J. Afr. Ass. Physiol. Sci. 8 (1): 16-22, July 2020

Journal of African Association of Physiological Sciences

Official Publication of the African Association of Physiological Sciences
http://www.jaaps.aapsnet.org

Research Article

Attenuation of impaired uterine contractile activity by ascorbic acid supplementation during early gestational variable stress exposure in Wistar rats

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Keywords:
Variable stress, early gestation, uterine contractility, ascorbic acid, oxidative stress

ABSTRACT

Background: This study investigates the effect of ascorbic acid supplementation during early gestational variable stress exposure on stress markers and *in-vitro* contractile responses of the uterus. **Methods:** Twenty nulliparous pregnant rats (180-200 g) were randomly divided into 4 groups of 5 each. Stressed animals were variably exposed to 6 non-habituating and painless stresses (sleep deprivation, predator exposure, immobility, rapid cage changes, noise, and foreign object). Treated groups received ascorbic acid supplementation orally (10 mg/kg bwt) with or without stress exposure while control group received normal saline only. Stress exposure and ascorbic acid treatment was during gestation days (GD) 1-8. Serum cortisol, oxidative biomarkers and *in-vitro* contractile responses of excised uterine tissue to acetylcholine, oxytocin, calcium chloride, potassium chloride, diclofenac, and magnesium were assessed. Statistical significance was taken at $p<0.05$. **Results:** Ascorbic acid supplementation in stressed pregnant group significantly deceased ($p < 0.05$) MDA activity. Catalase activity was enhanced in ascorbic acid supplemented stressed group while serum cortisol levels were significantly ($p < 0.05$) reduced in ascorbic acid supplemented stressed group when compared to stress only exposed group. Concentration dependent contraction responses to acetylcholine, oxytocin, calcium chloride, and potassium chloride were significantly reduced in stressed only pregnant rats. Ascorbic acid supplementation in stressed group reversed these reductions. However, doses of diclofenac and magnesium showed no significant effect on relaxation responses across all groups. **Conclusion:** Ascorbic acid supplementation during early gestational variable stress exposure attenuated impaired contractile functions of the uterus. Enhanced antioxidant enzymes and suppressed MDA activity appear to play a role in the modulation.

INTRODUCTION

Timing of maternal stress exposure has long been known to be critical in determining maternal and fetal wellbeing (Mueller and Bale, 2006). Variable stress exposure during early pregnancy is reported to contribute to male neurodevelopment disorders through impacts on placental functions and development (Muellar and Bale, 2008). Undue contractility of the uterus during early gestational period is inimical to pregnancy survival and fetal growth (Markiewicz *et al*., 2016). The normal regulation of myometrium contractility is important for maintenance of pregnancy (Tribe, 2001) and the quiescence and contractile unresponsiveness period during early gestation is reported to be critical to pregnancy survival (Mendelson *et al*., 2019). Recent reports have shown that oxidative stress in pregnancy (Sultana *et al.*, 2017) and metabolic syndrome (obesity) precipitating gestational oxidative stress usually affect reproduction and plasticity of the endometrium (Murakami *et al*., 2013)

Several studies have however, identified the beneficial impacts of ascorbic acid supplementation on stress in women. Mc Evoy *et al*, (2014) found that oxidative stress induced by pregnant smokers given ascorbic acid supplementation improved their newborn pulmonary function test. Dietary ascorbic acid supplementation in humans resulting in sustained high dose ascorbic acid release was reported to help reduce anxiety and mitigate blood pressure response to stress (McCabe *et al*., 2017). Maternal ascorbic acid supplementation was also able to

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prevent offspring DNA methylation changes associated with oxidative stress generated by maternal smoking during pregnancy (Shovey- Kendrick *et al*., 2017). Despite the foregoing, the beneficial impact of gestational ascorbic acid supplementation in literature remains inconclusive (Rumbold *et al*., 2015). This study investigates effect of ascorbic acid supplementation during early gestational variable stress exposure on uterine tissue responses to few utero-tonic agents.

METHODS

Animals

Twenty healthy, nulliparous female wistar albino rats, between 8 and 10 weeks of age and weighing 180 – 200 g were acquired from the Lagos State University College of Medicine Animal House. The animals were kept and maintained under conventional laboratory conditions (22 ± 3^{0} C temperature & 30 - 70 % humidity). 12 hours light/ dark period was maintained and animals were allowed standard pellet diet (Livestock Feeds Nig., Ikeja, Nigeria) and water. Standard animal handling and care protocol were adhered to throughout the study period.

Gestation day determination

Female animal at pro-estrus were paired with proven breeder male at ratio 1:1 male/female overnight. The appearance of sperm positive vaginal smear early the following morning, was used as indicator of positive mating (Salami and Raji, 2015). Gestation day (GD 1) was taken as the day following the observation of sperm positive vaginal smear (Salami and Raji, 2015).

Stress exposure procedure

Stressed group were exposed to daily, variable, unpredictable stress as described by Mueller and Bale, (2006) with slight modifications for predator exposure and noise (Salami *et al*., 2020). These include: rapid cage changes during the light period (at an interval of 20 minutes for two hours), noise exposure (≥100 decibels for 4 hours during light period), predator exposure (cat exposure in the same cage as the rats with a wire mesh separation for 30 minutes), immobility (restraining in a 50 ml tube for 30 minutes), sleep deprivation overnight (rats made to stand in a cage containing 700 ml of water at 23^{0}C overnight) and foreign object exposure (marbles) in cages overnight.

Experimental design and treatment

Twenty pregnant rats were randomly divided into four groups of 5 each. Group 1 and group 2 were treated orally with normal saline (vehicle) and ascorbic acid (10 mg/kg bwt) (McEvoy *et al.,* 2014) respectively, during early gestation days (EGD) 1-8. Groups 3 and 4 were exposed to variable stress during EGD 1-8, and group 4 was concurrently supplemented orally with ascorbic acid (10 mg/kg bwt). Ascorbic acid supplementation was via single, oral gavage, daily.

Drugs and Chemicals

Ascorbic acid and diclofenac used was from Kunimed Pharmachem LTD Nigeria. Drugs (Potassium Chloride (KCl), Acetylcholine (Ach), Phenylephrine (PHE), and Calcium chloride ($CaCl_2$) used for the dose response of the uterine strips was purchased from Tocris online store UK.

Serum collection

Blood samples were collected via cardiac puncture using syringe into plain sample bottles. Blood samples were then centrifuged by cold centrifugation (Uniscope Laboratory Centrifuge by Surgifriend Medicals, England) at 3000 rpm for 5 minutes. The serum obtained was stored at -4^{0}C for hormonal and oxidative biomarkers assay.

Tissue preparation for contractile activity of the uterus

The animals were anaesthetized with sodium pentobarbital and then sacrificed by cervical dislocation. The abdomen was surgically opened and the uterine horns exposed by means of blunt dissection and then freed of connective tissue. Each horn was cut out separately and transferred to a petri dish containing physiological salt solution (De Jalon). Strips of 2-3 cm long were suspended with cotton thread to the base of a 50 ml isolated organ bath containing the De Jalon solution. The other end of the strip was attached to an isometric force transducer (model 7004; Ugo-Basile Varese, Italy). This was coupled to a Data Capsule Acquisition System Model 17400, for isometric tension recording. The De Jalon's solution contained the following composition (mM): NaCl (154), $NaHCO_3$ (1.7), $MgCl_2$ (1.4), KCl (5.6), $CaCl_2$ (0.3) and glucose (5.55) (E.Merck, Darmstadt, Germany). The temperature of the organ/tissue bath was maintained at 37^{o}C and the solution was bubbled with a 95% O_2 + 5% CO_2 gas mixture (pH 7.35 – 7.40). The mounted uterine strips preparations were subsequently left to equilibrate for 30 min, under a passive tension of 1 g during which time the bathing physiological solution was changed after every 10 minutes.

Dose response of uterine tissue to acetylcholine

The uterine muscle strip was allowed to stabilize in the physiological solution for 30 minutes, after which the cumulative dose-response to acetylcholine (10^{-9} to 10^{-5}

Log M) was recorded using isometric transducer connected to the Data Capsule Acquisition system. The response was at the steady level before another dose was added.

Dose response to potassium chloride and calcium chloride

Dose response to potassium was determined by adding potassium (10 - 60 mM) cumulatively to potassium-free solution in the organ chamber. Dose response to calcium was determined by adding calcium chloride (10 - 60 - Log mM) cumulatively to calcium free solution in the organ chamber.

Dose response to oxytocin

Dose response of the uterine tissue to oxytocin (10^{-6} – 10^{-3} µg) was cumulatively determined and contractile responses were then recorded.

Table 1: Implication of ascorbic acid supplementation during early gestational variable stress exposure on serum MDA, SOD, catalase and cortisol level

N=5	MDA (µmol/ml)	SOD (µmol/ml)	CATALASE (µmol/ml)	CORTISOL (µg/dl)
Control	1.84 ± 0.21	467.55 ± 50.78	917.30 ± 82.7	1.00 ± 0.33
Vit C	0.73 ± 0.42	689.19 ± 75.91	1306.42 ± 229.99*	1.40 ± 0.08
VS	2.60 ± 0.30*	450.96 ± 17.5	644.91 ± 75.88	2.80 ± 0.61
VS+vit C	1.05 ± 0.27#	490.85 ± 82.45	1201.29 ± 12.64	1.50 ± 0.20#

*# $p<0.05$ when compared to control; # $p<0.05$ when compared to stressed only group

Dose response to diclofenac

Dose response to diclofenac (10 - 60 mM) after pre-contracting with phenylephrine (10^{-7}M) was determined cumulatively on the uterine tissue.

Dose response to magnesium chloride

Dose response of the uterine tissue to Magnesium chloride (20-80 mM) was determined cumulatively. All contractile responses were measured using the Ugo Basile data acquisition system.

Determination of serum catalase, superoxide dismutase, MDA and cortisol activity

Catalase activity was assayed in the serum colorimetrically according to the methods described by Sinha, *(*1972). Superoxide dismutase activity was determined by its ability to inhibit the auto-oxidation of epinephrine as described by Sun and Zigma (1978). Malondialdehyde (MDA), an index of lipid peroxidation was determined using the method of Uchiyama & Mihara (1978). Serum cortisol was determined using (ELISA kit, Monobind Inc, Lake Forrest, CA, USA) (Product code 3625-300) using the instructions in the manual. The normal within assay coefficient of variation was 6.4 % while the inter assay coefficient of variation was 7 %.

Statistical analysis

Data were analyzed using Graph pad prism (version 5) statistical software and one-way ANOVA and Newman Keuls post hoc test was determined. P values less than 0.05 were considered statistically significant.

RESULTS

Effect of early gestational variable stress with ascorbic acid supplementation on serum MDA, SOD, catalase and cortisol level

Ascorbic acid supplementation in stressed pregnant group significantly decreased ($p < 0.05$) MDA activity (Table 1). Catalase activity was enhanced in ascorbic acid supplemented stressed group while serum cortisol levels were significantly ($p < 0.05$) reduced in ascorbic acid supplemented stressed group when compared to stress only exposed group (Table 1).

Contractile responses of the uterus to acetylcholine, oxytocin, calcium chloride, potassium chloride, diclofenac and magnesium chloride during early gestational variable stress exposure with ascorbic acid supplementation

Contractile responses were significantly ($P<0.05$) reduced in stress only exposed pregnant rats. There were obvious impaired responses to acetylcholine, oxytocin, calcium chloride, and potassium chloride (figures 1, 2, 3, and 4 respectively). Ascorbic acid supplementation in stressed group reversed these reductions as shown in the figures. However, doses of diclofenac and magnesium showed no significant effect on relaxation responses across all groups (figure 5 & 6 respectively).

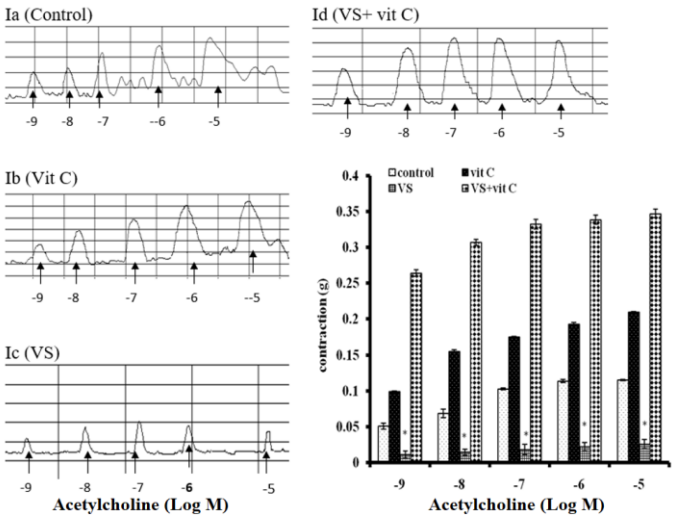

Fig. 1. Representative tracings (Ia-Id) and mean contractile responses (bar chart) induced by acetylcholine (10^{-9} to 10^{-5} M) across groups. Contractile responses of the uterine strips to acetylcholine after early gestational variable stress exposure with ascorbic acid supplementation. N=5, * p <0.05 when compared with control and ascorbic acid supplemented groups

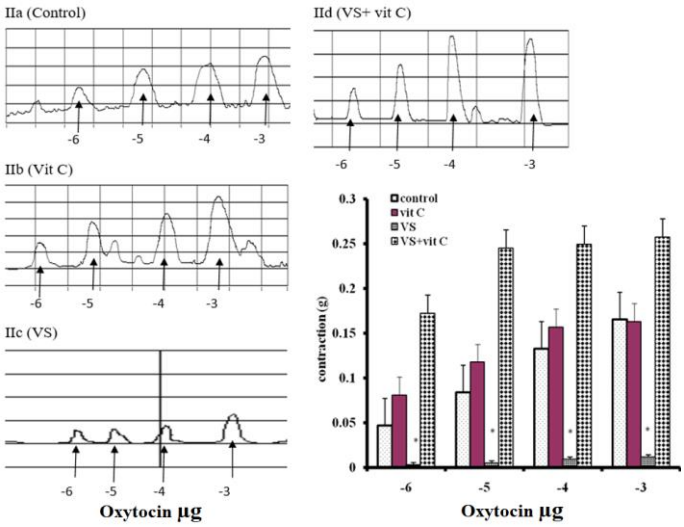

Fig. 2. Representative tracings (IIa-IId) and mean contractile responses (bar chart) induced by oxytocin (10^{-6} to 10^{-3} M) across groups. Contractile responses of the uterine strips to oxytocin after early gestational variable stress exposure with ascorbic acid supplementation. N=5, * p <0.05 when compared with control and ascorbic acid supplemented groups

DISCUSSION

Result from this study showed that ascorbic acid supplementation during early gestational variable stress exposure was able to ameliorate impaired utero-tonic functions. Contractions to utero-tonic agents like acetylcholine, oxytocin and calcium chloride were significantly enhanced in ascorbic acid supplemented groups when compared to control and stressed.

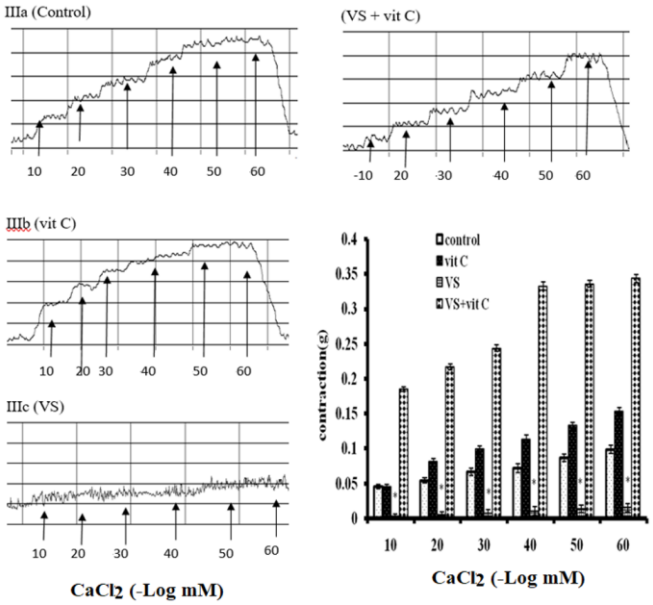

Fig. 3. Representative tracings (IIIa-IIId) and mean contractile responses (bar chart) induced by $CaCl_2$ (10 to 60 mM; -Log.) across groups. Contractile responses of the uterine strips to $CaCl_2$ after early gestational variable stress exposure with ascorbic acid supplementation. N=5, *p <0.05 when compared with control and ascorbic acid supplemented groups.

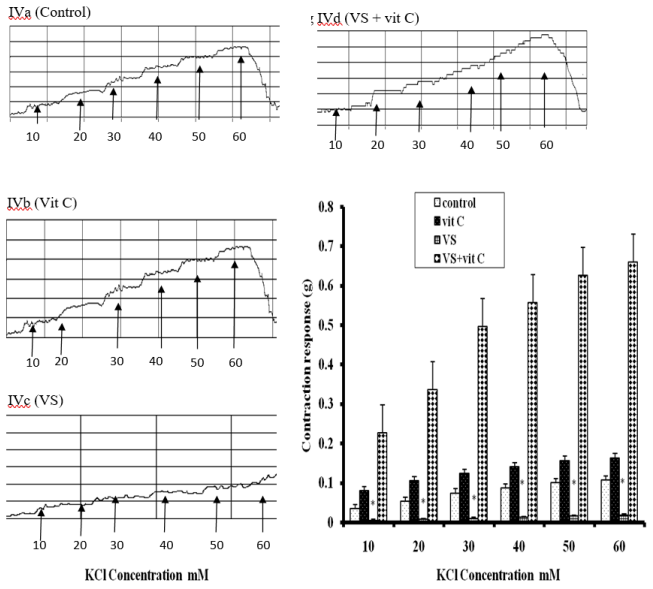

Fig. 4. Representative tracings (IVa-IVd) and mean contractile responses (bar chart) induced by KCl (10 to 60 mM) across groups. Contractile responses of the uterine strips to KCl after early gestational variable stress exposure with ascorbic acid supplementation. N=5, * p <0.05 when compared with control and ascorbic acid supplemented groups

group. Uterine smooth muscle strips contractions were significantly inhibited in variable stress only exposed

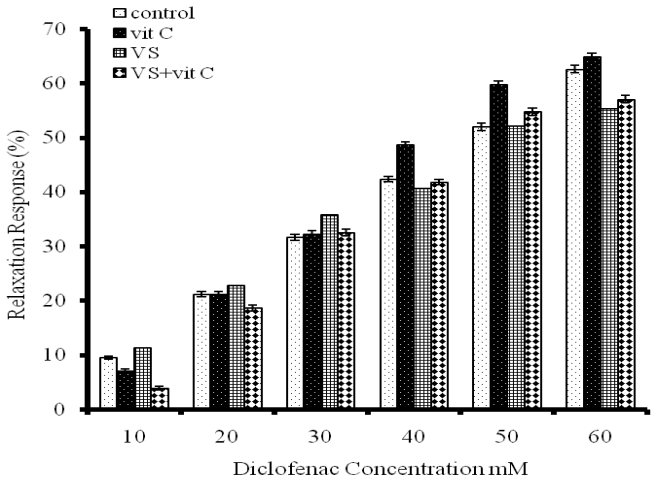

Fig. 5. Relaxation responses of the uterus strips to diclofenac after early gestational variable stress exposure with ascorbic acid supplementation. N = 5

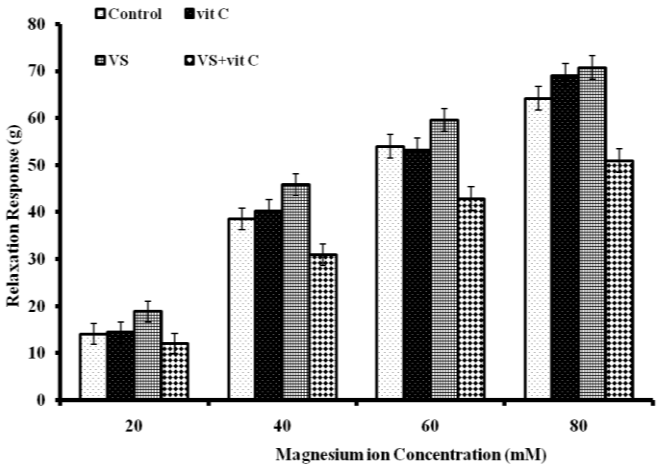

Fig. 6. Relaxation responses of the uterus strips to magnesium after early gestational variable stress exposure with ascorbic acid supplementation. N = 5

group. These results highlight the significance of calcium in the mediation of ascorbic acid action during supplementation. Calcium is a key mediator for contraction as its influx via different membrane gates (voltage gated Ca^{2+} channel (VGCC), receptor operated calcium channel (ROCC), store operated calcium channel (SOCC)) into cell is known to initiate depolarization of smooth muscle membrane and subsequent contraction (Catterall, 2011). Calcium release from intracellular sarcoplasmic reticulum can also increase intracellular calcium resulting in contractions (Reddish *et al.*, 2017). Oxytocin augments uterine contraction by increasing intracellular calcium concentration (McKillen *et al.*, 1999). Acetylcholine acting through its receptors, releases Ca^{2+} from intracellular store. It also, through acetylcholine receptors on uterus, initiates 2nd messenger activation of inositol 1, 4, 5 triphosphate and diacylglycerol to facilitate contraction of uterus (Kuo & Ehrlich, 2015). All the aforementioned mechanisms seemed to be enhanced by ascorbic acid supplementation as observed from the results obtained from this study with variable stress exposure appearing to impair these mechanisms. During early gestation, oxidative stress has been reported to increase the risks of miscarriage by reducing the plasticity of the endometrium (Alcala *et al.*, 2018). We suggest these appear to be responsible for the impaired contractile functions observed in this study.

Uterine contractile activity mediated by ascorbic acid supplementation in this study is similar to that reported by Salahdeen *et al.*, (2017) in *Tridax procumbens* leaf extract. *Tridax procumbens* is a plant with a reported potent antioxidant properties. Although the study of Salahdeen *et al* (2017) was in normal pregnant rats, this study has highlighted the potential of the antioxidant ascorbic acid in ameliorating impaired uterus function in response to early gestational variable stress exposure. This study also highlights the fact that oxytocin mediated contractile functions of the uterus was most impaired by variable stress exposure. This observation can be very fatal especially late in pregnancy when oxytocin mediated contraction of the uterus is expected to facilitate parturition. Oxytocin receptors are known to increase close to and during parturition (Andrews & Sreven, 2003). However, it is currently unknown if such receptor increase at that period will be enough to enhance uterine contraction more than that observed in this study.

Uterus strips relaxation responses to diclofenac, a non-steroidal anti-inflammatory drug in this study was enhanced at higher doses in stressed ascorbic acid supplemented group. Ascorbic acid supplementation without stress exposure also enhanced relaxation of uterus comparatively to normal pregnant rats. The enhanced relaxations as observed during early gestation in this study underpin the beneficial impact of ascorbic acid supplementation. Early gestation period is often a quiescent period necessary to prevent spontaneous abortion (Markiewicz *et al.*, 2016). Diclofenac inhibits COX1 or COX2 enzymes thereby decreasing prostaglandins release. This subsequently causes relaxation of uterine smooth muscle (Kothencz *et al.*, 2018).

Oxidative biomarkers

A key factor responsible for adverse pregnancy outcome is oxidative stress (Sultana *et al.*, 2017). Accumulation of OS is known to damage lipids, proteins and DNA of placental tissue causing premature ageing and pregnancy

complication (Sultana *et al*., 2017). Maternal obesity induced oxidative stress during gestation period is also reported as risk factor for adverse outcomes (Alcala *et al.,* 2018). Pre-eclampsia, intrauterine growth restriction and spontaneous abortion are reported possible outcomes. Recent studies have also suggested reduced regeneration capacity and plasticity of the endometrium (Murakami *et al*., 2013) in obese pregnant rats. Antioxidant supplementation is an interesting prophylactic and therapeutic tool that have yielded positive results in cellular and experimental animal models (Alcala *et al*., 2018). This study corroborated these findings as MDA activity was inhibited in ascorbic acid supplemented groups when compared to control and stress only exposed groups. Antioxidant enzymes activities were also enhanced in supplemented groups. This appears to be responsible for the improved plasticity and contractile function of the uterine tissues from ascorbic acid supplemented groups. This result has also shown the clinical relevance of ascorbic acid supplementation early in gestation. Impaired uterine contractile functions resulting from variable stress exposure that could be detrimental to pregnancy survival and fetal growth were significantly attenuated by ascorbic acid supplementation.

Conclusively, ascorbic acid supplementation during early gestational variable stress exposure attenuated impaired contractile functions of the uterus. Enhanced antioxidant enzymes and suppressed MDA activity appear to play a role in the modulation from this study.

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