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Effect of Prenatal Stress on Expression of Glutathione System in Neonatal Rat Brain

Prenatal Stresin Neonatal Sıçan Beyninde Glutatyon Sistemi Ekspresyonuna Etkisi

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ABSTRACT

AIM: Prenatal stress is known to adversely affect the fetal brain development and also neuronal loss. The mechanism(s) associated with prenatal stress induced developmental neurotoxicity remains obscure. Few studies point to the glutathione (GSH) antioxidant system which is an important molecular target for this toxicant. Hence the present study investigates the effect of prenatal stress on glutathione system in neonatal rat brain.

MATERIAL and METHODS: Three to four months old pregnant Wistar rats were subjected to restraint stress during early or late gestational period. The offspring were sacrificed on 40th day and their brain homogenate was subjected to antioxidant studies. The serum corticosterone and adrenal ascorbic acid levels were also estimated from offspring.

RESULTS: The prenatal stress has resulted in an increase in the serum corticosterone and reduced adrenal ascorbic acid levels in neonatal pups. Prenatal stress during early or late gestation life showed reduced glutathione, glutathione reductase (GSSG-Rd) and superoxide dismutase (SOD) activity in offspring brain homogenate.

CONCLUSION: These data suggest that stress during early or late gestation period affect glutathione system in developing neonatal rat brain, which is associated with elevated serum corticosterone and reduced adrenal ascorbic acid levels.

KEYWORDS: Prenatal stress, Glutathione, Glutathione reductase, Superoxide dismutase, Antioxidant enzymes

ÖZ

AMAÇ: Prenatal stresin fetal beyin gelişimi ve ayrıca nöron kaybını olumsuz şekilde etkilediği bilinmektedir. Prenatal stres tarafından indüklenen gelişimsel nörotoksisiteyle ilişkili mekanizma(lar) halen bilinmemektedir. Birkaç çalışma bu toksik madde için önemli bir moleküler hedef olan glutatyon (GSH) antioksidan sistemine işaret etmektedir. Bu nedenle bu çalışma neonatal sıçan beyninde prenatal stresin glutatyon sistemi üzerine etkisini incelemektedir.

YÖNTEM ve GEREÇLER: Üç ila dört aylık hamile Wistar sıçanları erken veya geç gestasyonel dönemde zaptetme stresine maruz bırakıldı. Yavrular 40. günde sakrifiye edildi ve beyin homojenatları antioksidan çalışmalara tabi tutuldu. Ayrıca yavrularda serum kortikosteron ve adrenal askorbik asit düzeyleri saptandı.

BULGULAR: Prenatal stres neonatal sıçanlarda artmış serum kortikosteron ve azalmış adrenal askorbik asit düzeylerine neden oldu. Erken veya geç gestasyonda prenatal stres, yavruların beyin homojenatında azalmış glutatyon, glutatyon redüktaz (GSSG-Rd) ve süperoksit dismutaz (SOD) aktivitesine neden oldu.

SONUÇ: Bu veriler erken veya geç gestasyon döneminde stresin gelişen neonatal sıçan beyninde glutatyon sistemini etkilediği ve sonuçta artmış serum kortikosteron ve azalmış adrenal askorbik asit düzeyleriyle ilişkili olduğunu düşündürmektedir.

ANAHTAR SÖZCÜKLER: Prenatal stres, Glutatyon, Glutatyon redüktaz, Süperoksit dismutaz, Antioksidan enzimler

INTRODUCTION

A substantial body of evidence indicates that prenatal stress adversely affects the brain development with neuronal loss and cognitive dysfunction. There are reports suggesting the possible mechanisms like increased brain corticosterone level (27), altered hypothalamo-pituitary-adrenal (HPA) axis (14), altered neurotransmitters (10) and the altered cellular homeostasis by oxidative damage (10) in brain which in turn involves neuronal death. Though the mechanisms involved in prenatal stress induced neuronal damage is not clearly understood, we hypothesized that the defective antioxidant system in the developing rat brain could be the cause, since brain is particularly vulnerable to oxidative stress due to its high rate of oxygen consumption. Though neurons are endowed with defense and repair mechanism to override the oxidative stress but existence of such defensive system in developing brain is obscure.

A number of studies suggest that prenatal stress is associated with alterations in the offspring HPA axis activity. Some studies find prenatal stress to be associated with higher basal glucocorticoid (GC) secretion. For example, baseline corticosterone release is elevated in rat pups whose mothers were stressed during gestation (12,15,27,29) or when stress hormones were administered to the mothers during pregnancy (7). Prenatal stress during the last week of pregnancy resulted in 64% increase in the level of plasma corticosterone in the offspring (27).

Enhanced plasma corticosterone level due to prenatal stress has a major impact on the developing brain (28). The possible involvement of oxidants and the relation to GC hormones in stress has been proposed (11,18). Enhanced GC inhibits glucose utilization, thereby compromising the activity of energy-dependent excitatory amino acid transporters. The ensuing increase in glutamate concentrations results in excessive stimulation of postsynaptic excitatory amino acid receptors and unregulated increase in intracellular Ca^{2+}concentrations. Consequently, Ca^{2+}dependent endonucleases and other Ca^{2+}dependent enzymes that contribute to the production of reactive oxygen species (ROS) are activated. In addition, mitochondrial respiration is compromised by overload of calcium and other GC-mediated effects, further contributing to ROS production. This could be the one of the factor associated with the cellular damage. Hence the cellular damage in the developing brain could be attributed to over production of oxidants. Hence the present study is focused to evaluate the antioxidant defense system in neonatal rat brain and also looking at possible role of fetal serum corticosterone.

MATERIAL and METHODS

Animals and housing conditions:

In-house bred male and female albino Wistar rats (3-4 months old) of weight 200-230gm were selected for the study. The rats were maintained in 12 hours light and dark cycle in temperature and humidity controlled environment. The rats were fed with standard food pellet and water *ad libitum*. Polypropylene cage with paddy husk as bedding materials was used for housing the rats. Breeding and maintenance of the animals were done as per the guidelines of Government of India for use of Laboratory animals (Government of India notifies the rules for breeding and conducting animal experiments, proposed in the gazette of India Dec 15, 1998: which was reproduced in Ind. Journal of Pharmacol 31:92-95, 1999). Institutional Animal Ethics Committee (I.A.E.C) approval was obtained before the conduct of the study (IAEC/KMC/11/06/2010) and care was taken to handle the rats in humane manner.

Mating of rats and animal groups:

Female rats were allowed to mate with one fertile sexually active male for four hours per day (separate male rats for each group). At the end of four hours, female rats were separated and vaginal smears taken to detect the presence of sperm for the confirmation of pregnancy and the rats were designated as day 0 of pregnancy for further counting the days. The pregnant rats were housed individually in separate cages with proper label indicating the day of conception and randomly allocated into three groups of six each. One male and one female pup from each mother (a total of 12 pups in each group) were considered for antioxidant studies.

Stressing procedure

The pregnant rats were stressed (restraint stress) using a wire mesh restrainer (23) for three times daily for 45 min. The wire mesh restrainer will have a wooden base and stainless steel wire mesh restrainer hinged to the base. A pad lock and latch will help to secure the rat in the restrainer. The restrainer with dimension 11 cm (L) x 6cm (B) x 6 cm (H) was used for rats with gestation day 1 to 10. Restrainer of 11cm (L) x 8 cm (B) x 8 cm (H) was used for rats with gestation day 11 to till delivery. This type of restrainer will only restrict the animal movement without any pain, discomfort or suffocation.

Animal groups

Group 1 (Control). The pups belonging to the pregnant rats who received only 0.5% carboxy methyl cellulose in a dose of 10ml/kg body weight (oral) throughout pregnancy.

Group 2. The pups belonging to the pregnant rats who received restrain stress from gestation day 1 to 10.

Group 3. The pups belonging to the pregnant rats who received restrain stress from gestation day 11 to till delivery.

All the mothers delivered at term (22-24th day of gestation). The offspring of the all groups were raised by their biological mothers until weaning (21st day after birth).

Chemicals

All chemicals and reagents are HPLC or analytical grade (Sigma, St. Louis, Mo, U.S.A.) procured from Sri Durga laboratories, Mangalore, India.

Experimental design:

At the 40th postnatal day one male and one female pups from each mother (n=12) were weighed and sacrificed by decapitation after obtaining blood sample under ether anesthesia. The whole brain was removed rapidly and rinsed with sucrose isotonic buffer. Tissue was weighed and homogenized (1:10w/v) in sucrose isotonic buffer (0.32 mM sucrose, 12.5 mMTris and 1 mM EDTA), pH 7.4 and dilution 1:19. The homogenate was centrifuged at 10,000g for 20 min at 4°C and aliquots of supernatant were separated and used for following biochemical estimations. Adrenal gland was removed and 10%(w/v) tissue homogenate was prepared in ice-cold 0.1M saline phosphate buffer (pH 7.4), centrifuged for 15min 10,000g at 4°C and the supernatant was used for estimation of adrenal ascorbic acid.

Quantification of corticosterone concentrations in blood serum:

Blood samples were taken between 8.00 and 10.00 AM

to avoid circadian variations of serum corticosterone concentrations. The animals were anesthetized individually in a glass jar containing saturated ether vapour and intracardiac blood was collected. Corticosterone was measured by using electro chemiluminescent method using an ECOBAS 411 automatic analyzer (Roche Diagnostics, U.S.A.) established at the Kasturba Medical College Laboratory, Mangalore, India. The concentration of corticosterone was expressed in ng/ml serum.

Adrenal gland weight and estimation of adrenal ascorbic acid:

The abdominal cavity was opened and the adrenal glands were recovered, carefully freed from adjacent tissues under a stereo dissecting microscope, and weighed individually by using high precision single pan electronic weighing balance (Adventure™).

Adrenal ascorbic acid was measured as described previously by Lyle et al. (20). In the adrenal gland homogenate, 6% Tricaboxylic acid (TCA) was added and centrifuged at 3000g for 15min. The supernatant was coupled with 2,4,N-dinitrophenyl hydrazine (DNPH) in presence of thiourea as a mild reducing agent. 2ml cold conc. H_2SO_4 (12M) was added drop by drop, which converts DNPH into a red compound, which was assayed spectrophotometrically with a Systronic-117 UV-Visible spectrophotometer at 520nm. The value was expressed in µg/mg protein. Protein was measured using the Lowery's method (13).

Estimation of reduced Glutathione:

Tissue GSH concentration was estimated according to the method described by Ellman (6). One ml of supernatant was precipitated with 1ml of metaphosphoric acid and cold digested at 4°C for 1h. The samples were centrifuged at 1,200g for 15min at 4°C. To 1ml of this supernatant, 2.7ml of phosphate buffer and 0.2ml of 5, 5' dithio-bis (2-nitrobenzoic acid, DTNB) was added. The yellow color that developed was read immediately at 412nm using a Systronic-117 spectrophotometer. The values were expressed in mg/gm protein. The total protein concentration of tissues was measured by the method of Lowry et al. (13).

Assay of Glutathione Reductase:

The GSSG-Rd activity was measured using the method originally described by Moron et al. (19). The reaction mixture consisted of 1.6ml of 0.067M potassium phosphate buffer (pH 6.6), 0.12ml of 0.06% NADPH, 0.12ml 1.15% GSSG, 0.1ml of enzyme source and water in a final volume of 2ml. All mixtures and solutions were prepared at room temperature. Control cuvettes then received 180µL of deionised water while sample cuvettes received 60µL of deionised water and 120 µL of GSSG solution. NADPH oxidation was followed for 5min and was recorded using a Systronic-117 spectrophotometer. The reduction of GSSG to GSH was determined indirectly by the measurement of the consumption of NADPH, as demonstrated by a decrease in absorbance at 340 nm as a function of time. The enzyme activity was calculated using extinction coefficient of chromophore ($1.36x10^4$ $(mol/l)^{-1}$ cm^{-1}) and expressed as nmol NADPH oxidized/min/mg protein. Protein content was determined by the method of Lowry et al. (13) with bovine serum albumin as standard.

Assay of Superoxide Dismutase activity:

Superoxide dismutase (SOD) activity was determined by the method of Marklund et al. (17). The reaction was performed in an mixture containing $5.6x10^{-5}$ M nitrobluetetrazolium (NBT), $1.17X10^{-6}$M riboflavin, $1x10^{-2}$M methionine in 0.05M potassium phosphate buffer, pH 7.8 with suitably diluted tissue homogenate in a total volume of 3ml. Illumination of solution was carried out in an aluminum lined foil box fitted with an 15v fluorescent lamp. The solution taken in a beaker was kept in the box and illuminated exactly for 10min. Control without the enzyme source was prepared. The absorbance was measured spectrophotometrically with a Systronic-117 UV-Visible spectrophotometer at 560 nm. SOD activity was expressed as specific activity of the enzyme in units per mg protein (U/mg protein). Protein content was determined by the method of Lowry et al (13).

Statistical analysis:

All the values were expressed as mean ± SE. The significance of differences among the groups was assessed using one way analysis of Variance (ANOVA) test followed by Bonferroni's multiple comparison test. Comparison of data between male and female group was assessed by unpaired "t" test. P values < 0.05 were considered as significant.

RESULTS

Gestational period stress did not had significant effect on gestational length ($p=0.077$, $F=2.231$) and litter size ($p=0.689$, $F=0.614$). There was absolute no mortality in any of the group till 21st postnatal day (Table I).

There was no sexually dimorphic effect in all the parameters studied, hence mean values for both male and female were collapsed. Early and late gestational stress did not show any difference in any parameters studied, as there is no statistical significant difference was observed between group-2 and group-3.

Effect of prenatal stress on basal corticosterone levels in the offspring

A significant increase in serum corticosterone level in the offspring of stressed mothers {group-2 ($p<0.01$) and group-3 ($p<0.001$)} compared to control rat pups (group-1) (Figure 1).

Table I: Effect of Prenatal Stress on Gestational Length and Litter Size

	Gestational length (days)	Litter size
Group-1	22.83±0.16	6.83±0.60
Group-2	23.33±0.21	7.33±0.55
Group-3	22.50±0.22	7.16±0.47

Effect of prenatal stress on adrenal weight and adrenal ascorbic acid in the offspring

Immobilization stress during early or late gestation did not had significant effect on adrenal gland weight (p=0.942, F=0.242). The mean adrenal gland weight for group-1 was 8.57±0.30, for group-2, 8.55±0.35 and for group-3, 8.42±0.39 mg respectively.

Adrenal ascorbic acid was significantly deleted in prenatally stressed offspring {group-2 (p<0.001) and group-3 (p<0.01)} compared to non-stressed control rat pups (group-1) (Figure 2).

Effect of prenatal stress in the levels of reduced glutathione (GSH) in the brain of offspring

Prenatal stress (early as well as late gestation period) caused a significant (p<0.001) depletion of GSH level in the brain homogenate (group-2 and group-3) compared to control (group-1). This clearly indicates that prenatal stress during early as well as late gestational period causes oxidative damage in the offspring brain (Figure 3).

Effect of prenatal stress in glutathione reductase (GSH-Rd) activity in the brain

The activity of brain GSH-Rd was significantly (p<0.001) decreased in prenatally stressed rat pups (group-2 and group-3) compared to control offspring (group-1) (Figure 4).

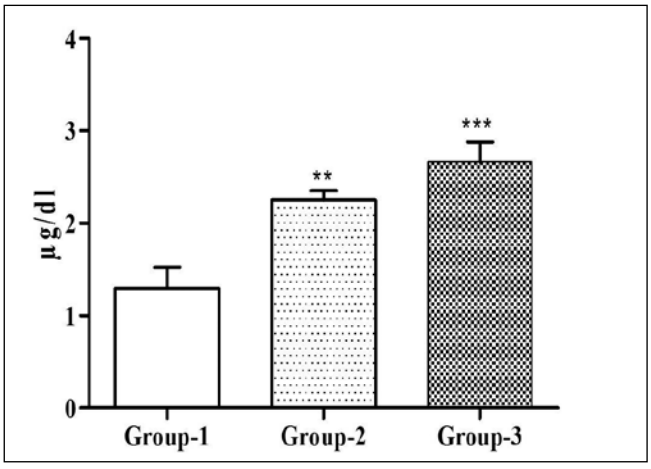

Figure 1: Serum Corticosterone level (µg/dl) in rats. Values represent Mean±SEM from animal groups like; **Group-1:** control, **Group-2:** pups received prenatal stress during day1 to 10 and **Group-3:** pups received stress during day 11 till delivery (n =12 per group). ***P < 0.001, **P < 0.01 vs. Control (Group-1).

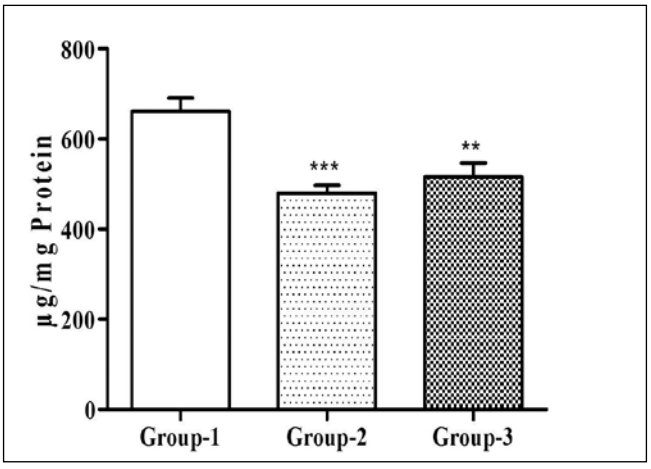

Figure 2: Adrenal ascorbic acid level (µg/mg Protein) in rats. Values represent Mean±SEM from animal groups like; **Group-1:** control, **Group-2:** pups received prenatal stress during day1 to 10 and **Group-3:** pups received stress during day 11 till delivery (n =12 per group). ***P < 0.001, **P < 0.01 vs. Control (Group-1).

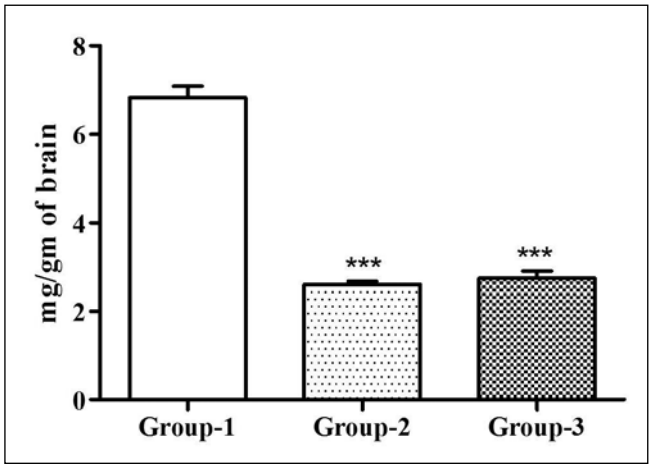

Figure 3: Brain reduced glutathione level (mg/gm protein) in rats. Values represent Mean±SEM from animal groups like; **Group-1:** control, **Group-2:** pups received prenatal stress during day1 to 10 and **Group-3:** pups received stress during day 11 till delivery (n =12 per group). ***P < 0.001 vs. Control (Group-1).

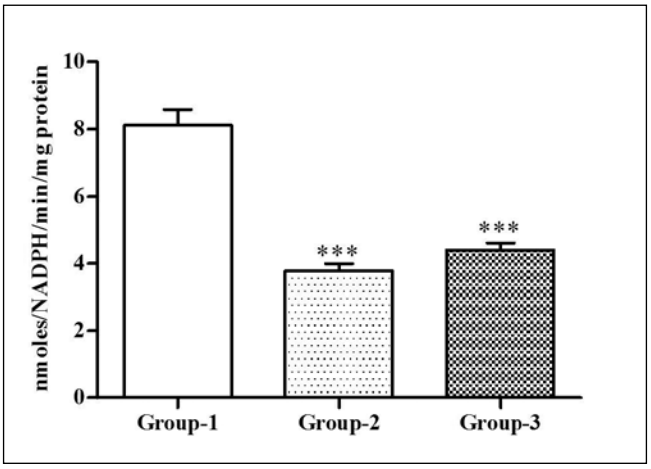

Figure 4: Brain Glutathione reductase activity (nmol NADPH oxidized/min/mg protein) in rats. Values represent Mean±SEM from animal groups like; **Group-1:** control, **Group-2:** pups received prenatal stress during day1 to 10 and **Group-3:** pups received stress during day 11 till delivery (n =12 per group). ***P < 0.001 vs. Control (Group-1).

Effect of prenatal stress in Superoxide dismutase (SOD) activity in the brain

The brain SOD activity was also decreased significantly in group-2 ($p<0.01$) and group-3 ($p<0.001$) when compared with control pups (group-1) (Figure 5) (Table II).

DISCUSSION

Antioxidant system is an important protective mechanism against ROS and, like many other biochemical systems; their effectiveness may vary with the stage of development and other physiological aspects of the organism (9).

The findings of this study confirm the deleterious effect of prenatal stress during different gestational period (early as well as late) on the antioxidant systems in the brain of offspring. In comparison to the control group, the prenatally stressed offspring showed a marked decrease in activities of GSH-Rd and SOD in the brain and also decreased in the levels of reduced GSH, validating the suppressed antioxidant efficiency in combating the prenatal stress induced free radical damage. Prenatally stressed offspring also showed an increased basal corticosterone secretion and decreased adrenal ascorbic acid level, our findings were consistent with earlier reports (12,15,30). Zhu et al. (2004) investigated the effect of prenatal stress during middle and late gestation on neuronal loss and oxidative stress. They measured free intracellular Ca^{2+} and intracellular reactive oxygen species formation. However this study does not involve the estimation of antioxidant enzymes in neonatal brain. In the present study we have evaluated major antioxidant enzymes and also tested the effect of early gestational stress.

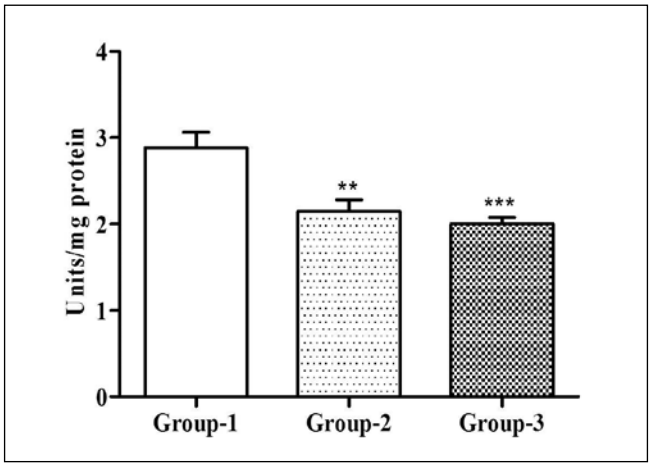

Figure 5: Brain Superoxide dismutase activity (U/mg protein) in rats. Values represent Mean±SEM from animal groups like; **Group-1:** control, **Group-2:** pups received prenatal stress during day1 to 10 and **Group-3:** pups received stress during day 11 till delivery (n =12 per group). ***P < 0.001, **P < 0.01 vs. Control (Group-1).

Table II: Percentage Change in the Antioxidant Activity in Rat Brain Homogenate, Serum Corticosterone and Adrenal Ascorbic Acid Level

	Group-2	Group-3
Serum Corticosterone	+74.41%	+106.2%
Adrenal Ascorbic Acid	-27.93%	-22.62%
GSH	-62.2%	-59.88%
GSH-Rd	-53.29%	-45.74%
SOD	-25.69%	-29.16%

'–' Sign indicates decrease, '+' Sign indicates increase over controls.

There is a variation in the hormonal level to stress during early and late gestation, i.e. increased basal corticosterone secretion was observed in the offspring who received prenatal stress during late gestation (106.2%) than the offspring who received prenatal stress during early gestation (74.41%) as mentioned in the Table II. But there was no variation observed in case of antioxidant status between prenatal stress during the early and late gestational period. This result indicates that prenatal stress during late gestation has a detrimental effect on developing HPA axis than prenatal stress during early gestation.

The reduced GSH content in the present experiment can be explained by: (i) The higher levels of free radicals that convert more reduced glutathione (GSH) to its oxidized form (GSSG) (21); and (ii) A decreased activity of glutathione reductase (3), the enzyme that regenerates reduced glutathione in a NADPH-dependent reaction. Dringen and Hirrlinger showed that the antioxidant glutathione is essential for the cellular detoxification of ROS in brain cells (4). In addition, the glutathione plays a vital role in the regulation of the redox state and prevention of the cell damage induced by oxidative stress (2).

Our data indicates that prenatal stress (during early as well as late) induced a significant inhibition of SOD and GSH-Rd activities. The possible reason for this finding could be the enhanced lipid peroxidation and the excess production of ROS. Increased fetal GC may cause increased activation of excitatory amino acid receptors and unregulated increases in intracellular Ca^{2+} concentrations, and consequently increased generation of oxidants and oxidative damage in brain. Prenatal stress caused an increase in the concentrations of the intracellular calcium and the production of ROS in the brain. Ca^{2+} influx plays a critical role in excitotoxicity, particularly via the NMDA receptors (1). The excess production of ROS, such as superoxide anion radical, hydroxyl radical, hydrogen peroxide, nitric oxide and peroxynitrate can oxidize key components of the cell including lipids, proteins, and nucleic acids (25). Brain is more vulnerable to the damage of ROS than are other organs because brain is enriched in oxidizable polyunsaturated fatty acid and redox-active metals, but antioxidant molecules are low (5).

A number of studies suggest that prenatal stress leads to higher fetal plasma cortisol and CRH (Corticotrophin Releasing Hormone) levels. This placental CRH and cortisol reaches the fetal brain and could influence the fetal brain development presumably by activating CRH receptors (24).

Stress during pregnancy also reduces the expression and activity of the glucocorticoid barrier enzyme, 11β-HSD2 (11β-hydroxysteroid dehydrogenase type 2) in the placenta, leaving the fetus less protected (16). This may be associated with alterations in the offspring HPA axis activity as noted in our study (increased cortisol level and decreased adrenal ascorbic acid).

The exposure of prenatal stress to pups is more detrimental in two accounts: porous blood brain barrier and deficiency of endogenous glutathione system. Chronic prenatal stress exerted adverse effects on blood-brain-barrier (BBB) functional development; the mechanism includes increased transvascular transport mediated by caveolae (8). The stress effects upon BBB could affect important neural developing processes, such as neurogenesis, myelination, and synaptogenesis (22), since direct exposure to blood-borne products can induce neuronal and glial damage (26).

In conclusion, the present study demonstrated that both early and late gestational stress induced a significant decrease in activities of GSH-Rd and SOD and GSH levels female and male offspring brain. These results suggest that prenatal stress can result in elevated oxidant generation and oxidative damage to the neurons and that the oxidative neuronal damage may be due to the overproduction of GC in male and female offspring. Thus, we hypothesize that the formation of glutathione system during development is related to the changes in the levels of free radicals. Hence this knowledge is of importance as this will provide the basis for cognitive and behavioral disorders in children of mothers who work and function under duress and stressed conditions during pregnancy. So, it is important to support endogenous defines mechanism by exogenous antioxidant agents to prevent the oxidative stress related consequences in developing brain. Such studies in an animal model would throw more light on probable alternate therapeutic measures to combat the prenatal stress induced behavioural dysfunctions.

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