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GESTATION RELATED HISTO-MORPHOMETRIC AND IMMUNOHISTOCHEMICAL CHANGES IN ACUTE REGULATORY PROTEIN OF STEROIDOGENESIS AND OXIDATIVE STRESS IN RAT ADRENAL GLAND CORTEX

Afrah Adil Hassan[1] and Huda R. Kamoona[2]*

[1]College of Dentistry, University of Misan, Iraq.
[2]College of Medicine , Al-Nahrain University, Baghdad, Iraq.
*e-mail : huda.r.kammona@gmail.com

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ABSTRACT : Adrenal gland took major part during pregnancy, it's cortex release hormones of cholesterol precursor that utilize StAR for its transfer across mitochondrial membrane, striodogenesis is associated with electron leakage lead to oxidative stress and both are changed during pregnancy. This study evaluate the histomorphotric changes of adrenal cortical zonal thickness in relation to steriodgenesis by immunohistochemical assessment of StAR protein and oxidative stress by assessment of malondialdehyde (MDA) through various stages of pregnancy in Rat. A significant variations among cortical zonal thickness was seen, with Significant variation in StAR and MDA expression among non-pregnant and pregnant groups at various gestational age (7^{th}, 14^{th} and 18^{th} days) at $p \leq 0.05$. All these changes verify specific gestational stage hormonal changes that emphasized by StAR and MDA topographic localization and immunohistological expression and buildup of the oxidative stress during gestation.

Key words : Adrenal gland, steroidogenesis , StAR, oxidative stress, MDA.

INTRODUCTION

Steroidogenesis is process by which steroids are generated from cholesterol (Hanukoglu, 1992). The steroid hormones are essential for many functions in the body, as in adrenal gland which responsible for syntheses of glucocorticoids ,and mineralocorticoids, which regulate metabolism and water balance, and production of androgens (Yadav, 2004). The first step in steroidogenesis is the cleavage of the cholesterol which derived from dietary lipids or be synthesized from cholesterol precursor by the cell of adrenal cortex (Porter and Herman, 2011), then provide a side chain to yield pregnenolone (Miller, 2005).

The intracellular transport of cholesterol involves complex mechanisms (Miller and Bose, 2011), the first and rate-limiting step in steroidogenesis is the conversion of cholesterol to pregnenolone by the mitochondrial cholesterol side-chain cleavage enzyme, cytochrome P450scc (Miller, 2013). This conversion is the rate-limiting step of steroid synthesis, which occurs inside the mitochondrion of the respective tissue (Rossier, 2006).

During pregnancy, it was reported that in spite of maternal adrenal gland does not change morphologically but, plasma adrenal steroid levels increase with advancing gestation, total plasma cortisol concentrations increase to three times than non-pregnant levels by the third trimester. The hyperestogenemic state of pregnancy lead to increased hepatic production of cortisol-binding globulin. This increase in cortisol-binding globulin due to an increase in plasma free cortisol and total free cortisol, additionally, an increase in maternal plasma ACTH concentration and the hyper responsiveness of the adrenal cortex to the ACTH stimulation (Lindsay and Nieman, 2005).

Despite the elevated free cortisol levels, pregnant women do not exhibit any overt signs of hypercortisolism, likely due to the antiglucocorticoid activities of the elevated levels of progesterone (Rainey *et al*, 2004).

StAR protein isthe rate-limiting step in steroidogenesis its act exclusively on the outer membrane of mitochondria, the biologically active form of StAR is the extra-mitochondrial 37kDa protein, while the intramitochondrial 30kDa StAR is inactive because of its intramitochondrial location (Miller, 2017). The absence of StAR resulting in a loss of most steroidogenesis in suprarenal gland and testicular steroidogenesis (Bose *et al*, 1996).

Mutation in StAR affects the production of estrogen, a steroid necessary for pregnancy, the first successful pregnancy in a StAR deficient woman was reported by Khoury *et al* (2009), also successful case has been reported by Sertedaki *et al* (2009). This particular mutation in StAR lead to failure to conceive, when estrogens administered pregnancy occur, estrogens were administered until the placenta could fully function. Other research has shown that steroidogenesis in the placenta is independent of StAR (Strauss *et al*, 1996).

Pregnancy is a state of oxidative process due to the production of reactive oxygen species (ROS) and saturation of endogenous antioxidant, that lead to an increase in ROS in the body (Lutoslawska *et al*, 2003). When occur in excess, however, there are adverse effects, such as lipid peroxidation and aggression to enzymes, DNA, carbohydrates and proteins and membranes of the tissues (Barreiros and David, 2006; Trindade and Rugolo, 2007).

Oxidative stress can influence the entire reproductive period of life of women (Agarwal *et al*, 2005), influencing the normal birth (Mocatta *et al*, 2004), preterm labor (Pressman *et al*, 2003).

Free radicals can react with the major classes of biomolecules being the most susceptible lipids (Halliwell and Gutteridge, 1989). The gradual reduction of oxygen to water produces a variety of unstable intermediates, highly reactive and potentially toxic, among them is the O_2 (superoxide anion), H_2O_2 (hydrogen peroxide) and OH (hydroxyl radical). These forms of partially reduced oxygen are continuously generated in all aerobic cells as a result of oxidation processes,are the main ROS in the body (Kyle and Farber, 1991).

During normal pregnancy there is a slight increase in oxidative stress, partially offset by the induction of antioxidant systems since the beginning of pregnancy, such as catalase, vitamin C, glutathione, among others (Raijmakers *et al*, 2001, Chaooell *et al*, 2002). An imbalance between the production of pro-oxidants and antioxidants, which facilitates lipid peroxidation of cell membranes lead to injury to the vascular endothelium that contribute to endothelial dysfunction (Serdar *et al*, 2003).

In rat defects in the transport of free cholesterol to CYP11A1 (P450scc) sites within the mitochondria where conversion of cholesterol to pregnenolonein side-chain cleavage (Liao *et al*, 1993), lead to loss of steroidogenic function that accompanied by a significant alteration in oxidative status of rat adrenal including enhanced oxidative stress, loss of enzymatic and non-enzymatic antioxidants and increased membrane lipid peroxidation (Cao *et al*, 2004).

Malondialdehyde is a highly reactive compound that occurs naturally and is a marker for oxidative stress, it results from lipid peroxidation of polyunsaturated fatty acids (Davey *et al*, 2005). Oxidative stress resulting from excessive generation of reactive species compared with antioxidant defense capacity that cause lipid peroxidation (Taylor *et al*, 2000).

Lipid peroxides are formed in the placenta due to membrane disruption by ROS (Patil *et al*, 2008). The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism (Del Rio *et al*, 2005).

MATERIALS AND METHODS

Asample of 40 adult female Rat (*Ratus ratus* Norvigeus) were divided into 4 Groups (10 animals) in each, G A : 7th day of gestation, B: at 14th days of gestation, GC: at 18th days of gestation & control group: (non-pregnant). All animals were treated according to National Institute of Health (NIH) guidelines for the care and use of laboratory animals, they were killed by deep authentication by chloroform, then adrenals were removed from its position in retroperitoneal cavity fixed in 10%NBF, processed for paraffin blocks and stained for H & E according to Bancroft and Layton (2013) and IHC for StAR (polyclonal antibody) (ab203193) and MDA (malondialdhyde) antibody (ab6463) were used. Histological slides that stained by H&E were assessed by image j software version (1.52a) for histomorphmetric analysis of zonal cortical thickness in relation to total cortical thickness. Immunohistochemical analysis done by aperio positive pixel count algorithm (V11) for assessment of total positivity of StAR and MDA at suprarenal cortex at various groups. Results were expressed as mean ±SE and assessed by ANOVA (single factor) among groups and Tukey test use to shows significance (between paired groups), significant result were expressed at P-value ≤ 0.05.

RESULTS

The histological tissue were organized into outer cortex and inner medulla, the cortex is subdivided into three zones, an outer : zona glomerulosa (ZG), middle : zona fasciculatea (ZF), an inner: zona reticularis (ZR). The cortical zones showed no sharp demarcation but depending on histological features of the cells that enable their identification. The cells of zona glomerulosa arranged into arched cords of cells, immediately beneath the capsule and zona fasciculatea occupying most of cortical thickness in which cells arranged as short cords of two

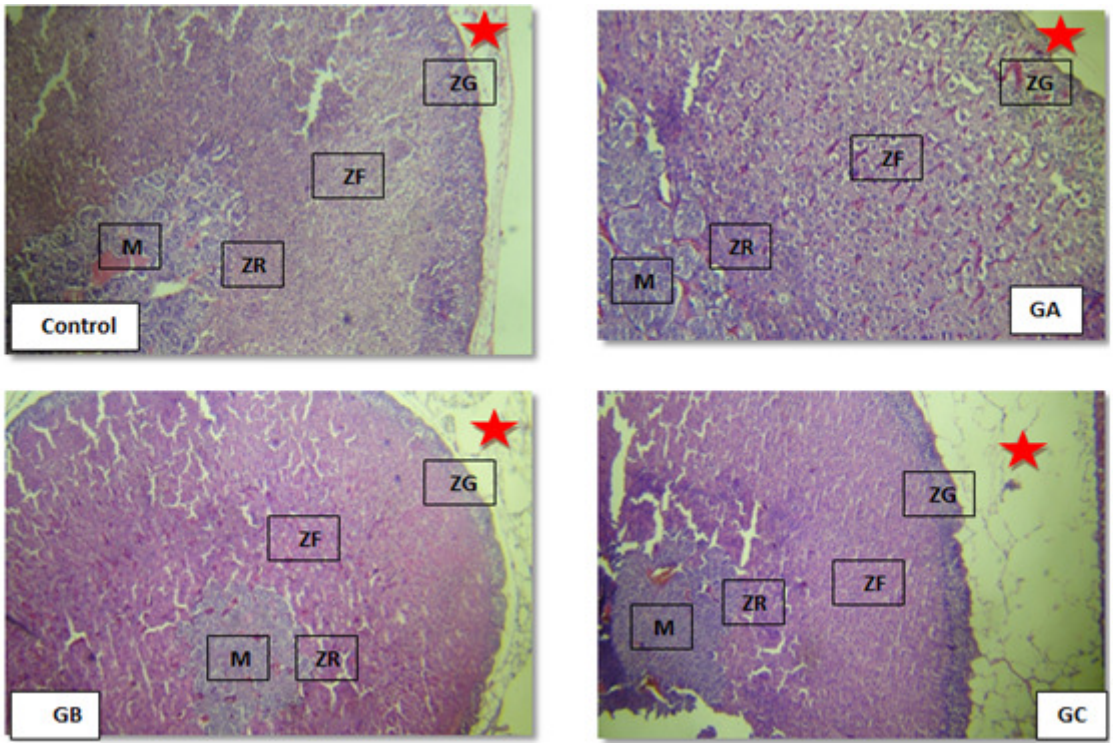

Fig. 1 : Histological variations in glandular cortex zonal proportions: zona glomerulosa, (ZG), zona fasciculata (ZF), zona reticularis (ZR), central medulla (M) and adipose tissue surrounding the gland (red star) in control and pregnancy groups, H and E, X4.

Table 1 : Histomorphometric analysis of cortical zones thickness in control and pregnancy.

Zones	Totally thickness of zones	Control	GA	GB	GC	p-value
ZG	**mean± SE**	0.746±0.347	0.889±0.208	0.930±0.464	0.918±0.246	0.001*
ZF	**mean± SE**	1.082±0.692	1.018±0.009	1.025±0.295	1.014±0.058	0.557
ZR	**mean± SE**	1.144±0.352	0.975±0.169	1.003±0.122	1.032±0.375	0.000*

*Significant P ≤ 0.05

Table 2 : Comparison of positivity of STAR in suprarenal cortex by Aperio scope image among group by ANOVA.

Age groups	Control	7day	14day	18day
Total positivity (mean±SE)	2.980±0.402	0.734±0.015	0.610±0.030	0.581± 0.012
p-value	0.000*			

*Significant P ≤ 0.05

cell thickness perpendiculars to the capsule and the innermost zona reticularis cells arranged as anastomosing cords in close contact to the medulla.

Pregnancy related variations in histological and Histo-morphometric features of adrenal cortex

In pregnancy, there is an increase in the adipose tissue that surround the gland and increase in capsular thickness, in addition pregnancy affect the cells of cortical zones lead to variation in cortical zonal proportion by cellular hypertrophy, cellular edema, increase in the vascularity and sinusoidal dilatation in whole cortex (Figs. 1, 2).

Histo-morphometric evaluation of zonal cortical thickness in relation to total cortical showed highly significant variations in the thickness of zona glomerulosa and zaona reticularis among control and pregnancy groups by ANOVA single factor analysis with p values: 0.001, 0.000 respectively (Table 1).

Immunohistochemical evaluation of StAR protein in adrenal cortex

The immunohistochemical evaluation of StAR showed variable distribution in control and pregnancy groups, it showed weak reaction in control group with mean value 0.205±0.008, to increase dramatically in GA (7^{th} day of gestation) to 0.734±0.015 being mainly localized in zona glomerulosa and less in other zones. In later gestation, it showed diffused distribution in the cortex , and highly significant variation in the immuno-histochemical expression (positivity) with p value 0.000 (Fig. 3, Table 2).

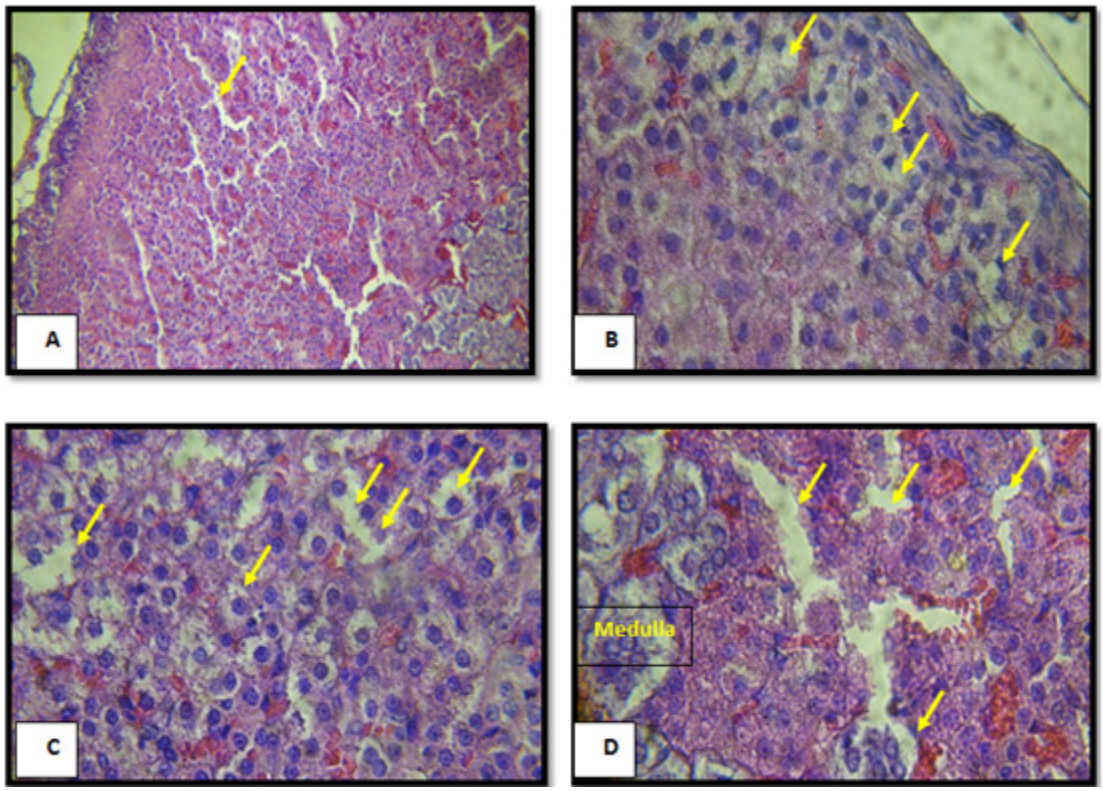

Fig. 2 : Pregnancy related changes in whole cortex vascularity (A), vascular dilatation and cellular edema (yellow arrows) in zona glomerulosa (B), zona fasciculate (C) and zona reticularis (D). H & E, X40.

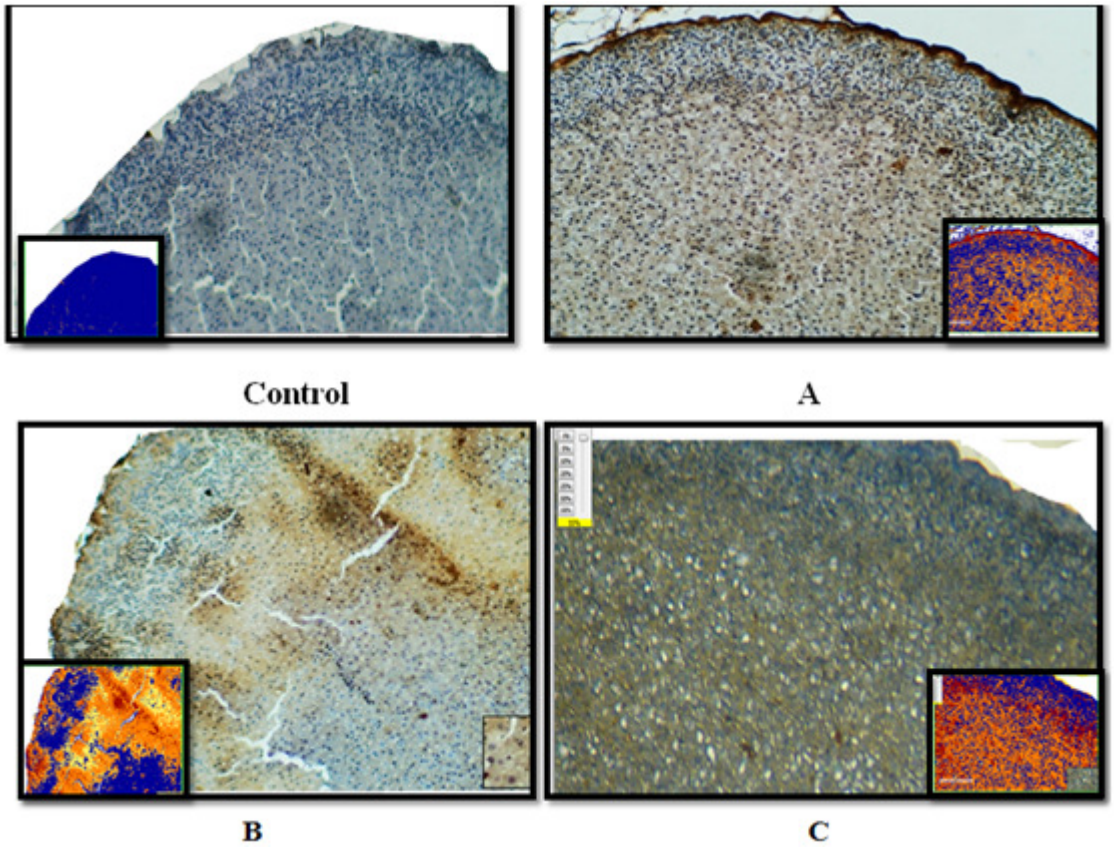

Fig. 3 : Immunohistochemical reaction of StAR in adrenal cortex in control and various gestational periods with their markup images by Aperio scope image analysis program. IHC. StAR, X10.

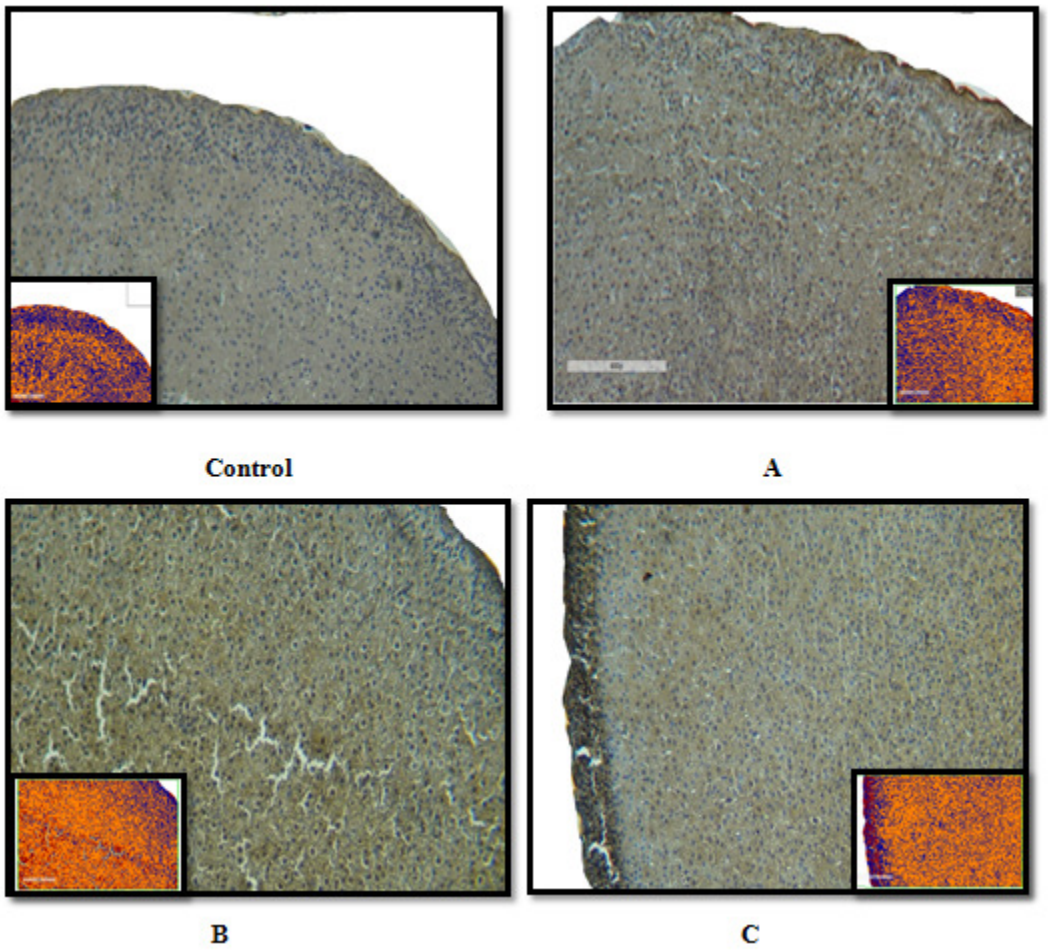

Fig. 4 : Immunohistochemical reaction of anti-MDA in adrenal cortex in control and various gestational periods with their markup images by Aperio scope image analysis program. IHC. MDA, X10.

Table 3 : Comparison of positivity of MDA in suprarenal cortex by Aperio scope image among group by ANOVA.

Age groups	Control	7day	14day	18day
Total positivity (mean±SE)	0.65±0.021	0.82±0.01	0.75±0.021	0.67±0.02
p-value	0.000*			

*Significant P ≤ 0.05

Immunohistochmical evaluation of MDA protein in suprarenal cortex

The immunohistochemical distribution of malondialdhyde as a marker of oxidative stress specifically lipid peroxidation showed wide distribution in all cortex with significant variation in the positivity of immunohistochemical expression assessed by single factor ANOVA with p.0.000, being lowest in control group and increase progressively to reach highest value at 18th day of gestation (Fig. 4, Table 3).

DISCUSSION

Maternal adrenal glands play a key role in supporting normal pregnancy, in particular the endocrine part, they undergo continuous changes in accordance with the different stages of pregnancy, these changes are presented by alterations in the gross anatomy, the histology and the hormonal levels secreted by the different parts of the gland (Molitch, 2015).

Despite the increase in the thickness of the adrenal cortex throughout the pregnancy, our data showed that the three cortical zones thicknesses were increasing differently. No previous studies have examined the morphometric changes in the maternal adrenal cortical zones thicknesses in pregnancy, these independent differential zonal cortical changes are induced by cellular hypertrophy, as was noticed in this study and by the high cellular proliferation rates that were reported before in the different cortical zones at the different stages of pregnancy (Andrea Bozzo *et al*, 2011).

Andrea Bosso and colleagues who studied the effects of chronic stress on the adrenal cortex cellular

proliferative profile in pregnant rats. They found noticeable variation in the proliferative profile among the different cortical zones in the second half of pregnancy with zonafasciculata cells showing the highest proliferative index. This agreed with our findings as zona fasciculate is the widest region in all groups of pregnancy as could be due to pregnancy induce stress that lead to this change in zona fasciculate thickness. While the borderline increment in zona glomerulosa in its relative thickness at the 7th day of pregnancy compared to the control group, that was significantly decreased at day 14 and day 18 of pregnancy. This decrement is more likely due to the significant increment of the relative thickness of zona fasciculate and zonareticularis.

The relative change in the thickness of zonaglomerulosa is likely related to the role of these to secrete mineral ocorticoids, in particular Aldosterone. This supported by Lindsay and Nieman (2005), who mentioned Aldosterone is produced early in pregnancy to support maternal physiological changes.

Zonaglomerulosa cells respond to the increased renin-angiotensin activity early in pregnancy that is induced by an increased production of renin by the kidneys and renin production by the ovaries and the utero-placental unit (Scaife and Mohaupt, 2017). Other factors that stimulate zonaglomerulosa cells to produce aldosterone early in pregnancy are the increased level of oestrogen that stimulates aldosterone production through stimulation of renin production and increased progesterone level that antagonises mineralocorticoids receptors which indirectly stimulates zonaglomerulosa cells to increase the production of aldosterone (Tkachenko *et al*, 2014).

Aldosterone, through its water and sodium retention function, plays a role in maintaining proper circulating maternal blood volume and blood pressure that is crucial for proper utero-placental perfusion. In addition, aldosterone production is important to control normal sodium level during pregnancy, which is crucial to facilitate many physiological changes that begin shortly after conception and support foetal and placental growth and development (Lindsay and Nieman, 2005; Soma-Pillay *et al*, 2016).

We used Steroidogenic acute regulatory (StAR) protein as a marker to indicate cellular engagement in steroidogenic activity. StAR protein governs the translocation of the cholesterol, steroid hormones substrate, from the external to the internal mitochondrial membrane to be processed into steroid hormones (Miller, 2017). StAR protein controls the rate-determining step in steroidogenesis and its synthesis varies according to the tissue activity (Men *et al*, 2016), this feature renders StAR protein as an excellent marker to study the spatio-temporal steroidogenic activity changes in the different layers of maternal adrenal cortex at the different stages of pregnancy.

Our result showed that the steroidogenic activity of the maternal adrenal cortex is significantly higher at different stages of pregnancy compared to its activity in non-pregnant adrenal glands, these results reflected the demands for steroid hormones increases in pregnancy for the crucial role played by these hormones in supporting maternal physiological changes in pregnancy.

The expression of StAR protein was progressively increasing in zonafasciculata and zonareticularis throughout the different stages of pregnancy, again, this gives a clue that the steroidogenic activity in these layers are increasing in the second halve of pregnancy and matches the progressive significant increment in zonafasciulata thickness. The second half of pregnancy is characterized by boosting Adrenocorticotrophic hormone through a positive feed-forward loop to increase the secretion of glucocrotoids by maternal adrenal zonafasciculata cells (Duthie *et al*, 2013).

The significant increase in StAR protein expression in maternal adrenal cortex throughout pregnancy compared to the non-pregnant controls indicates an increment in cholesterol utilization by adrenal cortical cells to synthesize steroid hormones by the process of steroidogenesis. Steroidogenesis involves a cascade of reactions that are catalyzed by cytochrome P450 enzyme isoforms. These reactions that are catalyzed by P450 enzymes are characterized by electron leakage that significantly contributes to the production of reactive oxygen species, which in turn increases oxidative stress (Prasad *et al*, 2014).

Interestingly, our data showed a significant progressive expression of malondialdehyde, was used as marker that indicates oxidative stress,throughout the different stages of pregnancy compared to the non-pregnant controls that indicates a progressive increase in in maternal adrenal cortical oxidative stress as pregnancy advances. Surprisingly, the oxidative stress is found to increase at the 18th day of pregnancy when StAR expression seems to be reduced. Korytowski and colleagues found that oxidative stress might compromise steroidogenesis in the Leydig cell model (Korytowski *et al*, 2013).

A recent study on lipopolysaccharide-treated porcine granulosa-lutein cells showed that oxidative stress can lower StAR expression by lowering the expression of

GATA4 and GATA6 transcription factors, an effect that can be restored by adding the antioxidants melatonin or vitamin C (Qu *et al*, 2019).

Despite the high level of oxidative stress during pregnancy, the maternal adrenal gland is still protected and return back to normal after parturition. The adrenal cortex is protected from the increased production of reactive oxygen species induced by steroidogenesis by non-enzymatic (vitamins A, C and E) and enzymatic (peroxiredoxins and glutathione peroxidases) antioxidants (Prasad *et al*, 2014).

The adrenal gland showed dramatic changes during pregnancy that varies during different stages of gestation, these changes reflected as an increase in adipose tissue investing the gland, vascular engorgement of cortical vessels, cellular edema, variation in relative zonal cortical thickness in relation to total thickness, in addition to changes in steroidogenic activity that showed specific topographic localization of (StAR) for each gestational stage with the increase in oxidative stress throughout pregnancy.

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