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Nitroso redox imbalance an important aspect of Pregnancy-induced- hypertension- a case control study.

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Abstract

Background- Pregnancy induced hypertension (PIH) includes Pre-eclampsia and eclampsia. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been associated with pre-eclampsia. Nitroso-redox imbalance could be considered the causative element in pregnancy induced hypertension.

Aim- To study the role of nitroso redox imbalance and antioxidants in Pregnancy induced hypertension.

Methods- Pregnant females with and without hypertension were evaluated for Serum Nitric oxide(NOx), Serum Nitrothiol, Serum Total Thiol, Serum Superoxide dismutase(SOD), Serum uric acid and were compared.

Results- Nitric oxide(NO_x- Nitrite and nitrate), thiol, superoxide dismutase levels were decreased while nitrothiol and uric acid levels were increased in cases as compared to control.

Discussion- Superoxide dismutase(SOD) seems to be the initiating factor for oxidative stress in PIH. Due to upregulation of xanthine oxidase, there is increased generation of uric acid and also superoxides. So more and more SOD is used up for the dismutation of superoxide radicals. Superoxides scavenge more and more Nitric oxide towards formation of peroxynitrite radicals, thus decreasing $NO^{\cdot}$ flux towards NO_x. These peroxynitrite radicals react with thiol to form nitrothiol, thus decreasing thiol levels and increasing nitrothiol levels in PIH.
Conclusion- Nitroso redox balance plays pivotal role as causative agent in PIH.

Key words- Oxidative stress, antioxidants, nitric oxide, nitrite, nitrate, superoxide dismutase, nitrothiol, thiol, uric acid, pregnancy induced hypertension, superoxide, nitrosative stress, nitroso redox imbalance

What is important? Oxidative and nitrosative stress is an imbalance between the systemic manifestation of reactive oxygen and nitrogen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. This imbalance plays vital role in Pregnancy induced hypertension.

Introduction

Pregnancy induced hypertension includes Pre-eclampsia and eclampsia. Mild Pre-eclampsia is defined as blood pressure greater than 140/90 mm Hg plus proteinuria (> 300 mg of protein in a 24 hr urine collection) after 20 weeks of gestation. Severe Pre-eclampsia includes blood pressure greater than 160/110 mm Hg, marked proteinuria [>1-2gm per 24 hr urine collection]. Eclampsia -meets the criteria of pre-eclampsia with tonic-clonic seizures. [1] Other maternal complications include pulmonary oedema, acute renal failure due to vasospasm. [2] Also the extent of fetal risk is higher.

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Pre-eclampsia has been described as a disease of theories because the cause is unknown. Some theories put forth are [3] endothelial cell injury, Compromised placental perfusion, imbalance between prostacyclin and thromboxane, dietary factors including vitamin deficiency , decreased intravascular volume and some genetic factors.

The maternal vascular endothelium appears to be an important target of factors triggered during preeclampsia. Both endothelium-derived relaxing and contractile factors play an important role in the regulation of arterial compliance, vascular resistance and blood pressure. When abnormalities in the production or action of these factors occur, the vasculature is predisposed to vasoconstriction, leukocyte adherence, oxidative stress and vascular inflammation [4,5]. Once immune cells adhere to the activated vascular endothelium, a series of cellular interactions occur inducing junction widening between cells and allowing immune cell infiltration into the vascular wall thereby invading local tissues. [6] As a result the endothelium becomes leaky allowing for extravasation of fluid, recognized clinically as edema.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been associated with pre-eclampsia [7,8,] Mitochondria is a major source of reactive oxygen and nitrogen species. Mitochondrial mass is increased in pre-eclamptic placentas. However, when cytotrophoblast cells are isolated from these defective placentas, the expression of mtDNA and nuclear respiratory factor 1 (NRF1) (linked to the mitochondrial mass) is abnormally low. [9] Proteomics analysis of severe pre-eclamptic placentas revealed altered mitochondria-related pathways such as fatty acid oxidation, ROS generation, and oxidative stress. [10]

Nitroso-redox imbalance could be considered the causative element in pregnancy induced hypertension.

Materials and methods

This study was conducted between October 2012 and March 2013. The study was undertaken after approval from Institutional Ethics Committee. Informed written consents were taken from patients. Sixty patients presenting with PIH in Obstetrics and Gynecology department without any complaint of urinary tract infection, bronchopneumonia, hepatitis, influenza or any infection served as cases. Sixty pregnant females without PIH and any infection served as control. Oxidative stress plays a dual role in infections. Free radicals protect against invading organisms and they can also cause tissue damage during the resulting inflammation. In the process of infection, there is generation of reactive species by nitric-oxide synthase. So, reactive nitrogen species are abundant in infectious conditions. Hence, all infectious conditions like urinary tract infection, bronchopneumonia, hepatitis, influenza etc., were excluded from this study.

Ethics approval and consent to participate- The study was undertaken after approval from Institutional Ethics Committee. Informed written consents were taken from patients.

Collection of Samples -The blood samples were collected from the Department of Gynecology. Total 5 ml sample was collected in plain red top tube containing no anticoagulant; serum was separated and used for the study. All of them were evaluated for Superoxide dismutase by Kajari Das method, serum uric acid by Uricase kit method , serum nitric oxide by Griess method , serum nitrothiol by Cook method , and serum total thiol by Habeeb method .

Statistical analysis

Unpaired Student 't' test was applied to compare the results between the normal pregnant and PIH . P value <0.001 was considered statistically significant.

Estimation of serum superoxide dismutase

Method- Kajari Das method[11]

Principle

Superoxide radicals are generated by photo reduction of riboflavin. These are allowed to react with hydroxylamine hydrochloride to produce nitrite. The nitrite in turn reacts with sulphanilic acid to produce diazonium compound which subsequently reacts with napthethylene to produce red azo compound whose absorbance was measured at 543 nm.

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The absorbance of the color formed was measured at 543nm.

Method- Reaction tubes were exposed to two 20W Philips fluorescent lamps fitted parallel to each other in aluminium foil coated wooden box for 10 minutes after addition of Griess reagent. (Fig 1) Absorbances were read at 520nm.

1 SOD unit = Amount of enzyme required to inhibit the nitrite formation of control by 50% at 37 ^{0}C for 10 minutes.

$$\text{SOD unit} = \frac{\text{OD of Control}}{\text{OD of Test}} - 1$$

Estimation of Uric acid

Uricase/PAP Kit method[12,13]

Principle

Uricase converts uric acid to Allantoin and hydrogen peroxide. The hydrogen peroxide formed further reacts with a Phenolic compound and 4 aminoantipyrine by the catalytic action of peroxidase to form a red colored quinoneimine dye complex.

Intensity of the color formed is directly proportional to the amount of uric acid present in the sample. Absorbances were read at 520 nm.

$$\text{Uric acid (mg/dl)} = \frac{\text{T-B}}{\text{S-B}} \times \frac{\text{concentration of Std x volume of Std}}{\text{volume of sample}}$$

Estimation of Serum Nitric Oxide by Griess Method [14]

Principle: Nitric-oxide is measured in terms of nitrite and nitrate. It involves formation of chromophore during the reaction of nitrite (NO -) with sulphanilamide and N (1-naphthyl) ethylenediamine (Griess reagent) forming pink coloured compound with a characteristic absorption spectra and λ max at 545 nm. Nitrate (NO -) does not undergo diazotization reaction with sulphanilamide. Hence, it is first reduced quantitatively to nitrite by nitrate reductase enzyme. Nitrite reacts with Griess reagent. The colour obtained is due to reaction of nitrite already present in the sample and nitrite formed by reduction of nitrate. Thus, both nitrate and nitrite i.e., nitric-oxide are measured.

Absorbances were read at 545 nm. Concentration was calculated from the standardization curve of nitrite in µM/ltr. (Figure 3)

Internal standard was used to determine the recovery of NO_3^- in the assay. If it was less than 90% new *E. coli* suspension was prepared.

Calculation:

$$\frac{\text{T-B}}{\text{S-B}} \times \frac{\text{concentration of Standard x volume of Standard}}{\text{volume of sample}}$$

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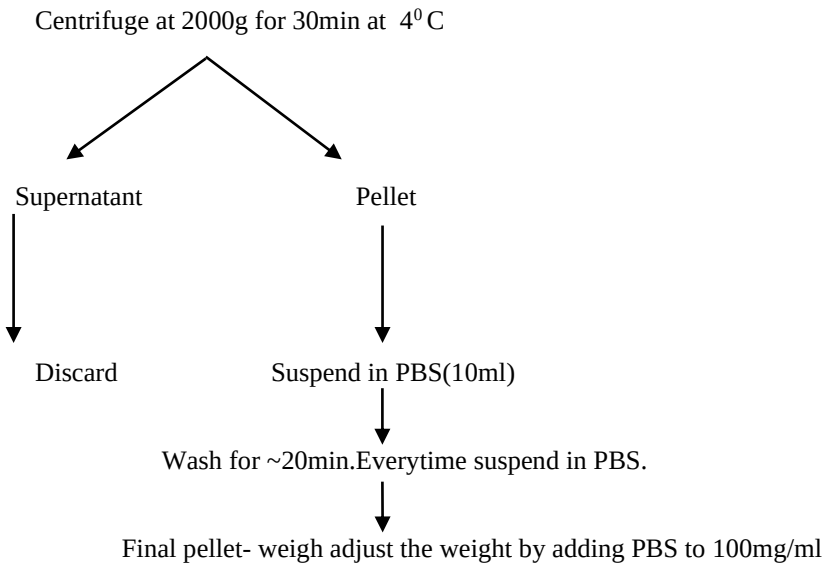
Procedure

1] Preparation of *E. coli* containing nitrate reductase



To one litre of culture medium *E. coli* were added from the agar plate. The container was sealed to make it air tight. The culture is incubated at 37^0 C for 20 hours with intermittent gentle shaking.

After the incubation period was complete the culture was centrifuged at 2000g for 30min at 4^0 C. The pelleted bacteria were then suspended and washed by centrifugation in cold phosphate buffer saline (PBS) of pH 7.4. The washing of bacteria was repeated 10 times to completely remove nitrate. Every time the bacteria were pelleted by centrifuge at 5000g for 10 min at 4^0 C. After final washing, the weight of pelleted bacteria determined and were suspended in cold PBS at 100mg/ml. The suspension was immediately suspended in 1ml aliquot in eppendrof tubes and stored at -100^0 C. Care was taken not to refrigerate the aliquots once they were thawed. The aliquot was used in the estimation of $N0_x$ concentration.

$$\text{rpm(alternate rotor)} = 1000 \times \sqrt{\frac{\text{RCF, original}}{11.18 \times r\,(\text{cm}),\ \text{alternate rotor}}}$$

Estimation of Nitrothiol in Serum by Cook Method [15]

Principle: The S-NO . bond is broken by metal ions like Hg to release NO. The released NO reacts with Griess reagent to form coloured chromophore. Absorbance measured at 496 nm. To avoid interference by nitrite, if present the reaction is not carried out at acidic pH.

Calculations were done using absorptivity. It was measured in µM/ ltr.

$E_{496} = 11500\ M^{-1}\ cm^{-1}$

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Estimation of Total Thiol by Habeeb Method [16]

Principle: The proteins are denatured by Sodium Dodecyl Sulphate (SDS) and urea. SDS also dissolves the membranes. Thus, all the –SH groups present in the mitochondrial proteins namely –SH group which are easily accessible and those present within the proteins are exposed which gives total thiol concentration. A 5,5'-Dithiobis (2-nitrobenzoic acid) also known as DTNB or Eliman's reagent reacts with –SH group to form 2-nitro-Sthiobenzoate (NTB) which is yellow coloured compound. The absorbance is read at 412 nm.

Calculations were done using absorptivity

ϵ_{412} =13600 M^{-1} cm^{-1}

RESULTS AND OBSERVATIONS

SOD -Fig 1

Control Mean-0.73+ 0.10
PIH Mean-0.46+0.06
t-test- 16.37
p value-<0.001-highly significant

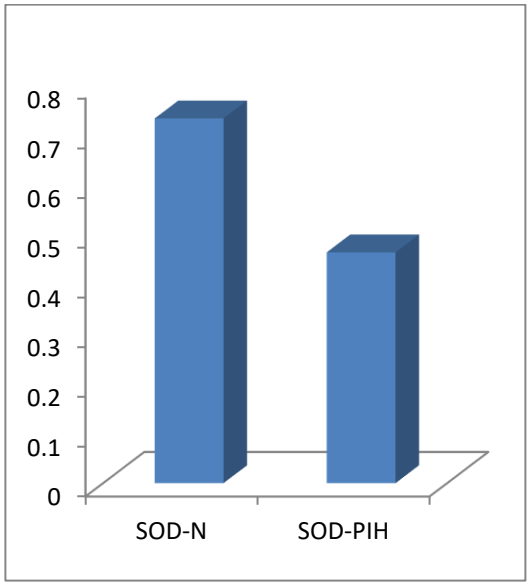

Fig 1. Mean SOD levels in control(N) and cases(PIH)

Nitric oxide-Fig 2

Control Mean-76.03 + 4.89

PIH Mean-61.81 + 5.35

t-test= 15.05

p value-<0.001-highly significant

Nitrothiol - Fig 2

Control Mean-2.12+0.53

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PIH Mean-6.20+1.47
t-test= -20.02
p value-<0.001-highly significant

Thiol- Fig 2

Control Mean-95.95+4.03
PIH Mean-83.08+4.03
t-test =17.33
p value-<0.001-highly significant

Uric acid- Fig 2
Control Mean-3.4+0.54
PIH Mean-5.66+0.88
t-test = -16.7
p value-<0.001-highly significant

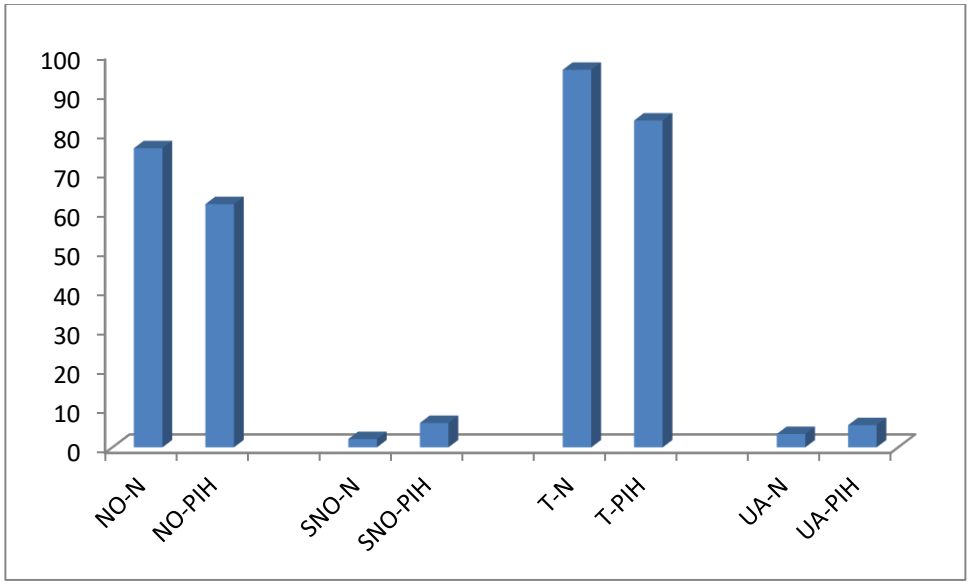

Fig 2. Mean values of Nitric oxide(NO), Nitrothiol(sno), Thiol(T), Uric acid(UA) in control(N) and cases(PIH).

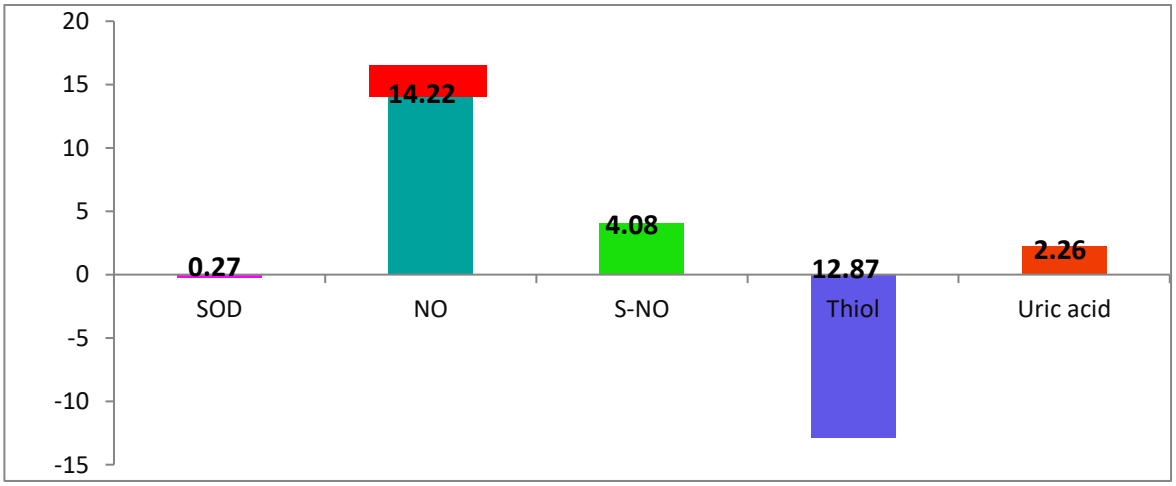

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Fig 3. Comparative change in mean of all parameters in PIH.

Discussion-

Oxidation reactions produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions.

Reactive oxygen species are formed under stressful conditions. SOD is one of the defences against this stress. [17,18] Superoxide dismutases (SODs) catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide (H_2O_2). Although H_2O_2 is not a radical, it is rapidly converted by fenton reaction into OH**.** radical which is very reactive [19]. Among various antioxidant mechanisms in the body, SOD is thought to be one of the major enzymes that protect cells from ROS. SOD is estimated in this study to check the antioxidant status in PIH. PIH patients are under oxidative and nitrosative stress, so there is increased generation of superoxide radicals. Due to increased superoxide radicals more and more Superoxide dismutase enzyme is used up to dismutate or inactivate superoxides. Moreover, built up of $O_2^{\cdot}$ leads to damage to SOD molecules and the enzyme is rendered functionless, leading to more accumulation of $O_2^{\cdot}$. [20,21] Figure no. 1 shows decreased superoxide dismutase activity in PIH as compared to control. The difference between mean of case and control is 0.27.

The endothelium (inner lining) of blood vessels uses nitric oxide to signal the surrounding smooth muscle to relax, thus resulting in vasodilatation and increasing blood flow. It relaxes vascular smooth muscle by binding to the heme moiety of cytosolic guanylate cyclase, activating guanylate cyclase and increasing intracellular levels of cyclic guanosine 3', 5'- monophosphate, which then leads to vasodilatation. [22,23,24] The decrease in mean of PIH was about 14.22 times that of control.(Figure 2) The decrease is highly significantly (p value <0.001). Decreased NO**x** levels directly suggest hypertensive condition. The NOx levels are also influenced by acute fluctuations in their renal tubular reabsorption. $NO^{\cdot}$ synthesis may be decreased in PIH or its metabolic conversion may be increased leading to decreased flux of $NO^{\cdot}$ towards NOx.

Nitrothiol plays a vital role in nitric oxide synthase (NOS) activity that modulates blood vessel tone. As a free radical, $NO^{\bullet}$ interacts readily with transition metals, other free radicals, and O_2 to form higher oxides of nitrogen. With respect to *S*-nitrosothiol formation, it should be noted that $NO^{\bullet}$ alone does not spontaneously react with thiol groups. Rather, the formation of a covalent bond between $NO^{\bullet}$ and the cysteine sulfur atom occurs through the synthesis of other reactive nitrogen oxide species. [25,26,27] However, the present study shows that they are increased in PIH as compared to control. In this situation it is possible that in PIH, $NO^{\bullet}$ synthesis may not be decreased but as more and more $NO^{\bullet}$ is used up in reaction with $O2^{\cdot}$, less of it will be oxidized to nitrite and nitrate. Thus, NOx (Nitrite and nitrate) will be less which will be interpreted as decreased synthesis of $NO^{\bullet}$. As more peroxynitrite (ONOO)- is generated, more thiols will be converted to nitrothiol. Hence, nitrothiol concentration is more and NOx concentration is less than control as shown in Figure no.2.

Plasma thiols are strong and resistant antioxidants that remove free radicals physiologically. Serum levels of protein thiols are among the indicators of antioxidant status in the body. Reduced thiol levels in this study suggests oxidative stress. The reducing environment of the cytosol maintains most protein thiols in the reduced state. Upon physiological increase in cellular oxidants, specific proteins could act as redox switches that regulate the conformation and activity of different proteins. This reversible post translational modification enables redox-sensitive dynamic changes in cell signaling and function. Excessive oxidative stress results in indiscriminate and irreversible oxidation of protein thiols, depletion of glutathione and cell death. [28,29]

Uric acid is a breakdown product of nucleic acid metabolism. This study shows increased uric acid in PIH as compared to control. Difference between mean uric acid levels in PIH and normal pregnant was 2.26. (Figure 2) Uric acid is a traditional marker of PIH. Hyperuricemia is a common finding in preeclamptic pregnancies. Hyperuricemia can be due to increased production, decreased excretion, or both. Uric acid is formed from xanthine by enzyme xanthine oxidase. [30] In PIH, there may be upregulation of xanthine oxidase leading to hyperuricemia. This increased xanthine oxidase in turn leads to increased generation of superoxides, thus adding to the oxidative stress. Hypertensive condition like PIH may be having some effect on kidney functioning, so in this context uric acid levels might be increased due to decreased excretion. Comparative change in mean of all parameters in PIH is shown in Fig 3.

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Thus, to summarise the entire scenario in PIH is shown diagramaticaly in Figure no. 3, where SOD seems to be the initiating factor. Due to upregulation of xanthine oxidase, there is increased generation of uric acid and also superoxides. So more and more SOD is used up for its dismutation. Thus, there is decreased SOD in PIH and increased $O2^{\cdot}$. Normally $NO^{\cdot}$ is rapidly converted to nitrite (NO_2) and then to nitrate (NO_3). When more and more superoxide radicals are formed Nitric oxide is directed towards formation of peroxynitrite radicals, thus decreasing $NO^{\cdot}$ flux towards NOx. Peroxynitrite increases the nitrosative stress. These peroxynitrite radicals react with thiol to form nitrothiol, thus decreasing thiol levels and increasing nitrothiol levels in PIH.

Limitation – More elaborative studies with proper clinical trials with antioxidants is needed. Also translating these advances into beneficial pharmacotherapy for patients is still an unmet goal.

Conclusion- There is increased free radical activity and compensatory decreased antioxidant levels in PIH. Whether such situation is a cause or effect or just a vicious cycle has to be elucidated.

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Conflict of interest- The authors declare that there are no conflict of interest.

References-

1. Alessia Mammaro, Sabina Carrara. Hypertensive Disorders of Pregnancy. J Prenat Med; 2009 Jan-Mar :3(1): 1–5.
2. Alilaj Minire , Muhametaj Mirton , Vishi Imri et al. Maternal Complications of Preeclampsia. Med Arh; 2013 Oct :67(5): 339-341.
3. Barbara Schiess. Inflammatory response in preeclampsia. Molecular Aspects of Medicine ;Volume 28: Issue 2, April 2007, Pages 210-219.
4. J Prakash. The kidney in pregnancy A journey of three decades. Indian J Nephrol 2012; 22:159-67
5. Hladunewich Michelle, Karumanchi S. Ananth , Lafayette Richard. Pathophysiology of the Clinical Manifestations of Preeclampsia . Clinical Journal of the American Society of Nephrology; May 2007:vol 2-543-549.
6. Marjaa J. VanWijk, Karolina R. Kublickiene, Kees Boeret et al. .Vascular function in preeclampsia ; Cardiovascular Research 2000;47.
7. Kapila B Gaikwad, Nitin G Joshi, and Sohan P Selkar. Study of Nitrosative Stress in 'Pregnancy Induced Hypertension'; J Clin Diagn Res. 2017 Mar; 11(3): BC06–BC08.
8. Rajaa Aouache, Louise Biquard, Daniel Vaiman et al. Oxidative Stress in Preeclampsia and Placental Diseases. Int J Mol Sci. 2018 May; 19(5): 1496.
9. Ludivine Doridot, Laurent Châtre, Aurélien Ducat et al. Nitroso-Redox Balance and Mitochondrial Homeostasis Are Regulated by *STOX1*, a Pre-Eclampsia-Associated Gene. Antioxid Redox Signal. 2014 Aug 20; 21(6): 819–834.
10. Zhonghua Shi, Wei Long, Chun Zhao et al. Comparative Proteomics Analysis Suggests that Placental Mitochondria are Involved in the Development of Pre-Eclampsia. PLoS One: 2013; 8(5): e64351.
11. Das K, Samantha L. and Chainy. A Modified Spectrophotometric Assay of Superoxide Dismutase using Nitrite. Formation of Superoxide Radicals. GBN (2000): I. J. Biochem Biophysics, 37, p. 201204.
12. Trinder ,P. Ann.Clin.Biochem. 1969, 6:24
13. Fossati,P. Prencipe,L;(1980)Clin.Chem.26:227
14. Mathew F, Glenda J, Jack L. Quantification of nitrite and nitrate in extracellular fluids. Metho Enzymol, (1996) 268: 237-246.
15. Cook JA, Kim SY, Teague D. Convenient colorimetric and fluorometric assays for S-nitrosothiols. Analytical Biochemistry : 1996, 238(2):150-8.
16. A.F.S.A.Habeeb, Methods Enzymol.25, 457,1972.
17. Maier CM, Chan PH. Role of superoxide dismutases in oxidative damage and neurodegenerative disorders. Neuroscientist. 2002 Aug;8(4):323-34.
18. Fattman CL, Schaefer LM, Oury TD. Extracellular superoxide dismutase in biology and medicine. Free Radic Biol Med. 2003 Aug 1;35(3):236-56.

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19. Lieberman Michael, Marks Allan D. Marks' Basic Medical Biochemistry: A Clinical Approach, 4th Edition Lippincott William & Wilkins; page no 439- 456,USA

20. Muth CM, Glenz Y, Klaus M, et al. Influence of an orally effective SOD on hyperbaric oxygen-related cell damage. Free Radic Res. 2004 Sep;38(9):927-32.

21. Dennog C, Radermacher P, Barnett YA et al. Antioxidant status in humans after exposure to hyperbaric oxygen. Mutat Res. 1999 Jul 16;428(1-2):83-9.

22. Aamer Sandoo, Jet J.C.S. Veldhuijzen van Zanten et al. The Endothelium and Its Role in Regulating Vascular Tone. The Open Cardiovascular Medicine Journal: 2010, 4, 302-312.

23. Zhou,Z. Martin, E. et al. Regulation of soluble guanylyl cyclase redox state by hydrogen sulfide. Pharmacol. Res*:* 2016, 111, 556-562.
24. Hofmann, F. A concise discussion of the regulatory role of cGMP kinase I in cardiac physiology and pathology. Basic Res. Cardiol: 2018, 113(4), 31.
25. Butler AR,Flitney FW. Williams DL. NO, nitrosonium ions, nitroxide ions, nitrosothiols and iron-nitrosyls in biology: a chemist's perspective. Trends Pharmacol Sci: 1995;16:18–22.
26. Espey MG. Thomas DD. Miranda KM. Focusing of nitric oxide mediated nitrosation and oxidative nitrosylation as a consequence of reaction with superoxide. Proc Natl Acad Sci U S A: 2002;99:11127–11132.
27. Gaston BM. Carver J.et al. S-nitrosylation signaling in cell biology. Mol Inter: 2003;3:253–263.
28. Stephen J.Wisdom, RhodaWilson, James H.McKillop. Antioxidant systems in normal pregnancy and in pregnancy-induced hypertension. American Journal of Obstetrics and Gynaecology. Volume 165, Issue 6, Part 1, December 1991, Pages 1701-1704.
29. Shahid Baba, Aruni Bhatnagar. Role of thiols in oxidative stress. Current Opinion in Toxicology. Volume 7;Feb 2018:133-139.
30. Changyi Chen, Jian-Ming Lü, Qizhi Yao. Hyperuricemia-Related Diseases and Xanthine Oxidoreductase (XOR) Inhibitors: An Overview. Med Sci Monit. 2016; 22: 2501–2512.

Abbreviations- $NO^{\cdot}$ - Nitric oxide,NO2- Nitrite,NO3- Nitrate, NOS - Nitric oxide synthase,.NOx - Nitrite and nitrate, O2 - Oxygen, $O2^{\cdot-}$ - Superoxide radical, ONOO- - Peroxynitrite anion, SOD - Superoxide dismutase, ROS - Reactive Oxygen Species, RSNO - Nitrosothiols, SH - Thiol group

Key words- Oxidative stress, antioxidants, nitric oxide, nitrite, nitrate, superoxide dismutase, nitrothiol, thiol, uric acid, pregnancy induced hypertension, superoxide, nitrosative stress, nitroso redox imbalance