell injury specially in the organs that have a fibrous network in their cytoarchitecture (Scheuer,1980) and the adrenal gland is one of them . Recently, it was reported that MA administration in adult mice was associated with the appearance of signs of fibrosis , where excess of collagen invaded the hepatie tissue (Sakr,2003) .

The apparent increase in lipid accumulation during MA treatment is expected to lead to an adverse effect on vascular system. Therefore, MA was reported to cause arterial occlusions and hypertension, followed by abnormal renin secretion. Moreover, a large degree of left ventricular hypertrophy was recorded as a side effect of MA administration (Brockman,

2004). Also, Aisner *et al*. (1987) reported that high doses of MA may cause congestive heart failure and blood pressure elevation. Even, low doses of MA (40 mg/day) were reported to cause vaginal bleeding and increased serum albumen levels (Lien and Ruffenach, 1996). Incidence of deep vein thrombosis was very high (88%) among patients treated with MA (Bennett, 2003), therefore, the latter author recommended that vascular examination of the patients should be performed regularly during treatment. Along with this line of evidence of the vascular problems, the present investingations showed that MA administration was associated with the appearance of malformed and fragmented blood corpuscles which can be suggested to lead to occlusions of the blood vessels. Recently, Brockman (2004) reported that thromboembolic events, hyperglycemia and hypertension are some of the unavoidable side effects of using MA for cancer anorexia cachexia, and all these events are expected to lead to vascular problems . On the contrary other authors (Schacter *et al*., 1989 and Qin *et al*., 1996) defended that MA is a safe drug and effective as a medical alternative to surgery for endometrial hyperplasia and has a beneficial influence on chronic obstructive pulmonary disease, respectively. Schacter *et al*. (1989) also considered MA as an ideal contraceptive as it inhibits sperm transport, but they didn't deny that it causes irregular vaginal bleeding. Moreover, Yeh *et al*. (2000) reported that there were no complications of MA treatment.

Alterations in the adrenal lipid content are usually considered as indication for the changes in secretory activity of the gland and changes in droplet size and number may reflect utilization (Weiss, 1988). Adding to this, cholesterol which is the precursor for steroid biosynthesis is stored within the lipid droplets. So it can be speculated that the marked increase of lipid content in the adrenocortical cells here in this study indicates suppressed steroidogenesis. Also, the frequent appearance of

myelin figures in MA-treated animals might indicate cellular degradation or change in lipid metabolism (Tseng, 1980). The same conclusion was reached by Wahba and Ezzat (1988) when they found an increase of lipid content of adrenal gland in chickens after the administration of cyproterone acetate. The appearance of vacuolization in deeper areas in zona reticularis can be considered as fatty degeneration because this layer particularly contained large masses of lipofuscin granules which can be dissolved during the long term dehydration process in preparing paraffin sections. This is why such vacuoles were not observed in electron microscopical preparations or in semithin sections where the time of dehydration is too short.

In the study made by Spellacy *et al.,* (1976), they reported that carbohydrate and lipid studies during six months of MA treatment in human showed no significant changes in lipid or carbohydrate metabolism. But it should be noted that the dose they used was very low for human (0.5 mg/day) if compared with the therapeutic dose (160 mg /day) which we used its equivalent for the mice in the present study

The present data showed that there is large amount of ascorbic acid in the adrenocortical cells and cells of zona fasciculata are more rich in ascorbic acid than the other zones of the cortex . It is well known that the highest concenrtration of stored ascorbic acid is in adrenal gland ; however, the liver stores the largest amount of ascorbic acid in the body because of its relative size to the rest of the body organs (Sackler ,1982) . The ascorbic acid plays a very important role in the synthesis of neurotransmitters and in corticosteroidogenesis . The correlation between ascorbic acid and the steroid dehydrogenase (D-5,3-hydroxysteroid dehydrogenase) was reported by Rao and Susheela (1979). They found that sodium fluoride caused a reduction in ascorbic acid content as well as a depletion of the steroid dehydrogenase activity . Also , ascorbic acid affects the activities of the enzymes responsible for B-hydroxylation of dopamine and amidation of peptide with c-terminal glycine for the biosynthesis of norepinephrine and

conversion of inactive precursor to active hormones respectively in adrenal gland (Padh, 1990). Therefore changes in ascorbic acid content have been used as an index of adrenocortical activity as it is reduced by ACTH   (Jones and Henderson , 1978) . Ezzat (1980) reported in his study on the effect of ACTH on adrenal gland of reptiles that this hormone caused marked depletion of ascorbic acid associating induction of corticosteroi-dogenesis . The current study showed that the ascorbic acid content increased prominently specially in zona fasciculata which is the major locus for the synthesis of corticosteroids . This might indicate that corticosteroidogenesis was suppressed because the ascorbic acid is not utilized but accumulated. In this concern, the present study is in agreement with the previous reports introduced about the possible adrenal suppression caused by MA treatment (Leinung *et al.,* 1995; Subramanian *et al*., 1997; Stockheim *et al*., 2000).

The acid phosphatase enzyme which is a marker for lysosomes showed apparent increase in MA-treated animals (specially in zona reticularis) if compared with the control or with those given selenium prior to MA-administration. This increase is related to the increased number of lysosomes noticed in zona reticularis, which might reflect a cytotoxic response due to the accumulation of excess lipofuscin granules in these cells. Also, it might be related to a possible role played by this enzyme in fat metabolism or in apoptotic changes preparing to cell death (Potten, 1987). Worthy to mention that one of the remarkable features noticed in MA-treated animals is the increase of acid phosphatase activity as well as increase of lipofuscin granules,specially in the cells of zona reticularis . A correlation between the increase of acid phosphatase activity and the accumulation of lipofuscin granules has been mentioned before (Ives *et al.,*1987) . A similar finding was also reported by Koike *et al*. (2000), where they found lysosomal storage within ceroid lipofuscin in mouse CNS neurons . Moreover, lipofuscin granules were considered to accumulate within the lysosomal system of a variety   of postmitotic cells throughout

life and this is considered a biomarker of ageing (Wassell *et al*.,2000) . Also, the latter authors confirmed that lipofuscin granules in retinal pigment epithelium induce lipid peroxidation in the presence of visible light , and thus contribute to aging or death of the cell .

Most of the enzymes involved in steroidogenesis are located in the microsomal fraction, which in steroid-secreting cells is composed largely of the ER, chiefly the smooth ER. However, enzymes for B-hydroxylation oxidation and sulfation necessary to produce glucocorticoids are located in the inner mitochondrial membranes and Golgi apparatus (Weiss, 1988). In the present study the Golgi apparatus was found atrophied in some of the adrenocortical cells in MA-treated animals, and this by its turn might lead to decrease in steroidogenesis and hence lower adrenal function. Similarly, atrophy of Golgi apparatus has been noted in hepatocytes subjected to a variety of toxic influences . On the contrary, Golgi apparatus showed atrophy in adrenocortical cells when they were stimulated during experimentally induced regeneration (Ghadially,1988) . Along with this line of reasoning it was found that one of the remarkable features noticed in the cells damaged due to MA treatment was the separation of the two nuclear membranes which means that these cells lost the functional nuclear pores which are potential pathways of nucleocytoplasmic exchanges, crucial for the biosynthesis of cell products (Ghadially, 1988). Moreover, MA was reported to have a genotoxic effect and generates DNA adducts (Werner *et al*.,1997) . Also the present recorded ultrastructural changes were expected, since previous reports stated that similar oral contraceptives ;ethinylestradiol and norethisterone acetate were found to cause ultrastructural lesions in different rat tissues, which by its turn may impair biosynthesis and energy production processes, and may simultaneosly enhance cellular catabolism (Czekaj *et al*.,1999) .

Herein it was demonstrated that the diameter of the adrenal cortex in MA-treated animals, showed a significant decrease when compared with the control animals or with those given selenium prior to MA administration . However the diameter of zona glomerulosa did not show any significant change in the two treated groups if compared with the control . On the other hand zona fasciculata and reticularis displayed a highly significant change (decrease in diameter)between the MA-treated group versus control and between MA-treated group versus MA+ selenium -treated group . But it has to be recorded that there were no significant change in case of comparing the control with the MA+ selenium treated group .

The decrease of the volume of the adrenocortical tissue is an indication of the decreased adrenocortical activity . This result is in agreement with previous results recorded in chickens treated with the antiandrogenic drug cyproterone acetate (Wahba and Ezzat ,1988) and is similar to those obtained in case of inhibition of the normal adrenocortical secretion caused by hypophysectomy in rat (Weiss,1988). Moreover, Tisell and Salander (1995) found that MA- administration at higher doses approximately 200 times greater than that used in the treatment of human prostatic disease was followed by involution of the adrenal glands when applied on rats .

In a trial to explain the present obtained results it can be concluded that MA works in two main ways . Firstly it has a direct toxic effect on the cells of the adrenal cortex which might lead to impairement of the functions performed by these cells . Secondly, MA as a synthetic progestin will raise the blood level of progesterone , so in turn , the adrenal-hypothalamic or pituitary axis will switch off as a negative feed back mechanism leading finally to adrenal hypofunction . This conclusion agrees with similar opinions mentioned by Naing *et al*. (1999), where they cocluded that adrenal suppression caused by MA is thought to be due to its effect at the hypothalamic level .

The data in this study indicated that selenium played a protective role to a great extent. In most of the adrenocortical cells the histological, histochemical and ultrastructural investigations showed great similarity between the control and those

given selenium prior to MA. This might be due to the protective role played by selenium to inhibit the chain reactions of lipid peroxidation and thus reduce the peroxide levels (Cuo,1996) which are normally expected to be very high in such fat ladden cells of the adrenal cortex . In agreement with the conclusion obtained from the present study that selenium has played a protective role on adrenal gland , it is found that most of the drugs prescribed for the treatment of adrenal stress or insufficiency contain selenium as a major ingredient (Wilson, 2003) . Selenium is an essential nutrient which acts as a cofactor for the glutathione peroxidases. Furthermore, glutathione peroxidases are considered as strong anticancer agents within the body ,therefore, the scientists focus now on selenium as an anticancer agent (Combs, 1997) . The latter author found significant lower risk to cancer associated with the use of a regular selenium supplement . Recently, it was reported that selenium found in natural food, like nuts, broccoli and mustard green is efficient in stimulating the immune system (Konlee, 2002) . Moreover, selenium metabolism itself may initiate changes that lead to programmed cell death of cancer cells and precancerous cells (Rang *et al*.,2003) . So it can be concluded that in the present experiment selenium might has played the same protective role to keep the adrenal gland in nearly normal structure and function . It can be suggested that selenium should be taken as supplement during the long term treatment with megestrol acetate .

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تثبيط الغدة الكظرية المستحدث بواسطة عقار خلات الميجسترول و الدور الوقائى المحتمل للسيلينيوم
دراسة نسيجية و كيميائية نسيجية و تركيبية دقيقة

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توجد بعض الشكوك حول امكانية حدوث انخفاض فى كفاءة الغدة الكظرية نتيجة لتعاطى عقار خلات الميجسترول المستخدم لعلاج سرطان الثدى و البروستاتا . وقد أشارت بعض التقارير الطبية الى أن العقار يمكنه أن يحدث تثبيط لوظائف الغدة الكظرية عند تعاطيه لفترات طويلة وقد صممت الدراسة الحالية لتقييم هذا الموضوع باستخدام الطرق الهستولوجية و الهستوكيميائية والتركيبية الدقيقة . وقد أظهرت النتائج المتحصل عليها من هذه الدراسة أن العقار قد أحدث فعلا العديد من الأضرار الخلوية والتحت خلوية فى معظم خلايا المناطق الثلاث الرئيسية لقشرة الغدة الكظرية . وقد أوضحت الفحوصات الهستوكيميائية أن استخدام هذا الدواء أدى الى زيادة المحتوى الدهنى ومخزون حامض الأسكوربيك و إنزيم الفوسفاتيز الحامضى , و كل هذه النائج قد تعكس حدوث حالة تثبيط لعملية تخليق الاستيرويدات . ولقد أشارت النتائج أيضا الى أن السيلينيوم له تاثير وقائى مضاد للسمية الخلوية لعقار خلآت الميجسترول على خلايا قشرة الغدة الكظرية . ولقد نوقشت النتائج و يستنتج أنه يجب اجراء متابعة لوظائف الغدة الكظرية أثناء تناول هذا العقار لفترات طويلة و يقترح استخدام السيلينيوم كدواء اضافى للحماية .