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GESTATIONAL HORMONES AND OXIDATIVE BIOMARKER CHANGES TO ASCORBIC ACID SUPPLEMENTATION DURING UNPREDICTABLE VARIABLE STRESS EXPOSURE IN PREGNANT WISTAR RATS

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Abstract

Harmful effect of gestational chronic variable stress (CVS) on maternal and fetal physiological indices is undisputed. However, benefits of ascorbic acid (AA) supplementation in pregnancy appear inconclusive. This study investigates implication of AA supplementation during CVS exposure in pregnancy on gestational hormones and oxidative biomarkers. Twenty (20) nulliparous pregnant rats were randomly divided into 4 groups of five rats each. Group 1 (control) and 2 were given orally normal saline (vehicle) and AA (500 mg/60 kg bwt) only respectively. Groups 3 and 4 were exposed to CVS. However group 4 was concurrently treated orally with AA (500 mg/60 kg bwt) Exposure and treatments were from gestational days 1-20. Serum superoxide dismutase (SOD), catalase, glutathione peroxidase, malondialdehyde (MDA) activity, follicle stimulating hormone (FSH), Luteinizing hormone (LH), progesterone and liver enzymes were determined at gestation day 21. Significance was taken at $p<0.05$. Ascorbic acid supplementation attenuates MDA activity in CVS exposed group. Furthermore SOD, catalase, and GSH were significantly increased in AA supplemented groups when compared to stress only and control groups ($p<0.05$). LH was significantly reduced in stress groups but slightly attenuated in AA supplemented group. Progesterone was significantly ($p<0.05$) increased in all treated groups when compared to control while FSH was significantly reduced ($p<0.05$). Liver enzymes (AST & ALT) were not significantly altered across groups. Derangement in oxidative stress biomarkers and gestational hormones during gestational unpredictable variable stress exposure appear substantially attenuated by AA supplementation. Antioxidant activity of AA militate aberrations in gestational physiological indices associated with unpredictable variable stress exposure in pregnancy.

Keywords: *unpredictable variable stress, gestation, ascorbic acid, oxidative biomarkers, gonadotrophins, progesterone.*

Introduction

Physiological well being of organisms depends on the maintenance of a relatively constant internal environment, or homeostasis. In several physiological conditions, the hypothalamic-pituitary-adrenal axis actively responds to both physical and psychological stress stimuli [1]. It has been reported that there could be acute or chronic stress response [2]. In acute single stress response, organism rapidly becomes adapted to the stressor(s); a circumstance that allows its return to the homeostatic state [3]. However, in chronic variable stress response, organisms usually do not develop adaptive response, and this often leads to health deterioration [4]. Glucocorticoids receptors whose presence help enhance cortisol effects on most cells are known to be up regulated in acute stress situation but down regulated during chronic cell [5]. Stress-related chronic diseases continuously places increasing burden on society [6, 7]. Oxidative stress in gynaecological situation has immense consequences on conception and healthy maintenance of same [8]. Gestational oxidative stress (OS) has been reported to affect multiple physiological processes [8]. Although OS is common feature in normal pregnancy, persistent overwhelming OS can lead to decline of maternal and fetal functions [9]. Exposure to continuous variable stress in pregnancy is an ever present reality due to varying environmental stressors. It is imperative to manage the effects of stress on our body and this is only possible if we have a robust understanding of the body's response to variable stress and possible interventions. The significance of gestational ascorbic acid supplementation in the literature is inconclusive [10]. However several studies have reported on its beneficial effects [11]. Several gestational parameters are known to serve as useful indices in assessing pregnancy [12]. This study investigates the impact of gestational ascorbic acid supplementation during CVS exposure on oxidative biomarkers, liver enzymes and gestational hormones.

Methods

Animals

Twenty nulliparous adult female Wistar rats (150 g-200 g) and 10 weeks of age were used. The animals were purchased from Lagos State University College of Medicine, animal house. The animals were allowed access to food, (standard pellet diet of livestock feeds, Nigeria) and drinking water *ad libitum*. Constant light/dark cycle (12hr light and 12hr dark), temperature (22 ± 2˚C) and relative humidity (70 %) was maintained. Procedures were done in conformity with the International Guiding Principles for Biomedical Research involving Animals [13].

Chronic variable stress procedure

Administration of chronic variable stress was performed as previously described [14]. Four different stressors were randomized and administered one per day. The order of four stressors varied across weeks. The duration of stress (20days) was selected to encompass a complete round of gestation. Stressors selected were non-habituating, painless and neither affect food or water intake. It include novel object (marbles) overnight, 15 min restraint (absence of movement, except whiskers and respiration, in a 50 mL conical tube during the light cycle), multiple cage changes (for 2hrs at 20 mins interval during the light cycle), and saturated beddings overnight causing sleep deprivation (700ml, 23 ^{0}C water) [14]

Experimental design and treatments

Proven male breeders were paired with virgin female in estrus at ratio 1:1 and were housed together overnight. The next morning a vaginal smear was obtained using a pipette. This was examined microscopically for the presence of sperm. Day after sperm positive vaginal smear was observed was taken as gestational day GD 1 [15]. Twenty nulliparous pregnant rats were randomly divided into 4 groups of five rats each. Group 1 (control) and 2 were given orally normal saline (vehicle) and ascorbic acid (500 mg/ 60 kg bwt) [16] only respectively. Groups 3 and 4 were exposed to CVS; however group 4 was concurrently supplemented orally with ascorbic acid (500 mg/ 60 kg bwt). All stress exposure and treatments were from GD 1-20.

Blood sample collection

Blood samples were collected from retro-orbital sinus on gestation day 21 after injection of sodium pentobarbital anesthetic agent (30 mg/kg bwt) [17].

It was then centrifuged in cold centrifuge (Uniscope Model SM 112 Surgifriend Medicals England) at 3000 rev per mins for 10 minutes. Serum was then aspirated into sample bottles and stored at -4° C.

Determination of serum SOD, GSH, Catalase and MDA activity

SOD activity was determined using a randox kit (Sigma Chemicals Ltd, USA) Glutathione (GSH) was determined using Ellman's reagent, as described by Sedlak and Lindsay [18] and Jollow *et al.*, [19]. Catalase activity was determined according to the method of Aebi [20]. Malondialdehyde activity was assayed by measuring the thiobarbituric (TBA) reactive products present in the test sample using the procedure of Uchiyama & Mihara [21].

Assay of AST and ALT

The serum levels of liver function biomarkers AST and ALT were assayed using the method of Moss and Henderson [22]

Assay of LH, FSH and Progesterone

Hormonal assays were done using ELISA hormone kits (Monobind Inc, Lake Forest CA USA) according to manufacturer instructions.

Statistical analysis

Results were expressed as means ± standard error of the mean (SEM). Data were analyzed using Graph pad prism version 5 statistical software. ANOVA was done with Neuman Keuls used as post hoc and p values less than 0.05 considered statistically significant.

Results

Effect of gestational chronic variable stress with ascorbic acid supplementation on serum malondialdehyde, SOD, catalase and GSH activity

There was a significant increase ($p<0.05$) in MDA activity of CVS exposed pregnant rats as compared to control. This was however attenuated in ascorbic acid supplemented CVS exposed group (Fig 1). Serum SOD, catalase and GSH levels increased ($p<0.05$) in ascorbic acid supplemented groups when compared to CVS only exposed group (Figures 2, 3, & 4)

Effect of gestational chronic variable stress with ascorbic acid supplementation on serum level of progesterone, LH and FSH

Serum progesterone levels were significantly increased ($p<0.05$) in pregnant rats exposed to CVS with or without ascorbic acid supplementation when compared to control (Fig 5). There was significant reduction ($p<0.05$) in LH levels of stressed pregnant rats with or without ascorbic acid supplementation (Figure 6). FSH levels were significantly decreased ($p<0.05$) in stressed and ascorbic acid supplemented groups when compared to control.

Effect of gestational chronic variable stress with ascorbic acid supplementation on the serum level of ALT & AST

Serum levels of both AST and ALT were not significantly altered across all treatment groups when compared to control (Figures 8 & 9)

Discussion

MDA, SOD, catalase and GSH activity

Ascorbic acid supplementation attenuates MDA activity in CVS exposed group in this study. Serum levels of antioxidants SOD, catalase, and GSH were also significantly increased in ascorbic acid supplemented groups when compared to stress only and control groups. These results unlike the study of Lukman *et al.*, [23] in normal pregnant humans underpin the importance of ascorbic acid supplementation during chronic variable stress exposure in pregnancy. Lipid peroxidation is known to be potentially harmful because of its uncontrolled, self-enhancing process causing disruption of membrane lipids and other cell components [24], which could lead to complications on foetal development and maternal health. Oxidative stress develops when the generation of reactive oxygen species overwhelms the scavenging capacity of antioxidants. Stress is one of the factors that generate this oxidative stress. A sharp peak in the expression of the markers of oxidative stress in the trophoblast was detected in normal pregnancies and this oxidative burst if excessive was speculated to be a cause of early pregnancy loss [25]. To cope with oxidative stress animal and human cells have developed ubiquitous antioxidant defense system

consisting of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase [26, 27]. The body's complex antioxidant system is influenced by dietary intake of antioxidant vitamins and minerals such as vitamin C, vitamin E, selenium, zinc, taurine, hypotaurine, glutathione, beta carotene, and carotene [28, 29, 30]. Ascorbic acid supplementation is particularly important in pregnant women as its deficiency has been reported to affect placental structure and facilitates placental infection both of which results in increased risk of premature rupture of placental membranes and premature births [11]. Specifically, ascorbic acid is a chain breaking antioxidant that stops the propagation of the peroxidative process. Furthermore, it also helps recycle oxidized vitamin E and glutathione [31, 40]. Several studies have also reiterated that vitamin supplements for pregnant mothers, not only reduce intra-uterine, foetal and neonatal infections, but also prevent complications such as pre-eclampsia, premature membrane rupture and preterm birth [32, 33].

Progesterone, LH and FSH

Plasma progesterone concentration is known to increase continually as pregnancy progresses. During the last 6 months of pregnancy, placental secretion by trophoblast cells is responsible for the marked increase in plasma concentration of progesterone. This is facilitated by the presence in the placenta of enzymes involved or needed for progesterone synthesis [34]. The essence of elevated plasma progesterone is to inhibit uterine contractility to prevent premature expulsion of fetus. This is attained by decreasing sensitivity of the myometrium to estrogen, oxytocin and prostaglandins. The significant increase in serum progesterone concentration in pregnant rats supplemented with ascorbic acid in this study may indicate ascorbic acid accumulation in the placental area augment the processes involved in progesterone synthesis. The ability of ascorbic acid accumulating in the granulosa, luteal and placental compartments have been earlier reported [35,36] On the other hand, pregnant rats exposed to stress only without ascorbic acid supplementation in this study also exhibited significant increase in serum progesterone concentration. This increase in progesterone might be due to the release of cortisol during stress as suggested by Alexandra *et al*., [37]. Stress induced increase in progesterone have been reported to serve multiple purposes, such as facilitating the negative feedback loop of the HPA axis via the progesterone metabolite allo-pregnanolone [38], reducing feelings of anxiety, tension, stress, or depression. It is not possible for now to situate in a definitive way reasons for the significantly increased progesterone level in this study. It also remains to be seen if the increase in progesterone as reported in stressed group in this study is due to stress alone or is assisted by endogenous ascorbic acid synthesis. Rodents particularly rats used in this study have the capacity to synthesize ascorbic acid *in-vivo.* Unlike humans, rats do not rely entirely on exogenous sources for ascorbic acid synthesis as they have the enzyme L-gulonolactone oxidase [39] which catalyzes the last step of L-ascorbic biosynthesis *in vivo*.

In normal pregnancy, GnRH secretion and by extension LH and FSH secretion is strongly inhibited by the high plasma concentration of progesterone [34]. Furthermore, since progesterone secretion is continually increased throughout pregnancy, LH and FSH continually remain low. In this study, the reason for the significantly high serum LH level in pregnant rats supplemented with ascorbic acid is currently unclear. However pregnant rats exposed to CVS with or without ascorbic acid supplementation had significantly reduced LH & FSH. Stress is known to cause elevated circulating levels of glucocorticoids which act directly on the hypothalamus to suppress GnRH production [40]. Although glucocorticoids levels were not measured in this study, glucocorticoids have been reported to stimulate expression of RFamide-related peptides (RFRP); a gonadotrophin-inhibitory hormone which reduces GnRH production and also directly lower pituitary secretion of LH and FSH [41, 42].

AST & ALT

Among the most widely used liver enzymes in the determination of liver functions are the aminotransferases. These enzymes are normally predominantly contained within liver cells and to a lesser degree in the muscle cells. If the liver is injured or damaged, the liver cells spill these enzymes into the blood, raising the AST and ALT enzyme blood levels signaling liver damage/disease.

Serum levels of AST and ALT were not significantly increased across treatment groups in this study. However, the fact that AST and ALT levels were not statistically altered does not preclude the possibility of developing liver damage as indicated in the study of Gao *et al.,* [43]. Acute stress (though in male rats) was reported in the study to significantly alter LDL, HDL and expression of hepatic genes associated with lipid metabolism without significant impairment in AST and ALT levels. Therefore, determining the expression of these genes in future study is imperative.

Conclusion

Ascorbic acid supplementation ameliorates the oxidative stress of chronic variable stress exposure during pregnancy. Hormonal aberrations were also militated. These appeared achieved by enhancement of the antioxidant enzyme levels and suppression of reactive oxygen species activity. Future studies will focus on impact of ascorbic acid supplementation on variable stress exposure during early/late gestation period and the implication on *in-vitro* contractile function of excised uterine tissue.

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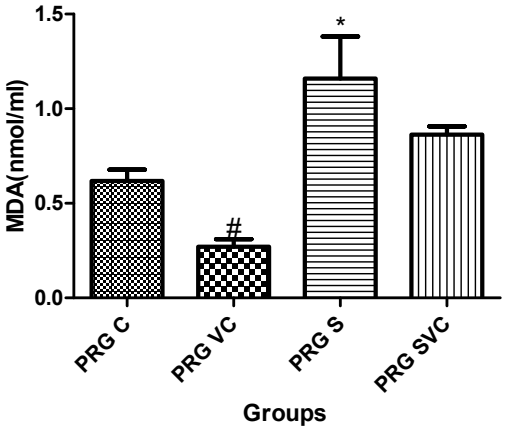

Figure 1. Effect of gestational chronic variable stress with ascorbic acid supplementation on serum level of malondialdehyde activity in Wistar rats.

PRG C – Pregnant Control rats, PRG VC –Pregnant rats with ascorbic acid supplementation, PRG S- Pregnant rats with chronic variable stress, PRG SVC –Pregnant rats with chronic variable stress and ascorbic acid supplementation
*=p<0.05 when compared to control
#=p<0.05 when compared to pregnant rats with chronic variable stress
Values are expressed as mean ± SEM

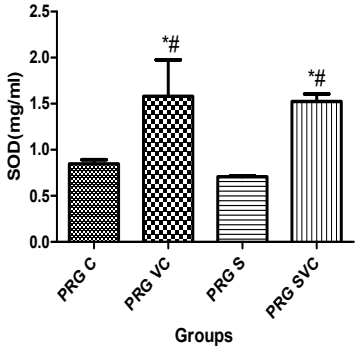

Figure 2. Effect of gestational chronic variable stress with ascorbic acid supplementation on serum level of SOD in Wistar rats

PRG C – Pregnant Control rats, PRG VC –Pregnant rats with ascorbic acid supplementation, PRG S- Pregnant stressed rats, PRG SVC –Pregnant stressed rats with ascorbic acid supplementation
*=p<0.05 when compared to control
#=p<0.05 when compared to pregnant rats with chronic variable stress
Values are expressed as mean ± SEM

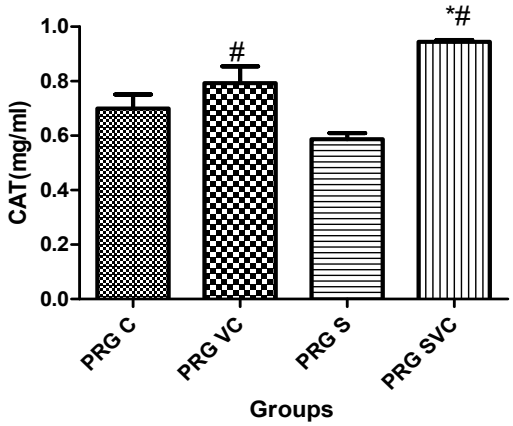

Figure 3. Effect of gestational chronic variable stress with ascorbic acid supplementation on serum level of catalase wistar rats

PRG C – Pregnant Control rats, PRG VC –Pregnant rats with ascorbic acid supplementation, PRG S- Pregnant stressed rats, PRG SVC –Pregnant stressed rats with ascorbic acid supplementation
*=p<0.05 when compared to control
#=p<0.05 when compared to pregnant rats with chronic variable stress
Values are expressed as mean ± SEM

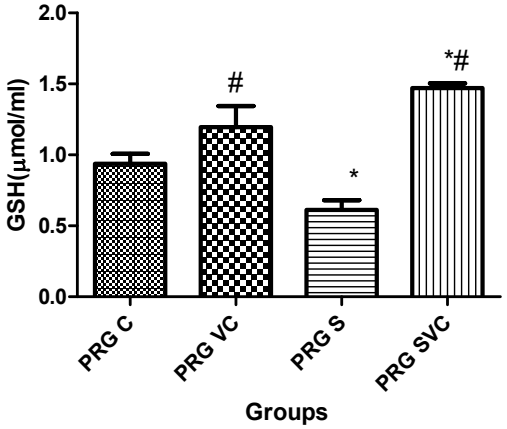

Figure 4. Effect of gestational chronic variable stress with ascorbic acid supplement on serum glutathione level of pregnant Wistar rats

PRG C – Pregnant Control rats, PRG VC –Pregnant rats with ascorbic acid supplementation, PRG S- Pregnant stressed rats, PRG SVC –Pregnant stressed rats with ascorbic acid supplementation
*=p<0.05 when compared to control
#=p<0.05 when compared to pregnant group with chronic variable stress
Values are expressed as mean ± SEM

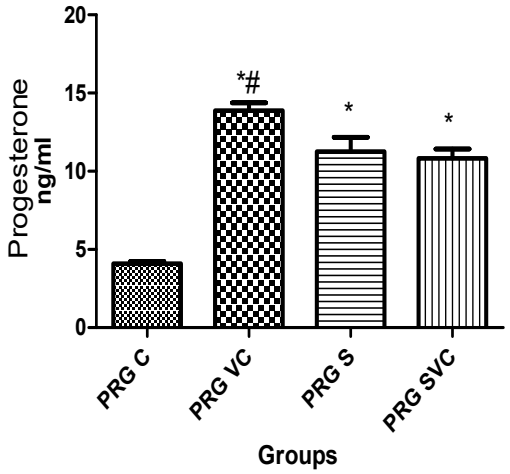

Figure 5. Effect of gestational chronic variable stress with ascorbic acid supplementation on serum level of progesterone in Wistar rats

PRG C – Pregnant Control rats, PRG VC –Pregnant rats with ascorbic acid supplementation, PRG S- Pregnant stressed rats, PRG SVC –Pregnant stressed rats with ascorbic acid supplementation
*=p<0.05 when compared to control
#=p<0.05 when compared to pregnant group with chronic variable stress
Values are expressed as mean ± SEM

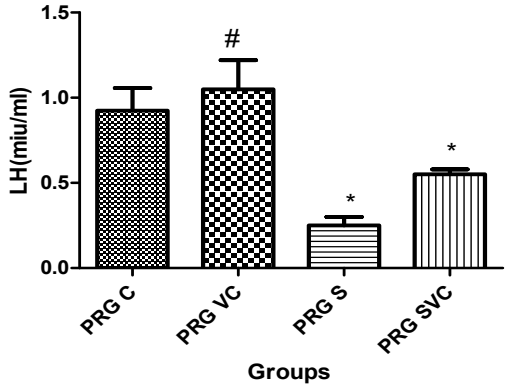

Figure 6. Effect of gestational chronic variable stress with ascorbic acid supplementation on serum level of LH

PRG C – Pregnant Control rats, PRG VC –Pregnant rats with ascorbic acid supplementation, PRG S- Pregnant stressed rats, PRG SVC –Pregnant stressed rats with ascorbic acid supplementation
*=p<0.05 when compared to control
#=p<0.05 when compared to pregnant rats with chronic variable stress
Values are expressed as mean ± SEM

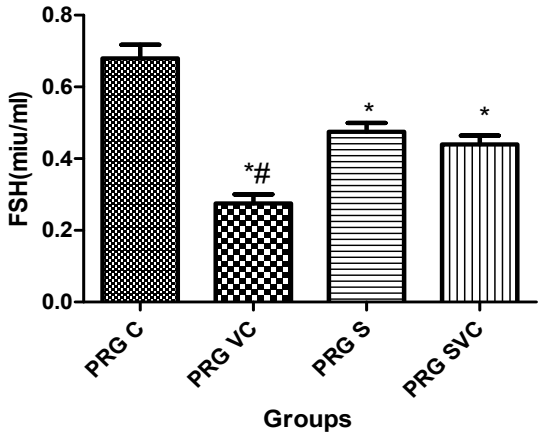

Figure 7. Effect of gestational chronic variable stress with ascorbic acid supplementation on serum level of FSH in Wistar rats

PRG C – Pregnant Control rats, PRG VC –Pregnant rats with ascorbic acid supplementation, PRG S- Pregnant stressed rats, PRG SVC –Pregnant stressed rats with ascorbic acid supplementation
*=p<0.05 when compared to control
#=p<0.05 when compared to pregnant rats with chronic variable stress
Values are expressed as mean ± SEM

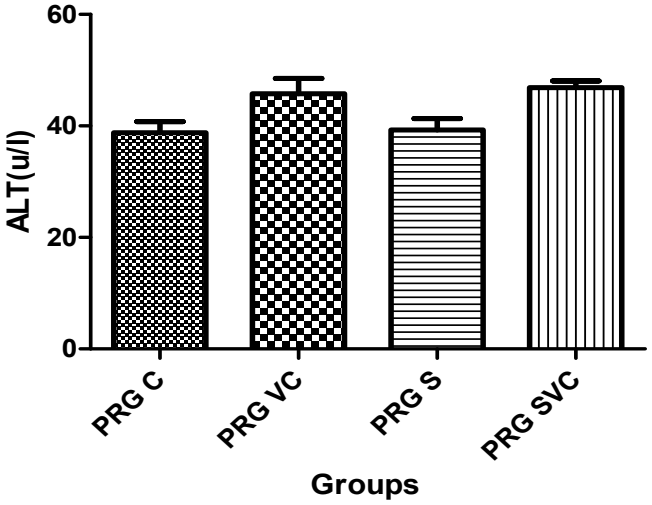

Figure 8. Effect of gestational chronic variable stress with ascorbic acid supplementation on the serum level of ALT in Wistar rats

PRG C – Pregnant Control rats, PRG VC –Pregnant rats with ascorbic acid supplementation, PRG S- Pregnant stressed rats, PRG SVC –Pregnant stressed rats with ascorbic acid supplementation
Values are expressed as mean ± SEM

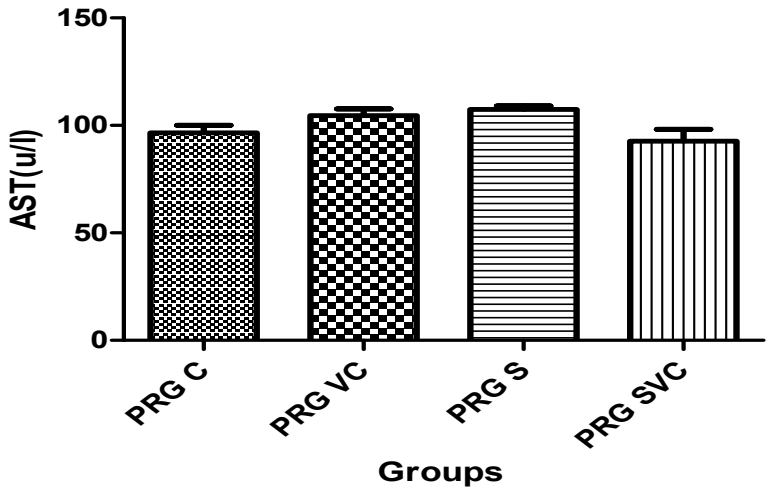

Figure 9. Effect of gestational chronic variable stress with ascorbic acid on the serum level of AST in Wistar rats

PRG C – Pregnant Control rats, PRG VC –Pregnant rats with ascorbic acid supplementation, PRG S- Pregnant stressed rats, PRG SVC –Pregnant stressed rats with ascorbic acid supplementation
Values are expressed as mean ± SEM