Interaction Between Maternal Chrysin Intake and Development of Adrenal Gland in Rat Fetus during Organogenesis

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ABSTRACT

Background: Chrysin is a flavone that occurs naturally in plants and possesses antioxidant and anti-inflammatory effects. **Objective:** This study was carried out to examine the effect of chrysin on the adrenal cortex of maternally treated rat fetuses. **Material and methods**: Twelve pregnant rats were assigned into two groups (6 rats each). The first group acted as a control and received a vehicle 1% w/v Tween 80 (the drug's solvent) (GDs 7-14), whereas rats of the second group were treated orally with chrysin (50 mg/kg body weight/day) daily from day 7 till day 14 of gestation. Animals were sacrificed, and samples of the adrenal glands were taken, processed, and prepared for the histological and ultrastructural examination. **Results:** The three cortical zones, Zone glomerulosa, Zone fasciculata, and Zone reticularis in the cells of the adrenal cortex sections from maternally treated rat foetuses showed cytoplasmic vacuolation. Some of these cells' nuclei showed indications of karyolysis. Cortical cells had clear alterations at the ultrastructural level, including cavitated and deformed mitochondria as well as dilated smooth and rough endoplasmic reticular membranes. Accumulation of lipid droplets and lysosomes was seen in the vacuolated cytoplasm. These cells' nuclei had karyolysis visible. **Conclusion**: It appears that oral administration of chrysin caused damaging effects on adrenocortical cells of maternally treated rat fetuses. Therefore, it should be taken into mind and viewed with great worry that chrysin, although one of the flavonoids, should be utilized with caution during pregnancy.

Keywords: Fetus, Estrogen, Adrenal cortex, Chrysin, Histology, Ultrastructure.

INTRODUCTION

Plant flavonoids have recently piqued both scientific and public attention due to claims that they provide health benefits [1]. Chrysin is a naturally occurring flavone that is frequently present in plants. Its anti-inflammatory and antioxidant properties have been researched [2].

Most research indicated that chrysin inhibits aromatase activity. Aromatase, also known as cytochrome P450, had a significant influence on cell growth and the cell cycle and allowed for the production of excess estrogens from testosterone. Because of their enhanced protein stability and lower bioavailability, chrysin solid lipid nanoparticles allow for long-term therapeutic delivery [3]. According to a study by **Balta** *et al.* [4] chrysin has a therapeutic impact on liver fibrosis brought on by carbon tetra chloride (CCl4). Also, Chrysin has high bioavailability in the low gastrointestinal tract so it is effective in treating conditions affecting the ileum and colon such as cancer and local infections [5]. Chrysin not only provides a defense against interstitial fibrosis and renal impairments brought on by Cyclosporine A (CsA) [6], but also inhibits D-gal-induced oxidative stress, inflammation, apoptosis, and ovarian damage [7], suggesting that chrysin may play a role in the prevention of premature ovarian failure.

Physiological changes during pregnancy, along with the demands of a growing human fetus, such as increased metabolic demands, and lack of essential vitamins and minerals might be harmful to both parties' health [8]. There is a strong correlation between maternal malnutrition and the mortality and morbidity of fetuses [9]. Pregnant women may turn to consume nutritional supplements, vitamins, and antioxidants in an attempt to enhance their health and the health of their fetuses; nevertheless, antioxidants may harm the fetuses.

The adrenal cortex is a crucial mammalian endocrine organ that produces a variety of hormones. Pregnancy must be maintained to encourage fetal growth and nervous system development [10]. Additionally, it is crucial for the fetus's growth and development after delivery. The adrenal cortex's improper functional growth will result in an abnormal production of steroid hormones, which will lead to the development of associated disorders [11].

Despite the previously mentioned therapeutic benefits of chrysin, the developmental period at which exposure takes place is a significant matter; for example, its usage during pregnancy is an important subject that requires further investigation. So, the study tried to illustrate whether the beneficial impacts of chrysin as proven in adults extend to fetuses or not by focusing on the embryonic development of one of the most essential organs, the adrenal glands of the fetuses. To our best knowledge, there were scarce studies that demonstrated the possible harmful effects of chrysin exposure during pregnancy. Therefore, this investigation aimed to explore the potential undesirable outcomes of chrysin exposure during pregnancy on the histological and ultrastructural characteristics of the adrenal gland of maternally treated rat fetuses.

MATERIALS AND METHODS

Mature Wistar albino rats (Rattus norvegicus) utilized in this study were obtained from Theodor Bilharz Research Institute (TBRI), Imbaba, Giza, A.R. Egypt. The Wistar rats were 150–160 g in weight on average. Males and females were maintained in separate plastic cages, each containing two rats, to avoid overpopulation. They were kept in the laboratory for

one week before the initiation of the experiments for acclimatization. Cubes containing uncooked proteins, minerals, and fibers were fed to rats. The animals were given unlimited access to fresh veggies that were vitamin-rich as well as milk and tap water. Mating was performed by housing one male with two females overnight. The presence of a vaginal plug or spermatozoa in the vaginal smears indicated successful mating [12]. The first day of gestation was the morning on which sperm-positive smears were collected.

The experimental design

Before each experiment, the medication Chrysin (Alfa Aesar Germany) was newly suspended in 1% tween 80 solution in water [13]. Six rats each were divided between the two groups of pregnant animals as follows:

The first group was a control group, in which each pregnant rat received a vehicle 1% w/v Tween 80. Pregnant animals of the second group were administered with 50 mg/kg/day of Chrysin [14], suspended in 1% w/v Tween 80. All the animal groups were treated by oral gavage daily for 8 days during pregnancy from day 7 to day 14 of gestation (GDs 7-14).

On day 19 of pregnancy, rats from the experimental and control groups were killed. After the fetuses were removed, the uteri were hauled out and dissected before being put in a standard saline solution. Small adrenal gland fragments from fetuses of untreated and maternally treated animals were fixed in aqueous Bouin's fixative for 24 hours to prepare for light microscopy. The samples were then dried, cleaned with terpineol, and set in paraffin wax. Haematoxylin and eosin were used to stain a series of transverse slices that were each around 5 thick. These were subsequently inspected under a microscope, and when needed, photomicrographs were taken. Small sections of the adrenal glands were preserved for electron microscopic examinations in 2.5 percent glutaraldehyde for 4 hours and 2 percent paraformaldehyde in 0.1M cacodylate buffer (pH 7.4). The samples were post-fixed in osmium tetroxide buffer for an hour at 4C.

Following two dehydrating changes of ethyl alcohol in an escalating order, two clearing changes of propylene oxide lasting five minutes each were carried out. After that, Epon-epoxy resin was used to implant the specimens. Toluidine blue was used to stain semithin slices of (0.5) thickness, which were then inspected under a bright field light microscope to check for general orientation. Lead citrate and uranyl acetate were used to dye the very thin slices [15]. The Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, used a Joel 1200 EX 2 transmission electron microscope to inspect and take pictures of sections.

RESULTS
A-Light microscopic observations:
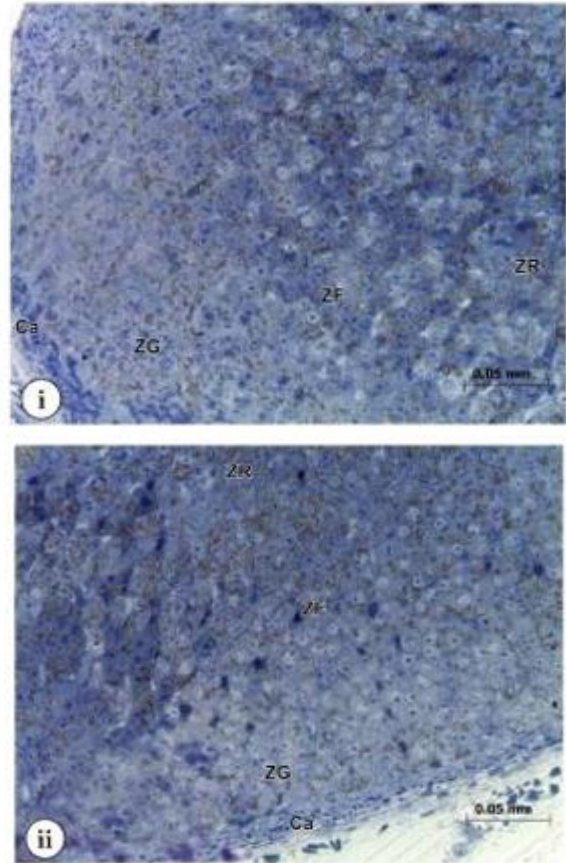
Figures (i & ii): show toluidine blue-stained photomicrographs of semithin slices of the adrenal gland from the control group **(Fig. i)** and treated group (Fig. ii), revealing the capsule (Ca), zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticulata (ZR).

I-The control group:

Histological analysis of the exterior thin connective tissue capsule that enveloped the outer cortex and the inner medulla was visible in the hematoxylin and eosin-stained sections of the adrenal gland of the rat fetus. Similar to the adult gland, the cortex is segmented into three zones: the zona glomerulosa (ZG), zona fasciculata (ZF), and zona reticularis (ZR), however, the zonation is considerably less distinct. The medulla, which is still growing, and the zona reticularis are poorly defined, although the zona glomerulosa and zona fasciculata are rather well delineated. The ZG, which is made up of collections of tiny cells, presents beneath the thin capsule that encapsulated the adrenal gland. The ZF, is an intermediate zone located beneath the ZG, and consists of large cells grouped in cords and separated by blood sinusoids. Cells from the future ZR and immature medulla are mixed in the gland's deepest part (**Figs.1 &2**).

Figures 1-2: photomicrographs of adrenal gland slices from a control rat fetus that is 19 days old.
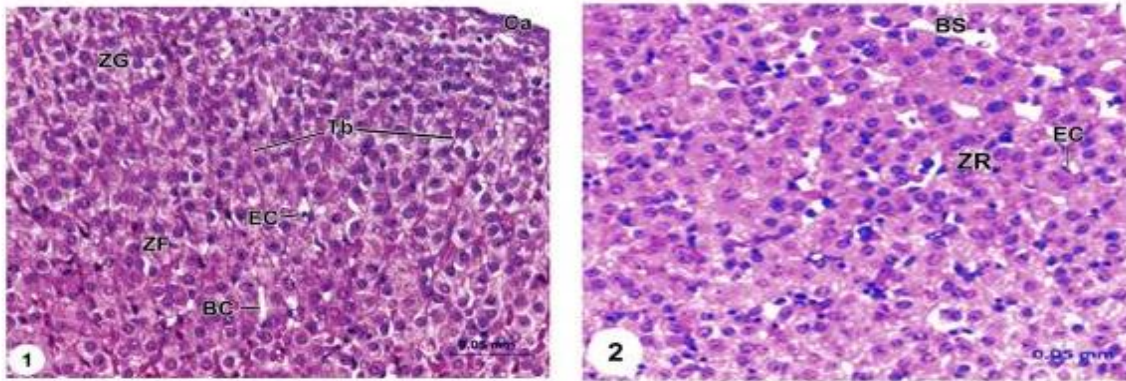
Fig. (1): The capsule (Ca), which is made up of fibrous components, extends from the zona glomerulosa cells (ZG), which are divided by trabeculae (Tb). Zona fasciculata cells (ZF) were also seen to be grouped in long radial cords divided by narrow blood capillaries (BC) lined with endothelial cells (EC).

Fig. (2): Zona reticularis cells (ZR) are arranged in an irregular network of tangled cords, which are divided by endothelial cells (EC) lined wide blood sinusoids (BS).

II -Chrysin-treated group:

The adrenal cortical tissues of rat foetuses maternally treated with chrysin for 8 days during gestation (GD 7-14) displayed hypertrophy with cytoplasmic vacuolation in the majority of the cells of the cortical zones, namely the glomerulosa, the fasciculata, and the reticularis (Figs. 3-5). Figures 3, 4 & 5 showed that these damaged cells' nuclei demonstrated karyolysis. In addition, there were dilated and leaking blood sinusoids (Figs. 4&6).

Figures 3-6: Photomicrographs of adrenal gland sections from 19-day-old rat foetuses maternally exposed to 50 mg/kg of Chrysin:
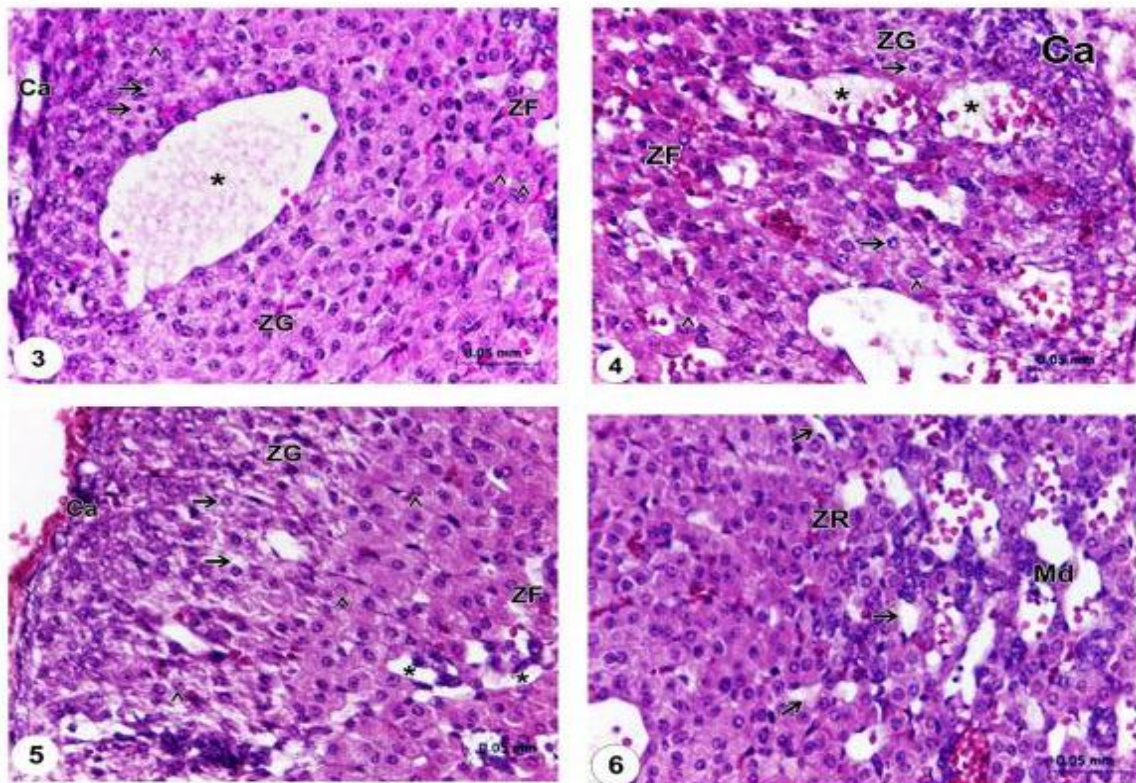

Fig. (3): Arrows indicate cytoplasmic vacuolation in zona glomerulosa cells (ZG) and karyolysis in their nuclei (arrowheads). This illustration also shows a clogged blood vessel with stagnant blood (*).

Fig. (4): Hypertrophied zona glomerulosa cells (ZG), fasciculata cells (ZF), and reticularis cells (ZR), each of which has variable-sized cytoplasmic vacuolation (arrows) and affected nuclei that show symptoms of karyolysis (arrowheads). Note the enlarged and clogged blood sinusoids with the blood stagnating (*).

Fig. (5): Cytoplasmic vacuolation (arrows) in Zona glomerulosa cells (ZG) and karyolysis in their nuclei (arrowheads). In the zona reticularis, dilated blood sinusoids (*) are also visible.

Fig. (6): Between the medulla cells (Md) and the zona reticularis (ZR), blood sinusoids enlarge (arrows).

B- Ultrastructural observations:

I-The control group:

The adrenal cortical tissues of the control rat fetus underwent electron microscopy analysis, which revealed their typical ultrastructural characteristics. Fibroblasts with recognizable long nuclei and collagen fibers make up the capsule that surrounds the adrenal gland (**Fig. 7**). **Figure 8** shows zona glomerulosa cells with various mitochondrial architectures, including oval or spherical forms with distinctive tubule-saccular cristae, numerous tiny Golgi vesicles, and plenty of lipid droplets. These cells are distinguished by having nuclei that are spherical or oval, coated in nuclear envelopes, and made up of nucleoli, homogeneous euchromatin material, and peripheral abundant heterochromatin (**Fig. 8**).

As seen in **figure 9**, cells in the zona fasciculata have a lot of spherical mitochondria with visible :

tubular cristae, a smooth endoplasmic reticulum, a lot of lysosomes, and a lot of cytoplasmic lipid droplets. Large, globular nuclei with prominent nucleoli and abundant peripheral heterochromatin characterize these cells. Endothelial cells fill the blood capillaries that run between these fasiculata cells.

The cells of the zona reticularis, the deepest layer of the cortex, are distinguishable from other cell types by their abundance of smooth endoplasmic reticulum, lysosomes, and lipid droplets of various sizes. They also include spherical mitochondria with electron-dense tubular cristae. Their ovoid or spherical nuclei feature prominent nucleoli and abundant heterochromatin and euchromatin (Fig. 10).

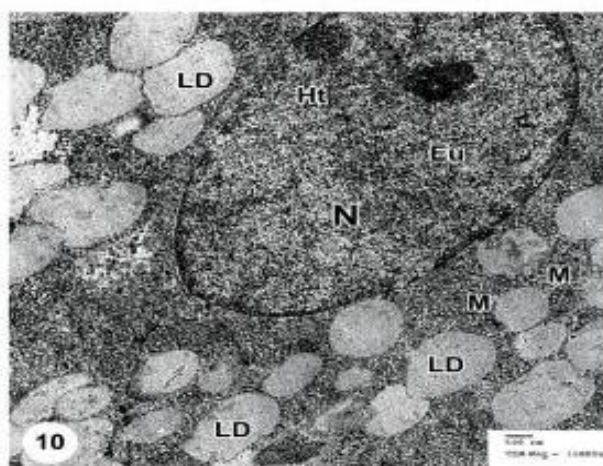**Figs. (7–10):** Electron micrographs of adrenal gland tissues taken from a control rat fetus that is 19 days old and show
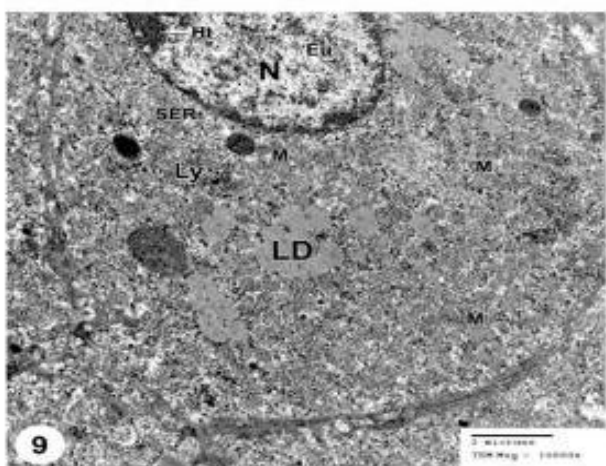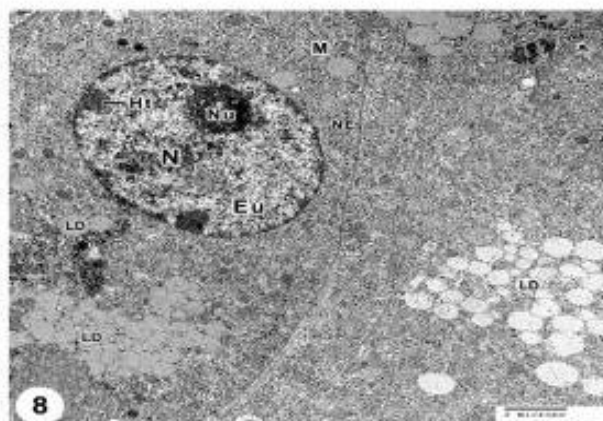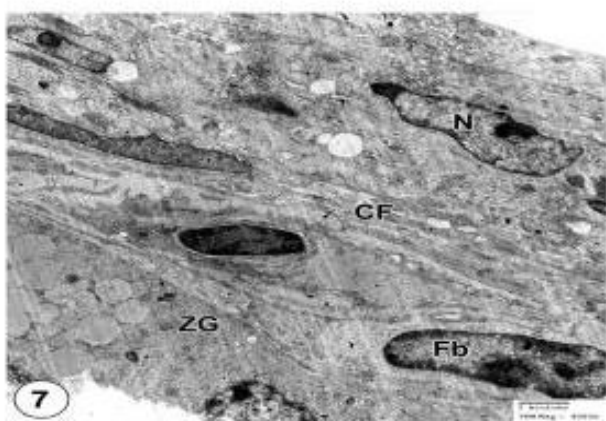

Fig. (7): An area of the adrenal cortex capsule, which is composed of collagen fibers(CF) and fibroblasts (Fb) with distinctively long nuclei (N). Additionally seen are a few zona granulosa (ZG) cells.

Fig. (8): Numerous lipid droplets (LD), mitochondria (M) with tubule-saccular cristae, a spherical nucleus (N) surrounded by a nuclear envelope (NE), a nucleolus (Nu), marginated heterochromatin (Ht), and euchromatin (Eu)may all be seen in zona glomerulosa cells.

Fig. (9): The cytoplasm of fasciculata cells is filled with a large number of mitochondria (M), which may be identified by their tubular cristae, smooth endoplasmic reticulum (SER), lysosomes (Ly), and lipid droplets (LD). Euchromatin (Eu) is flocculent and peripheral heterochromatin (Ht) are both seen in the spherical nucleus (N).

Fig. (10): a reticularis cell with an oval nucleus with outlying heterochromatin (Ht) and euchromatin (Eu) as well as spherical mitochondria (M) with tubular cristae, and lipid droplets (LD).

II- Chrysin-treated group:

Ultrastructural examination of the cortical tissue of the treated group showed a large number of cells exhibiting various degrees of degeneration. Increasing numbers of intercellular vacuoles and collagen fibers were seen in the adrenal cortex capsule, which also showed how fibroblasts' uniquely irregular nuclei make up the bulk of the capsule (**Fig. 11**). The cytoplasm of the zona glomerulosa cells showed a noticeably decrease amount of lipid accumulation (**Fig. 12**). The sizable number of lysosomes is seen along with degenerated and hypertrophied mitochondria with a gradual loss of their cristae in addition to degenerated rough endoplasmic reticulum (**Figs. 12&13**). Zona fasciculata cells displayed swollen mitochondria which lost their internal cristae in addition to variable numerous lipid droplets (**Figs. 14&15**).

The endoplasmic reticulum was found to be dilated and also many lysosomes were seen (Fig. 15). Similarly, zona reticularis cells have hypertrophied mitochondria with large numerous of lipid droplets and degenerated rough endoplasmic reticulum. In addition, deformed nuclei surrounded by irregular nuclear envelops were observed (**Fig. 16**).

Figs. (11–16) show electron images of the adrenal gland tissues of 19-day-old rat fetuses that had received 50 mg/kg of Chrysin from the mother. Displaying:
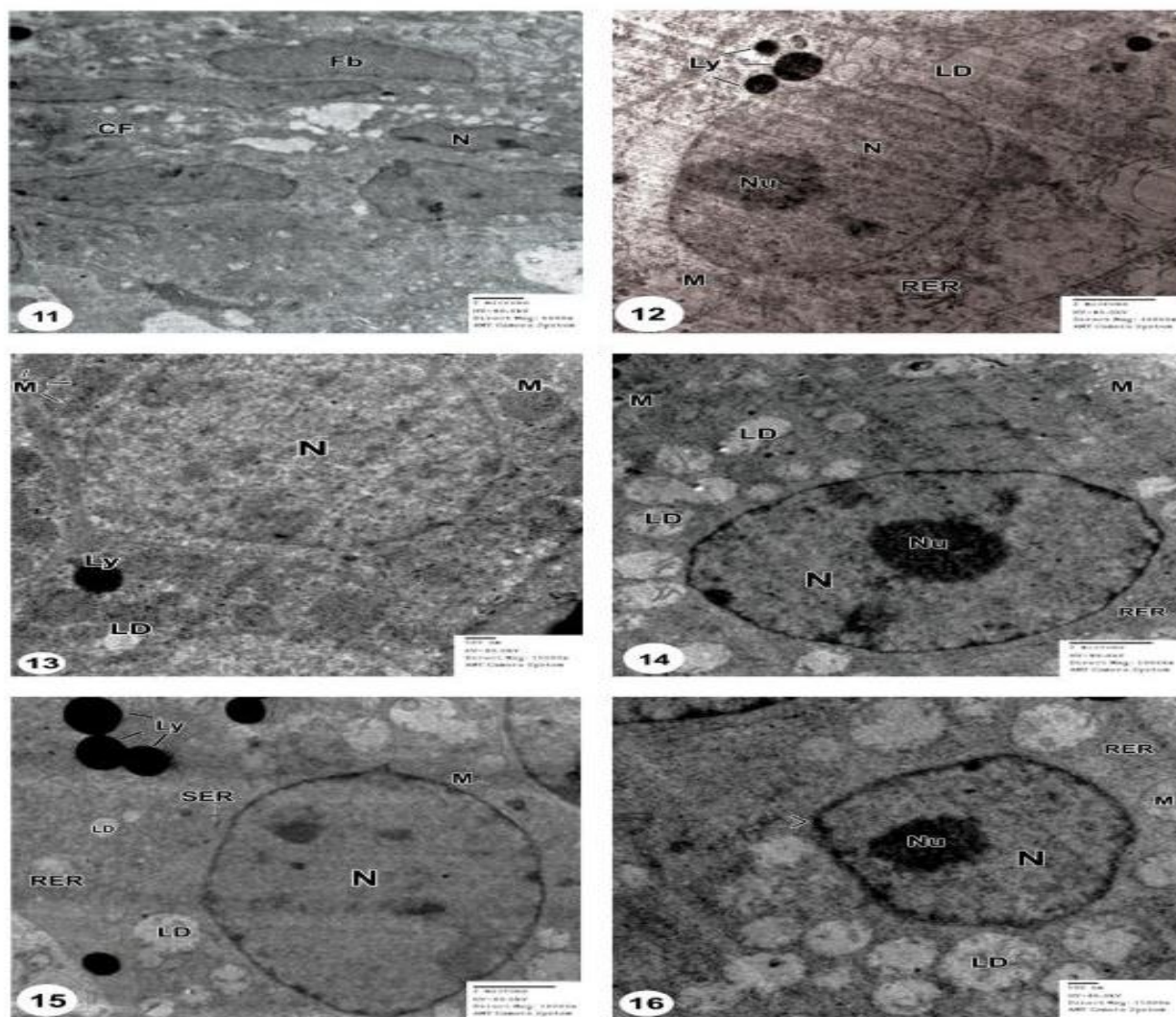

Fig. (11): A section of the adrenal cortex capsule, which is mostly made up of collagen fibers (CF) and fibroblasts' (Fb) distinctly irregular, elongated nuclei (N).

Fig. (12): Depicts a globulosa cell with lysosomes (Ly), degraded rough endoplasmic reticulum (RER), hypertrophied mitochondria (M), and lipid droplets (LD). and spherical nucleus (N) with a nucleolus (Nu)

Fig. (13): Depicts a glomerulosa cell with lysosomes, hypertrophied mitochondria (M), and few lipid droplets (LD).

Fig. (14): A fasciculata cell with variable-sized lipid droplets (LD), hypertrophy of the mitochondria (M), degeneration of the rough endoplasmic reticulum (RER), and an oval nucleus (N) with a nucleolus (Nu).

Fig. (15): Shown another fasciculata cell which also has hypertrophied mitochondria (M), degraded smooth and rough endoplasmic reticulum (SER), fluctuating lysosomal quantity (Ly), and a rounded nucleus (N).

Fig. (16): shows an overloaded reticularis cell with lipid droplets(LD) of various sizes, vacuolated mitochondria (M), a degenerating rough endoplasmic reticulum (RER), and a deformed nucleus (N) with a thickening nuclear membrane (arrow head) and a nucleolus (Nu).

DISCUSSION

The cortex and medulla, two endocrine tissues that are ontogenetically, anatomically, and functionally separate, make up the adrenal gland. The medulla is ectodermal in origin and formed from the neural crest, whereas the cortex is mesodermal in origin and generated from a proliferation of the coelomic epithelium [16]. The cortex and the medulla, two tissues with independent embryological origins, form the bilateral structures that make up the rat fetal adrenal glands at around embryonic day 13 (E13) [17].

On E16, zona fasciculata/reticularis and cortical zona glomerulosa cell morphologies are evident, as shown by the expression of P450 cytochrome aldosterone synthase (P450aldo) and 11-hydroxylase (P45011), respectively [16]. Contrary to most other organs, the embryonic adrenal gland frequently has rapid cell proliferation, and the fetal adrenal gland develops during pregnancy [18].

In the majority of animals, the adrenal cortex is divided into three morphologically and functionally different layers. Mineralocorticoids are formed in the zona glomerulosa, whereas glucocorticoids and androgen precursors are created in the zona fasciculata and zona reticularis. However, mouse and rat adrenals lack a functionally unique zona reticularis and do not produce androgens in the adrenal cortex due to tissue-specific deficiencies of the enzyme P450c17, which is required for androgen synthesis [19].

According to **Kaludjerovic and Ward** [20], the fetomaternal endocrine system, which is made up of the placenta, the mother's adrenal cortex, and the fetus's adrenal cortex, controls the synthesis of estrogens throughout the development of the fetus. Furthermore, they assert that exposure to environmental estrogens like isoflavones can lower placental estrogens production in addition to the synthesis of the hormones cortisol and dehydroepiandrosterone (DHEA) in the fetal adrenal cortex.

A greater percentage of the waste products utilized to produce placental estrogen come from the fetal adrenal gland [18]. According to **Ishimoto and Jaffe** [10], appropriate fetal adrenal cortex development is essential for the generation of placental steroid hormones including estrogen, cortisol, and aldosterone as well as fetal maturation. According to **Coulter et al.** [21], environmental estrogens may interfere with the embryonic adrenal cortex's capacity to naturally adjust to estrogen. While environmental estrogens are not particularly dangerous for adults, exposure during crucial stages of human development might have negative effects [20].

The present study agrees with the study obtained by **Mc-Lachlan** [22] who suggested that the placental estrogens generated by the placenta may hinder growth and development in the fetal zone of the adrenal cortex. Estrogen may be able to modulate DHEA-S synthesis by regulating the size of the fetal zone, which would then affect the production of estrogens itself [22].

The D and C rings of the substrate are similar to the A and C rings in the aromatase active site, claim **Kao et al.** [23]. As a result, by binding to the active site of aromatase, flavones, and steroids may compete for it. The most efficient inhibitor of flavone aromatase is chrysin because the hydroxyl group at position 7 is essential for inhibition [24]. Numerous enzymes are required for the manufacture of estrogens, including the aromatase enzyme, which changes androgens into estrogens [25].

The results of the present investigation demonstrated that the administration of chrysin to pregnant rats between days 7 and 14 of gestation resulted in appreciable modifications in the histological structure of the tissue lining the adrenal cortex of developing rat fetuses. The glomerulosa, fasiculata, and reticularis cells all exhibit hypertrophy and have large vacuoles in their cytoplasm, making these lesions, particularly evident in the three zones of the outer cortex that have hypertrophy. It is easy to see that the nuclei have undergone karyolysis. These outcomes are in line with other studies that examined the negative effects of midazolam [26].

The present investigation also illustrated that administrating chrysin to maternally treated rat fetuses altered the vasculature in the adrenal cortex. Blood arteries and sinusoids' dilatation and congestion were among these modifications. According to **Sandritter et al.** [27], fibroid degeneration or necrosis is caused by a major transient shift in vascular permeability, which causes blood plasma to leak rapidly into the artery wall and the surrounding connective tissue. These modifications can cause clogging or injury to the vascular tissues. The current findings support the earlier findings attained by all previous authors.

According to the findings of the current study, maternal treatment of fetuses with 50 mg/kg/day of chrysin for 8 days during pregnancy (GDs 7–14) resulted in obvious negative modifications to the adrenal cortex's ultrastructure. These changes were seen in the three cortical zones' cytoplasmic organelles, especially the occurrence of lamelliform cristae, which are faulty mitochondrial cristae, and an increase in the matrix's density. Following acute treatment with Apis mellifera (honey bee) venom, **Florea and Cracium** [28] noted this abnormality in the rat adrenal cortex. According to scientists, the complex fusion of the tubular mitochondrial cristae that resulted in the anomalous lamella mitochondrial cristae suggests that this is the first step of cristae alteration before they are eliminated or closed into the circular, concentric mitochondrial malfunction. Due to their participation in the coordinated activities of the enzyme 3ß-hydroxysteroid dehydrogenase (3ß HSD), which is dispersed between the mitochondria and the

smooth endoplasmic reticulum, these organelles play a crucial role in steroidogenesis inside the cortex [29].

Significant damage to the endoplasmic reticulum and mitochondria may be enough to limit steroid production. The rate-limiting step in the synthesis of steroid hormones, according to **Rainey *et al.* [30] and Isola *et al.* [31],** takes place in the mitochondria at cholesterol side-chain cleavage enzyme (CYPIIA), the first enzyme in the steroidogenic pathway. Since the cascade of processes leading from cholesterol to progesterone has been suppressed, the formation of lipid droplets seen in this study may be explained as a secondary effect [32].

This theory is in line with the findings of **Tarantio *et al.* [33],** who found that hepatotoxic chemicals are produced, largely by the enzyme cytochrome P450 (CYP), along with other routes such as mitochondrial failure and apoptosis, during the pathogenesis of drug-induced liver damage. Fetal rat adrenocortical cells can have morphological abnormalities and change how they produce steroid hormones when exposed to endocrine disrupters. These modifications might be a factor in the adrenal gland's maldevelopment [34].

CONCLUSION

The results of the current study demonstrated that, despite having significant benefits for body health when administered to adult rats, chrysin during pregnancy, particularly during the organogenesis period, causes obvious histopathological and ultrastructural changes in the adrenocortical cells of albino rat fetuses. Accordingly, our findings advise against administering chrysin during the development of the fetus's adrenal glands.

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