

Hindering Effect of Resveratrol on Oxidative Changes and Na⁺ K⁺-ATPase Activity in Rat Hepatocytes Exposed to Prenatal stress

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ABSTRACT

Introduction: The fetal programming hypothesis states that conditions during pregnancy, including stress, will have long-term effects on adult health, probably via epigenetic mechanisms. **Methodology:** Pregnant rats were subjected to restrain stress either during early or late pregnancy with and without resveratrol. Blood and liver tissues were collected from 40 days old offsprings of the above rats to study the prenatal effect on corticosterone, and stress development. **Results:** It was found that levels of corticosterone advanced protein and lipid oxidation products, GSHRx, increase significantly in offsprings of stressed rats and decreased on intervention with resveratrol, whereas total antioxidants, vitamin C, GSH, SOD and Na⁺K⁺-ATPase decreased with stress and increase on resveratrol intervention as compared to controls. **Conclusion:** The alterations may be due to the effect of stress on HPA axis. Results also support the prevention/protective effect of resveratrol on oxidative stress and may be used as a measure to prevent the metabolic changes in adult life due to prenatal stress.

Key words: Prenatal stress, Resveratrol, Cortisol, Oxidative changes, Na⁺K⁺-ATPase activity.

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History

- Submission Date: 15-03-2017;
- Review completed: 04-05-2017;
- Accepted Date: 18-05-2017

DOI : 10.5530/pj.2017.5.98

Article Available online

<http://www.phcogj.com/v9/i5>

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INTRODUCTION

Fetal and early postnatal development likely constitutes the most vulnerable time period of human life, in regard to adverse effects of environmental hazards. Subtle effects during early life can lead to functional deficits and increased disease risks later in life. The programming hypothesis, has gathered much support from both experimental and epidemiological studies. The prenatal and early postnatal environment affects gene expression and epigenetic changes may constitute an important mechanism for the programming effects. All of this information suggests that the timing of exposure to environmental chemical is crucial in determining the toxicity effects.

Extensive epidemiological outcomes and experimental studies have developed links between low birth weight and an increased prevalence of organ and metabolic disorders in adult life.¹ In humans, exposure to in-utero stressful environmental factors reduces birth weight and forecasts the subsequent occurrence of hypertension, cardiovascular disease, dyslipidemia, insulin resistance/type2-diabetes mellitus, and neuro-endocrinal and behavioral alterations in adulthood.² Dietary changes and/or exposure to cortisol in the course of prenatal challenges that might strengthen these long-term effects as well as variations in maternal behavior have been proposed as key mediators of developmental programming of adult pathophysiology.^{3,4}

Adverse early-life experiences, including maternal exposure to stress during pregnancy, can 'programme' persistent changes in several physiological systems and behaviours, probably via epigenetic mechanisms. In rodents, prenatal stress is associated with negative pregnancy outcomes such as low birth weight, reduced litter sizes and lower survival rates.⁵ In the adult offspring, it is associated with heightened, enhanced or prolonged hypothalamo-pituitary-adrenal (HPA) axis responses to acute stress, impaired glucose homeostasis, insulin resistance, diet-induced obesity,⁶ impaired neural development and cognitive deficits.⁷

Prenatal stress (PS) also induced emotional and cognitive disturbances in adult and aged animals including, "anxiety," depressive-like behavior and altered reactivity to stress of the hypothalamic-pituitary-adrenal (HPA) axis. Importantly, prenatal stress reduces offspring weight at birth and resulted in long-term metabolic, behavioral, and neuroendocrine changes consistent with a prenatal programming of the adult biology and pathophysiology. These outcomes specify the impact of maternal stress on developing fetus to adjust multiple aspects of its tissue development which alter the adult phenotype. These changes reflect those seen in animals, prenatally exposed to glucocorticoids⁸ and mostly

Cite this article : Rao GM, Sahu SS, Shetty V. Hindering effect of resveratrol on oxidative changes and Na-K-ATPase activity in rat hepatocytes exposed to prenatal stress. Pharmacog J. 2017;9(5):615-20.

comparable to human studies associated with low-birth-weight.⁹ Significant increases in calcium content and oxidant generation were induced by late gestational stress in the hippocampal CA3 region in female rats. Our previous study data suggest that prenatal stress can cause oxidative stress and consequent damage to neurons, leading to neuronal loss in the brain of offspring during development.¹⁰

Extensive animal experiments have been performed to determine whether maternal stress disturbs the function of the HPA-axis. The main glucocorticoid is cortisol in humans and primates, effect of stress is on liver. In mammals, it has been suggested that stressful pregnancy induced modification of the offspring's phenotype, ultimately results in the development of long-term diseases.¹¹

Liver, an important site for several metabolic activities in the body,¹² not only acts as strainer to out the bad from the good from intestine, but also plays an important role in synthesis, detoxification, to control various metabolic activities including blood glucose levels. So any physiological alterations in liver alters all these activities. Moreover diseased liver releases some toxic molecules, will *worsen* the condition.

Some of the naturally available/dietary components, because of their antioxidant components can ameliorate the oxidative stress. Resveratrol is a stilbenoid (natural phenol) and phytoalexin, a rich component of some plants, such as Japanese knotweed and red grapes. Studies have shown resveratrol to have a multitude of health benefits, including anti-cancer, antioxidant, anti-inflammatory, estrogen modulating and caloric restriction mimetic activities.¹³

In the present study we made an attempt to evaluate the effect of prenatal restraint stress on liver of adult offspring of rat dams exposed to chronic restraint stress and the effect of resveratrol.

SUBJECTS AND METHODS

Inbred albino *Wister* rats weighing around 200-230 gm were the subjects of the study and were maintained in humidity and temperature controlled environment in 12 h. dark and light cycle. They were fed with standard food pellet and water ad libitum. Breeding and maintenance were according to the guidelines of Government of India for use of Laboratory animals. Institutional ethics committee approval was obtained before the conduct of the study.

Sexually active male and female rats in 3:1 ratio were allowed to mate for 4hrs per day. After 4hours, pregnancy was confirmed by vaginal smears for sperms and the day was considered as 0 hr of pregnancy for further calculations. The pregnant rats were housed in individual cages and randomly allocated into six groups.

The pregnant rats were subjected to restrain stress for 45 min each, 3times / day using a mesh restrainer, which only restricted the animal movement without any pain or suffocation.

Selected group of animals were treated with 10 mg/kg body weight of resveratrol in 0.5% carboxy methyl cellulose by oro-gastric gavage.

The pregnant rats were allocated into following groups:

Grouping:

1. Normal Control: received only 0.5% carboxymethyl cellulose
2. Resveratrol Control: received resveratrol
3. Early gestational stress(EGS): exposed to restrain stress from 1-10 days
4. Late gestational stress(LGS): received restrain stress from day 11 till delivery
5. Resveratrol +Early gestational stress: Resveratrol (entire gestation period)+ stress from 1-10 days
6. Resveratrol + Late gestational stress: Resveratrol (entire gestation period) + stress from day 11 till delivery

After delivery (22-24th day of gestation), the offspring were raised by their biological mothers until weaning (ie till 21 days). On 40th postnatal day the animals were anesthetized by chloroform and sacrificed by decapitation.

The animals were anesthetized to collect blood sample for cortisol estimation, and was collected between 8 to 10 am to avoid circadian variations of serum cortisol concentration. Corticosterone was measured by chemi-luminescent method. The concentration of cortisol was expressed as ng/ml of serum. Liver tissue was collected, weighed and homogenized with respective buffers, for Na-K-ATPase – sucrose buffer and for other parameters–saline phosphate buffer. The homogenates were centrifuged at 10,000g for 20 min at 4°C and aliquots of supernatants separated and used for biochemical estimation.

Biochemical Parameters

Lipid peroxidation: in liver supernatant was measured in terms of thiobarbituric acid-reactive substances (TBARS) and determined using the method as described by Buege JA and Gayathri *et al.*^{14,15} Proteins present in the supernatant was precipitated with 2.5ml of ice cold trichloroacetic acid (TCA) and was centrifuged at 3000 g for 10 min. To 2ml of the supernatant, 0.67% of thiobarbituric acid was added and kept in boiling water bath for 10min and cooled. The pink chromogen obtained by the reaction of malonaldehyde (MDA), the product of lipid peroxidation with thiobarbituric acid, was read immediately at 532 nm using spectrophotometer.

Estimation of total antioxidants (TAO): Total antioxidant activity in the liver was determined according to the method described by Koracevic *et al.*¹⁶ Fe-EDTA complex reacts with hydrogen peroxide by Fenton - reaction, results in the formation of hydroxyl radicals. These reactive oxygen species act on benzoate, resulting in the release of thiobarbituric acid-reactive substances (TBARS). The capacity of brain homogenate to suppress this TBARS formation was considered as the total antioxidant activity of the tissues. The rate of inhibition of color development is proportional to the concentration of anti-oxidative activity. The decrease in the absorbance was read at 535 nm. The total antioxidant activity was expressed as antioxidant activity in mmol/lit.

GSH: Tissue reduced glutathione (GSH) concentration was estimated according to the method described by Ellman.¹⁷ One ml of supernatant was precipitated with 1ml of metaphosphoric acid and cold digested at 4°C for 1h. The samples were centrifuged at 1200g for 15min at 4°C. To 1ml of this supernatant, 2.7 ml 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 412 nm using a spectrophotometer.

Glutathione Reductase

The Glutathione Reductase (GSSG-Rd) activity was measured using the method originally described by Horn and Burns.¹⁸ The reaction mixture consisted of 1.6ml of 0.067M potassium phosphate buffer (pH 6.6), 0.12 ml of 0.06% NADPH, 0.12 ml 1.15% GSSG, 0.1 ml of enzyme source (supernatant) and water in a final volume of 2ml. All mixtures and solutions were prepared at room temperature. Control cuvettes then received 180 µL 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 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 (1.36x10⁴ (mol/l)-1 cm-1) and expressed as nmol NADPH oxidized/min/g tissue.

Superoxide Dismutase activity: Superoxide dismutase (SOD) activity was determined by the method of Beauchamp and Fridovich.¹⁹ The reaction

Table 1: HPA axis activity: (n=6)

	Serum Corticosterone ($\mu\text{g/dl}$)	Adrenal Ascorbic acid ($\mu\text{g/g tissue}$)	Adrenal weight (gm)
Control	1.29 \pm 0.22	566.1 \pm 30	8.571.29 \pm 0.3
Resveratrol(Resv)	1.49 \pm 0.17	599.2 \pm 16.1	8.21 \pm 0.39
EGS	2.35 \pm 0.17***	480.04 \pm 16.8***	8.55 \pm 0.35
LGS	2.66 \pm 0.18** b	515.4 \pm 31.4** b	4.21 \pm 0.39
EGS +Resv	2.25 \pm 0.10#	566.6 \pm 17.8	8.15 \pm 0.38
LGS + Resv	1.88 \pm 0.07\$	568.4 \pm 30	8.29 \pm 0.39

p<0.01, *p<0.001, #p<0.05 Vs control and Resveratrol

bp< 0.01 LGS + Resveratrol

\$p< 0.01 Vs EGS

Table 2: Liver enzymes (U/L), n = 6/group: Mean \pm SEM

	Control	Resveratrol (Resv)	EGS	LGE	EGS+ Resv	LGS+Resv
ALT	21.5 \pm 2.09	21.95 \pm 2.12	22.6 \pm 2.19	22.73 \pm 2.22	22.06 \pm 2.09	22.92 \pm 2.28
AST	9.93 \pm 0.87	9.95 \pm 0.9	10.23 \pm 1.09	9.97 \pm 0.94	10.19 \pm 1.06	10.06 \pm 0.99
ALP	5.4 \pm 0.29	5.52 \pm 0.24	5.78 \pm 0.26	5.86 \pm 0.29	5.55 \pm 0.25	6.03 \pm 0.32
GST	224 \pm 32.8	249 \pm 32.54	378 \pm 28.4*	360 \pm 27.32*	256 \pm 33.15	273 \pm 31.96

ANOVA significance (Benferoni's test) * p< 0.05 vs control and Resv group.

Table 3: Oxidative stress and antioxidant Parameters (n=6)

	Control	Resveratrol (Resv)	EGS	LGS	EGS+ Resv	LGS+ Resv
LPO $\mu\text{mole/g tissue}$	0.82 \pm 0.06	0.91 \pm 0.06	1.67 \pm 0.09***	1.72 \pm 0.13***	1.13 \pm 0.09*	1.02 \pm 0.06
AOPP $\mu\text{mole/g tissue}$	3.32 \pm 0.12	3.5 \pm 0.14	9.06 \pm 0.16***	8.26 \pm 0.08***	4.92 \pm 0.06*	6.04 \pm 0.07**
TAO mmols/g tissue	1.58 \pm 0.05	1.99 \pm 0.07	0.66 \pm 0.01***	0.93 \pm 0.03**	1.56 \pm 0.05	1.35 \pm 0.03*, a
GSH mg/g tissue	3.45 \pm 0.02	3.02 \pm 0.01	2.97 \pm 0.01#	2.59 \pm 0.01*	3.8 \pm 0.019	2.83 \pm 0.01
GRx U/L	11.46 \pm 1.8	12.39 \pm 2.0	9.46 \pm 1.52*	9.03 \pm 1.4*	10.01 \pm 1.8	10.84 \pm 2.0
SOD U/L	3.93 \pm 0.08	3.98 \pm 0.09	3.03 \pm 0.07*	2.93 \pm 0.06*	3.32 \pm 0.07	3.41 \pm 0.07
Na-K-ATPase(U/g issue)	4.2 \pm 0.12	4.26 \pm 0.12	3.2 \pm 0.09**	2.96 \pm 0.08**	3.75 \pm 0.1*	3. 62 \pm 0.09*

*-p<0.05, **p<0.01, ***p<0.001= vs control and resveratrol

p<0.05 vs control

a p<0.01 = Vs resveratrol alone

was performed in a mixture containing 5.6×10^{-5} M nitro blue tetrazolium (NBT), 1.17×10^{-6} M riboflavin, 1×10^{-2} M methionine in 0.05M potassium phosphate buffer, pH 7.8 with suitably diluted liver homogenate in a total volume of 3ml. Illumination of solution was carried out in an aluminium lined foil box fitted with a 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 UV-Visible spectrophotometer at 560 nm. SOD activity was expressed as specific activity of the enzyme in units per mg protein (U/mg of tissue).

Serum corticosterone

Blood samples were collected 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 chloroform vapor and intra-cardiac blood was collected. Corticosterone was measured by

using electro chemiluminescent method and concentration of cortisol was expressed in ng/ml serum.

ALT, AST and Alkaline Phosphatase (ALP) in liver homogenate were estimated using Randox kits.²⁰⁻²²

Glutathione S-transferase (GST)

GST was assessed by the method of Habig *et al.*²³ The activity of the enzyme was determined by observing the change in absorbance at 340 nm. The reaction mixture contained 0.1 ml of GST, 0.1 ml of CDNB and phosphate buffer in a total volume of 2.9 ml. The reaction was initiated by the addition of 0.1 ml of the homogenate. The readings were recorded every 15 seconds at 340 nm against distilled water blank for a minimum of three minutes. The assay mixture without the homogenate served as the control to monitor non-specific binding of the substrate.

Na⁺ K⁺-ATPase activity

Na⁺ K⁺-ATPase activity was determined by the method of Wyse *et al.*²⁴ ATPase activity was measured by adding 0.2ml of liver homogenate to the reaction mixture containing 1M NaCl, 1M KCl, 0.1M MgCl₂, 0.2 M EDTA and 0.5M Tris-HCl buffer, pH 7.4, in a final volume of 500 μL. The reaction was started by the addition of 100 μL of 30 mM ATP (disodium salt, vanadium free). Control was assayed under the same conditions with the addition of 200 μL of 10 mM ouabain. Reaction was terminated by the addition of 1mL 10% ice cold trichloroacetic acid (TCA) after 60 min. Then the mixture was centrifuged for 10 min at 1000×g to remove the precipitate. Na⁺- K⁺ ATPase activity was calculated by the difference between the two assays. Released inorganic phosphate (Pi) was measured by the method of Fiske and Subbarow²⁵ (U/g tissue= mg of Pi released/min).

Statistical analysis

Data is presented as the mean ± SE. Statistical analysis for multiple comparisons was performed by one way analysis of variance (ANOVA) with Bonferroni's corrections.

RESULTS

Results are presented in Tables 1 to 3. There was a significant increase in the corticosterone levels in the off-springs exposed to prenatal stress. Though there was a tendency to return to normal in those exposed to LGS + resveratrol, the level remained significantly high compared with that of controls. A significant decrease in the adrenal ascorbic acid was also observed in the animals exposed to gestational stress. However, resveratrol treated groups showed a normal level. Adrenal weight of the animals remained unaltered when compared with the controls. ALT, AST and ALP levels in liver of the study group remained in the normal control range. The GST levels were significantly increased in those animals exposed to prenatal stress and were returned to the normal range in the resveratrol treated groups as depicted in table II.

There was a significant increase in the lipid oxidation products as well as protein oxidative products in the groups exposed to prenatal stress when compared with normal control and resveratrol alone treated groups. The groups treated with resveratrol along with prenatal stress remained in the normal control range. However there was a significant decrease in the AOPP levels in those animals exposed to late gestational stress along with resveratrol. The antioxidant levels showed a consistent decrease in those exposed to LGE. A recovery in the antioxidant level was observed in the resveratrol treated groups exposed to stress when compared with normal control group.

DISCUSSION

The release of glucocorticoids controls the homeostasis - steady or healthy state of each organ. A remarkable exacerbation of liver injury has been observed in corticosterone pretreated mice. During stress, there can be change in chemical composition of the cells like increased free radical formation, oxidative processes, variation in ion concentration and immunological properties which contribute to liver cell death and worsening of liver disease. In the part of the brain that controls the liver, stress was found to impair blood flow and may lead to or trigger liver damage. Vagus nerve from the brain to the liver when stimulated may actually improve or reduce the negative effect of stress on the liver.

Enhanced plasma corticosterone level due to prenatal stress has a major impact on the developing organs. The present study results showed a significant change in the cortisol levels. Offspring's exposed to prenatal stress during both types of gestational stress. The lipid peroxidation results of the study indicate the increased oxidative stress in the study

subjects. The variation in the antioxidant parameters observed in the study also supports the view. The degree of unsaturation of the membrane lipids is related to lipid peroxidation susceptibility of polyunsaturated fatty acids. These membrane effects could contribute to modulate the functional activity of membrane-bound enzymes. TBARS, product of lipid peroxidation, underwrites the loss of some cellular function through the changes in the membrane-bound enzymes and native ion channels.²⁶ Impairment of Na⁺- K⁺-ATPase activity could be due to the loss of its optimal communication with the membrane components, as a consequence of increased lipid peroxidation. It may be a reflection of the direct/indirect action of increased levels of corticosterone via other signal path ways. A direct inhibition by peroxynitrite, occurring in liver plasma membranes, should not be discredited. Nevertheless, other effects liable to influence the variation of Na + K+-ATPase, could be protein oxidation and this factor should not be discarded because the present study results show a significant increase in the levels of protein oxidation products. An excess of oxidative agents is involved in lipid peroxidation, which additionally increases mitochondrial permeability and alters their function.²⁷ Moreover, ROS are responsible for the release of reactive aldehydes such as 4-HNE, which inactivate the mitochondrial respiratory chain and hinder electron flow from the mitochondrial respiratory chain. As a result, the production of ROS and oxidative stress in mitochondria increases, and energy production decreases. These processes have direct effect on the activity of Na⁺- K⁺ ATPase and could be another reason for the observed decrease in the activity of the enzyme.

Enhanced GC inhibits glucose utilization, thereby compromising the activity of energy-dependent transporters and unregulated increase in intracellular Ca²⁺ concentrations. Consequently, Ca²⁺-dependent enzymes that can contribute to the production of reactive oxygen species (ROS) are activated. Along with these, mitochondrial respiration is compromised by overload of calcium and other hormone mediated effects, further contributing to ROS production. This could be associated with cellular damage. Hence the cellular damage in the developing organs could be attributed to over production of oxidants.²⁸ It has been reported that resveratrol has an inducing effect on genes for oxidative phosphorylation and mitochondrial biogenesis and modulation of gluconeogenesis and glycolysis in liver. These could be the explanations for the observed reversed effects on the parameters in resveratrol treated groups, by increasing the energy level and decreasing the free radical formation.¹¹

Glutathione (GSH) plays an important role in cellular defense against oxidative stress, since it eliminates in a non-enzymatic way, hydroxyl radicals and singlet oxygen, and serves as a cofactor for enzymes such as glutathione peroxidase and glutathione S-transferase.²⁹ The main function of GST is to detoxify xenobiotics in the liver by catalyzing the nucleophilic attack by GSH on electrophilic carbon, sulfur or nitrogen atoms of nonpolar xenobiotic substrates, thereby preventing their interaction with crucial cellular proteins and nucleic acids and thus susceptibility to various types of diseases. Numerous studies have implicated such GST level variations seen in asthma, atherosclerosis, allergies, and other inflammatory diseases.³⁰ An increase in the levels of GST enzyme observed is a sign of hepatic tissue damage. To support the increase in oxidant status of the subjects exposed to prenatal stress, there is a decrease in the total antioxidant along with the GSH levels and GRX activity.

Our previous study data suggests 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.³¹ 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

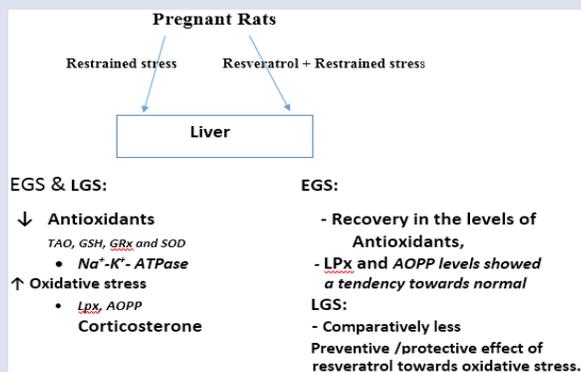
fetus less protected. This may be associated with alterations in the offspring HPA axis activity as noted in our study (increased cortisol level and decreased adrenal ascorbic acid).

The study report shows a tendency towards development of oxidative stress due to the prenatal stress. This in-turn could result in the metabolic changes in the liver which will affect the normal metabolic programs in adulthood. Changes in the intrauterine environment have been suggested as a mechanism for programming the offspring to develop diseases that manifest later in life. The changes observed in the neonates of the present study could represent permanent modifications in the mechanisms governing neonatal growth, glucose metabolism and sodium regulation due to steroid-induced in utero effects. However, what is not clear yet, is whether these changes alter the ability to respond to other stimuli and contribute to the development of adult onset diseases. The tendency to develop stress was comparatively less in animals treated with resveratrol. The data suggests that prenatal stress can cause oxidative stress and consequent changes in the physiology and metabolic functions of offspring during development. To conclude, even though all of the interactions between stress and the liver are not completely understood, the study results support the ameliorating effect of resveratrol on oxidative stress development and may be a preventive measure towards the metabolic changes in adult life due to prenatal stress. It can be noted that the link between liver disease and stress needs to be identified so that physicians and other healthcare practitioners would be better able to treat liver disease by helping their patients learn coping and relaxation skills.

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GRAPHICAL ABSTRACT



HIGHLIGHTS OF PAPER

Prenatal stress

- Increased the levels of corticosterone
- Leads to the development of oxidative stress in liver
- Decreases the Na⁺-K⁺-ATPase levels, may altered the ionic environment of the tissue and may worsen the condition
- Resveratrol has preventive/reversible action on the development of stress to some extent, especially on EGS.

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Cite this article : Rao GM, Sahu SS, Shetty V. Hindering effect of resveratrol on oxidative changes and Na-K-ATPase activity in rat hepatocytes exposed to prenatal stress. Pharmacog J. 2017;9(5):615-20.