ESTATUS ANTIOXIDANTE Y RADICALES LIBRES EN RECIEN NACIDOS CON MALFORMACIONES.

Bedabrata Mukhopadhyay y cols. Journal of Indian Association of Pediatric Surgeons 2015;Vol 20(4):179 83

ANTECEDENTES: Varios estudios en modelos animales han demostrado que el estrés oxidativo durante el embarazo puede desempeñar un papel importante en causa de defectos congénitos. Las anomalías congénitas afectan a unos 270.000 recién nacidos que mueren durante los primeros 28 días de vida cada año a partir de diferentes defectos congénitos. Por lo tanto, en la actualidad muchos trabajos de investigación estarán orientados a reducir la mortalidad infantil por anomalías congénitas.

OBJETIVO: El objetivo fue medir el oxidante y el nivel de antioxidantes en el suero de los recién nacidos con la anomalía congénita y comparar estos niveles con la misma edad y sexo recién nacidos normales. Se trata de identificar cualquier papel del estrés oxidativo como causa de anomalías congénitas.

MATERIALES Y MÉTODOS: Este estudio de casos y controles incluyeron 159 participantes: 106 recién nacidos con la anomalía congénita y 53 recién nacidos sanos. Tanto en los neonatos con anomalías congénitas y controles de neonatos sanos normales se midieron marcadores de estrés oxidativo como nivel sérico de malondialdehído (MAS) y de proteínas carbonilicas (PC) , así como la actividad de los antioxidantes como la vitamina C, glutatión. Estos parámetros se compararon estadísticamente.

RESULTADOS: Los niveles de MAS y los niveles de PC fueron significativamente mayores ($p < 0,0001$), y la vitamina C y los niveles de glutatión reducido fueron significativamente menores ($p < 0,0001$), en los recién nacidos con malformaciones congénitas que en la peroxidación lipídica newborns.

CONCLUSIÓN.

El aumento de la peroxidación de lípidos y de carbonilación de proteínas podría desempeñar un papel importante en la patogénesis de la anomalía congénita. El deterioro del balance radicales libres / antioxidante está dando lugar a un aumento del daño por radicales libres en recién nacidos con malformaciones congénitas.

Original Article

Free radicals and antioxidants status in neonates with congenital malformation

Bedabrata Mukhopadhyay, Ajay Narayan Gongopadhyay[1], Anjali Rani[2], Roshni Gavel, Surendra Pratap Mishra

Departments of Biochemistry, [1]Pediatric Surgery and [2]Obstetrics and Gynecology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Address for correspondence: Dr. Surendra Pratap Mishra, Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India. E-mail: drsurendram2@gmail.com

Access this article online

Website: www.jiaps.com

DOI: 10.4103/0971-9261.161037

Quick Response Code:

ABSTRACT

Background: Several studies using animal models have shown that oxidative stress during pregnancy may play an important role in causing birth defects. Congenital anomalies affect an estimated 270,000 newborns who die during the first 28 days of life every year from different birth defects. Hence, at present many research works are going on to reduce the infant mortality from congenital anomaly.[1] **Objective:** The objective was to measure the oxidant and antioxidant level in the serum of newborn babies with the congenital anomaly and compare these levels with age and sex matched normal neonates. This is to identify any role of oxidative stress in the causation of congenital anomaly. **Materials and Methods:** This case-control study included 159 participants: 106 newborns with the congenital anomaly and 53 healthy newborns. The markers of oxidative stress like serum malondialdehyde (MDA) level, protein carbonyl (PC) level, and the activity of antioxidants such as Vitamin C, glutathione were measured in both cases (neonates with congenital anomaly) and controls (normal healthy neonates). These parameters were statistically compared. **Results:** MDA levels and PC levels were significantly higher ($P < 0.0001$), and Vitamin C and reduced glutathione levels were significantly lower ($P < 0.0001$), in newborns with congenital malformation than in healthy newborns. Conclusions: Increased lipid peroxidation and protein carbonylation might play an important role in the pathogenesis of congenital anomaly. Impairment of the free radical/antioxidant balance is leading to increased free radical damage in neonates with congenital malformation.

KEY WORDS: Congenital anomaly, glutathione, malondialdehyde, protein carbonyl, Vitamin C

INTRODUCTION

Congenital anomalies can be defined as structural or functional anomalies, including metabolic disorders, which are present at the time of birth.[1,2] An excess of free radicals may result in abnormal embryogenesis and congenital malformations. Increase in free radical production will cause diminished antioxidant capacity and increased generation of superoxide radicals.[3] The congenital anomalies taken in our study are trache-esophageal fistula (TEF), anorectal malformation (ARM), intestinal atresia (IA) [Figure 1]. These are some of the congenital malformations with a structural defect that are surgically correctable. Study of development

Cite this article as: Mukhopadhyay B, Gongopadhyay AN, Rani A, Gavel R, Mishra SP. Free radicals and antioxidants status in neonates with congenital malformation. J Indian Assoc Pediatr Surg 2015;20:179-83.

Source of Support: Nil, **Conflicts of Interest:** None declared.

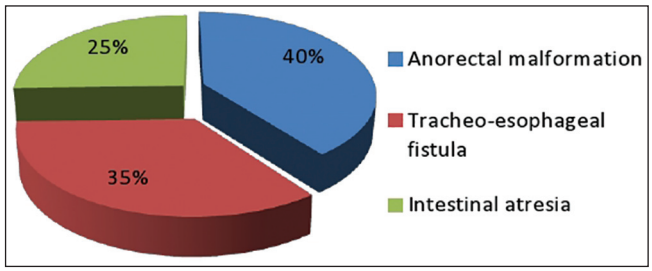

Figure 1: Percentage distribution of congenital anomalies.

tell us about the anatomical origin of these defects, like congenital TEF can arise due to the failure of fusion of the trache-esophageal ridges during the 3rd week of embryological development.[4] Impairment of the free radical/antioxidant balance may lead to increased free radical and decreased antioxidant levels in esophageal atresia.[5] Premature neonates with increased levels of total oxidant status were associated with severity of necrotizing enterocolitis with activated oxidant stress mechanisms.[6]

MATERIALS AND METHODS

Selection of patients and controls

Our study includes 53 blood samples from normal neonates taken as control and 106 blood samples from neonates with congenital malformation admitted for surgery. All control neonates selected for study were born of healthy, non-diabetic, normotensive mothers who showed no evidence of any acute or chronic infection. Diagnosis of malformation was done at birth in the majority of cases (only a few were detected by ultrasonography prenatally). Ethical clearance was taken from our University Ethical Committee before beginning of the study. Informed consent was also taken from mother or guardian before collection of each blood sample.

Collection of samples

Taking all aseptic precautions, about 3-4 ml of venous blood was drawn with a disposable syringe. All samples were collected in the morning. Of 3-4 ml, 0.5-1 ml of blood to be used for glutathione estimation was stored in the ethylenediaminetetraacetic acid vial and kept in a refrigerator at 4°C for later use. The remaining blood thus collected was allowed to stand for 30 min at room temperature for the retraction of clot. This was then centrifuged at 3000 r.p.m. for 10 min to separate the serum. The serum sample was stored at −20°C in the refrigerator for analysis. Care was being taken to avoid hemolysis of the sample. All chemicals used were of the highest purity available and were of the analytical grade. For measuring absorbance, SL 164 double beam ultraviolet — visible spectrophotometer (ELICO) was used in all cases.

Analysis of samples

1. Estimation of serum malondialdehyde (MDA) - MDA is one of the products of lipid peroxidation. It can be estimated by the thiobarbituric acid (TBA) test.[7]
2. Estimation of serum protein carbonyl (PC) content - PC content was estimated with dinitrophenylhydrazine test.[8]
3. Estimation of ascorbic acid - the ascorbic acid was estimated with 2, 4-dinitrophenylhydrazine test.[9]

Reduced glutathione - glutathione level was estimated using 5, 5'-dithio bis-(2 nitro) benzoic acid test.[10]

Statistical analysis

Statistical analysis between group 1 (controls) and group 2 (patients) was performed by the Java Stat user interface guidelines. The data were expressed as mean ± standard deviation (S.D), $P < 0.05$ is considered significant, and mean ± S.D, $P < 0.0001$ is highly significant.

Observations

The present study was carried out to evaluate the role of oxidative stress in causation of congenital malformation, as evidence has been accumulating about the role of free radicals in some of the malformations such as TEF, ARM, IA. Two important parameters of oxidative stress were evaluated: Serum PC level was estimated to check for oxidative damage to proteins; serum MDA level was estimated as a marker for lipid peroxidation. Two important antioxidant levels were also measured: Ascorbic acid, glutathione.

In the present study, the oxidative stress status of total 106 neonates with congenital malformation is being compared with 53 normal healthy neonates.

Serum malondialdehyde

Serum MDA level was estimated by TBA test as described under materials and methods. Estimation was done in all patients ($n = 106$) and control subjects ($n = 53$). The final result was expressed as µmol/l. Figure 2a shows the mean serum MDA in patients (1.54 ± 0.19) and controls (1.25 ± 0.20). The mean level was much higher in patients as compared to controls. This MDA level, when compared between cases and controls was found to be highly significant ($P < 0.0001$).

Serum protein carbonyl

Serum PC level was estimated by extraction of PC group with dinitrophenylhydrazine, as described in materials and methods. Estimation was done in all patients ($n = 106$) and control subjects ($n = 53$). The final result was expressed as n mol/mg protein. Figure 2b shows the mean PC levels in patients (5.77 ± 0.65) and controls

(3.89 ± 0.59). The mean level was much higher in patients as compared to controls. This PC level when compared between cases and controls was found to be significant ($P < 0.0001$).

Serum ascorbic acid

Serum ascorbic acid level was estimated by oxidizing it and then derivatizing the product with dinitrophenyhydrazine, as described in materials and methods. Estimation was done in all patients ($n = 106$) and control subjects ($n = 53$). The final result was expressed as mg/dl. Figure 2c, g and h shows the mean serum ascorbic acid in patients (0.689 ± 0.10) and controls (1.485 ± 0.23). The mean level was much lower in patients as compared to controls. This ascorbic acid level when compared between cases and controls was found to be significant ($P < 0.0001$).

Reduced glutathione

The glutathione estimation was performed by the method described by Beutler *et al.*, using 5, 5'-dithio bis-(2 nitro) benzoic acid, as described in materials and methods. Estimation was done in all patients ($n = 106$) and control subjects ($n = 53$). The final result was expressed as mg/ml. Figure 2d shows the mean glutathione levels in patients (37.75 ± 5.52) and controls (51.80 ± 6.39). The mean level was much lower in patients as compared to controls. This difference in glutathione level when compared between cases and controls was found to be significant ($P < 0.000$).

DISCUSSION

Information on the mechanisms of birth defects due to oxidative stress is not only important academically, but also may throw some light on the therapeutic approach. A free radical may be defined as a molecule or molecular fragment containing one or more unpaired electrons in its outermost atomic or molecular orbit and are capable of independent existence.[11] Reactive oxygen species (ROS) and reactive nitrogen species include radicals such as superoxide, hydroxyl, lipid peroxide, nitric oxide, etc.[12] Though some oxidizing agents such as hydrogen peroxide (H_2O_2), hypochlorous acid are not radicals but reactive species, they can become involved in free radical reactions. Antioxidants are compound that quenches the formation of ROS. Oxidative stress has been defined as harmful because oxygen free radicals attack biological molecules such as lipids, proteins, and DNA. However, oxidative stress also has a useful role in physiologic adaptation and in the regulation of intracellular signal transduction. Therefore, a more useful definition of oxidative stress may be "a state where oxidative force exceeds the antioxidant systems due to loss of the balance between them."[13] If a radical reacts with a non-radical, another free radical is produced. This property enables free radicals to participate in chain reactions. Radicals may also serve as oxidants and reductants. Peroxidation of membrane lipid produces MDA as a by-product. Conditions such as, photochemical reactions, radiation injury, drugs, hypoxia, inflammatory conditions, viral infections, and carcinogenic process produce abundant ROS in the human body resulting in a state of oxidative stress. Our body has antioxidant defense mechanisms to handle this oxidative burden. Hence, direct and indirect evidence of oxidative stress may be ascertained by either detection of radicals themselves or by detection of biological damage caused by these radicals (such as MDA, PC etc.) or by assessing the antioxidant defense mechanism (such as superoxide dismutase, catalase, peroxidase, ceruloplasmin, reduced glutathione, ascorbic acid, and transferrin).

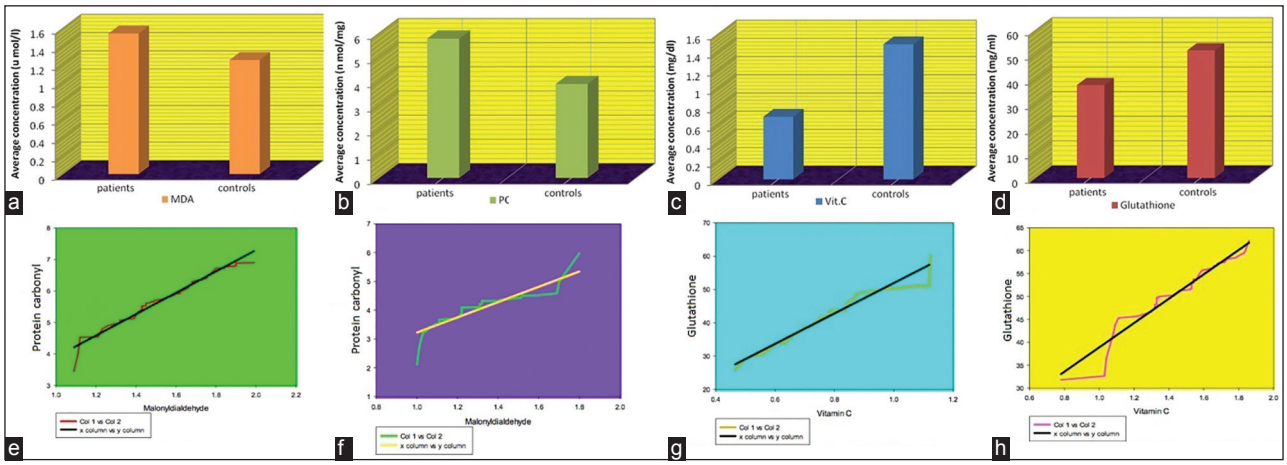

Figure 2: Figure 2 a, b, c, d represents the status of MDA, PC, Vit.C and Glutathione in cases and controls, respectively. Figure 2 e, and f represent the correlation between PC and MDA. Figure 2 g and h represents the correlation between Glutathione and vitamin C. PC- Protein carbonyl, Vit.C- Vitamin C, MDA- Malondialdehyde

Several studies have shown that antioxidants such as Vitamin E, Vitamin C, combination of antioxidants, lipids, folic acid etc., may have some preventive effect on birth defects.[14,15] In the present study, parameters of oxidative stress and levels of certain antioxidants were evaluated in 106 patients with congenital malformation and 53 control neonates. The patients and control were age and sex matched. Following exclusion criteria were kept in consideration, mothers were:

1. Not having any acute infection, especially those which are documented to cause congenital malformation,
2. not taking any drug,
3. healthy in the prenatal period without any complication,
4. not having any chronic disease such as diabetes mellitus, hypertension, hyperlipidemia, etc.

Vitamin C, a radical scavenging antioxidant, present in all cells, can also act as a reducing agent. This free radical scavenger protects the cell against the toxic oxygen free radicals. Ascorbic acid is a redox catalyst, which can reduce, and thereby neutralize, ROS like H_2O_2.[16]

In our study, the significant decrease in the levels of Vitamin C occurs with the progression of lipid per oxidation. The lowered values of Vitamin C indicates that the severity of the infection. Vitamin C level decreases by scavenging the free radicals and prevent the lipid peroxidation. The findings in the present study shows decreased serum ascorbic acid levels in neonates with congenital malformation.

We observed a significant decrease in the levels of erythrocyte glutathione (non-enzymatic antioxidant defense system) in neonates with congenital malformation compared to controls. The decrease in the levels of these non-enzymatic antioxidant parameters may be due to the increased turnover, for preventing oxidative damage in these patients suggesting an increased defense against oxidant damage.

Lipids, especially polyunsaturated fatty acids, are very susceptible to free radical attack, which can initiate lipid peroxidation. The determination of MDA is one of the most commonly used methods for monitoring lipid peroxidation. Serum MDA levels were significantly elevated ($P < 0.000$) in neonates with congenital malformation as compared to controls. The increase in the levels of MDA in our study indicates that lipid peroxidation is taking place in neonates with congenital malformations.

Serum PC levels were significantly elevated ($P < 0.0001$) in neonates with congenital malformation as compared to controls. PC is most commonly measured products of protein oxidation in biological samples. Among the various oxidative modifications of amino acids in proteins, carbonyl formation may be an early marker of protein oxidation. PC increases in neonates with congenital malformations.

SUMMARY AND CONCLUSION

The aim of this study was to estimate the levels of oxidants indirectly by measuring serum MDA, serum PC, and antioxidants (serum ascorbic acid, blood glutathione) in all patients and controls. The study shows that the pathophysiology of congenital malformation involves oxidative stress. We found that the products of lipid and protein oxidation are significantly higher in patients with congenital malformation. Plasma MDA and PC levels were significantly higher in patients with a congenital malformation than in the control neonates, and this is in agreement with some previous reports.

The result suggests the necessity for therapeutic co-administration of antioxidants with conventional drugs to such patients before corrective surgery (as surgery itself causes stress) and also antioxidant precautions should be taken by mothers during pregnancy to prevent congenital malformations in their neonates. Therefore, treatment with antioxidants may be useful as a preventive therapy to inhibit the oxidative damage producing congenital malformation.

ACKNOWLEDGMENT

We thank Prof. R. G. Singh, Director of Institute of medical sciences, Banaras Hindu University for giving us the opportunity for conducting all the research work in his institute. We are grateful to our dean Prof. R. K. Goel for financial and moral support. Prof. D. Dash, Department of Biochemistry helped us with the availability of all the research related instruments of his laboratory along with technical and moral support.

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