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Oxidative Stress and Antioxidant Enzyme Activities in Newborns with Oesophageal Atresia and Their Mothers

M Melek[1], H Demir[6], S Bilici[1], B Beger[1], U Çobanoğlu[2], I Meral[3], S Keskin[4] and E Ozmen[5]

[1]Department of Paediatric Surgery, [2]Department of Thoracic Surgery, [3]Department of Physiology, [4]Department of Biostatistics, and [5]Department of Gynaecology, Medical Faculty, University of Yuzuncu Yil, Van, Turkey; [6]Department of Biochemistry, Art and Science Faculty, University of Yuzuncu Yil, Van, Turkey

OBJECTIVE: To measure the oxidant/antioxidant status of newborn babies with oesophageal atresia and their mothers, compared with healthy control subjects. METHODS: This case–control study included 40 participants: 10 newborns with oesophageal atresia and their mothers, and 10 healthy newborns and their mothers. Whole blood malondialdehyde (MDA) levels and the activities of antioxidant enzymes (catalase, carbonic anhydrase [CA], glucose-6-phosphate dehydrogenase [G-6-PD], and superoxide dismutase [SOD]) were measured. RESULTS: MDA levels and CA activity were significantly higher, and catalase, SOD and G-6-PD activities were significantly lower, in newborns with oesophageal atresia and their mothers than in healthy newborns and their mothers. Although CA activity was similar between the newborns and mothers in the patient group, it was significantly lower in newborns than in mothers in the healthy group. CONCLUSIONS: Increased lipid peroxidation might play an important role in the pathogenesis of oesophageal atresia. Impairment of the free radical/antioxidant balance may lead to increased free radical and decreased antioxidant levels in oesophageal atresia.

KEY WORDS: Oesophageal atresia; Newborn infant; Oxidant; Antioxidant; Malondialdehyde; Antioxidant enzyme activity

Introduction

Congenital tracheo-oesophageal fistula with oesophageal atresia is a rare disease in newborns. It describes a group of congenital anomalies in which the oesophageal continuity is interrupted, with or without continuity of the trachea.[1] The presence of additional anomalies provides clues to the probable aetiology.[1,2] Quan and Smith[3] described the VATER association, which consists of a combination of vertebral, anorectal, tracheo-oesophageal and renal or radial anomalies. This association has been broadened to include cardiac and extremity defects, which has consequently been described as the VACTERL association.[2,3] The aetiology of oesophageal atresia is probably multifactorial but precise causative factors remain unknown. Many studies have investigated its aetiology and several

theories have been suggested.[4 – 10]

It is well known that the production of reactive oxygen species (ROS; including superoxide anions [O_2^-], hydrogen peroxide [H_2O_2] and hydroxyl anions) beyond the ability of the antioxidant defence systems to scavenge them may result in oxidative stress.[11] Oxidative damage to cellular macromolecules is implicated in many diseases including neurodegenerative disorders, cancer, cirrhosis, cardiovascular disease, atherosclerosis, cataracts, diabetes and inflammation. Compounds that can scavenge free radicals have great potential for ameliorating these diseases.[12] The first molecular targets for oxidative damage in cells are proteins, and their side chains can be carbonylated by reactive carbonyl compounds.[13] In addition, oxidative damage to lipids leads to the formation of products such as malondialdehyde (MDA).[14]

Catalase is a ubiquitous antioxidant enzyme that is present in nearly all living organisms. Catalase and glutathione peroxidase are equally active in the detoxification of H_2O_2 in human erythrocytes.[15,16] Superoxide dismutase (SOD) catalytically scavenges O_2^-, protecting against their potential cytotoxicity. It catalyses the dismutation of highly reactive O_2^- to O_2 and H_2O_2, a less reactive ROS.[17] Carbonic anhydrase (CA) is a zinc-containing metalloenzyme that catalyses the reversible hydration of carbon dioxide.[18,19] Glucose-6-phosphate dehydrogenase (G-6-PD) is the key enzyme in the first step of the hexose monophosphate pathway *in vivo*, and is widespread in all tissues and blood cells.[20,21]

The aims of the present study were to investigate MDA levels and the activities of the antioxidant enzymes CA, catalase, SOD and G-6-PD in newborns with oesophageal atresia and their mothers, compared with healthy newborns and their mothers.

Patients and methods

STUDY POPULATION

Patients and healthy subjects for this case–control study were recruited from the outpatient clinics and hospital wards of the Department of Paediatric Surgery, Medical Faculty of Yuzuncu Yil University Hospital (Van, Turkey) between January 2007 and December 2010. Newborns with oesophageal atresia who were diagnosed in the neonatal intensive care unit, and their mothers, were selected for the patient group. The control (healthy) group included healthy newborns who were delivered spontaneously at term, and their mothers. Newborns in both groups were diagnosed and evaluated during the immediate 48-h postpartum period; blood samples were collected and a diagnosis of oesophageal atresia was made in the first 48 h postpartum. Among healthy neonates, those with mothers who had any drug or substance addiction, who smoked or who had any trauma or diseases during pregnancy were excluded. Neonates (both healthy and those with oesophageal atresia) with prolonged hyperbilirubinaemia, those with infection or whose mother had known infection, and any cases known to have received any drug therapy in the first 48 h of life, were also excluded. In the patient group, mothers with a difficult delivery history and their newborns who died during the immediate 48-h postpartum period were excluded from the study.

The study was approved by the Ethics Committee of the Medical Faculty of Yuzuncu Yil University (Van, Turkey). Written and verbal consent was given by family members of all of the newborns who participated in the study.

BIOCHEMICAL ANALYSES

Venous blood samples were obtained from the antecubital fossa veins of all participants

and processed immediately. Whole blood samples (1 ml) were treated with 1.8 mg ethylenediaminetetra-acetic (EDTA) as an anticoagulant. Serum samples were prepared by allowing blood to coagulate, and the serum was then separated by centrifugation and stored at –80 °C in deionized polyethylene tubes until analysis.

Levels of MDA were estimated by measuring thiobarbituric acid-reactive substances in whole blood samples according to a modified method of Jentzsch *et al.*[22] Briefly, 0.2 ml of whole blood was added to the reaction mixture containing 1 ml of 1% orthophosphoric acid and 0.25 ml alkaline solution of thiobarbituric acid (pH 7.5) (final volume 2.0 ml), followed by heating at 95 °C for 45 min. The results were expressed as nmol MDA per ml of plasma/whole blood.

A colourimetric technique was employed to determine CA activity, assayed by the hydration of carbon dioxide with bromothymol blue as an indicator.[23] All assays were performed at 3 °C in a water bath with a cooling coil.

The biochemical analysis of catalase activity in erythrocytes was performed according to the method described by Aebi.[24] First, the supernatant was prepared by centrifuging whole blood at 2800 ***g*** and discarding the upper part of the liquid. Next, 0.9% saline was added, the sample was centrifuged again at 44 800 ***g*** and again the the upper part of the liquid was discarded. This procedure was repeated and then distilled water was added to the remainder pellet and mixed. Then, 0.1 ml of remaining supernatant was added to a quartz cuvette containing 2.95 ml of 19 mmol/l H_2O_2 solution prepared in potassium phosphate buffer (0.05 M, pH 7.00). The change in absorbance at 240 nm was monitored for 5 min using a spectrophotometer (Shimadzu UV-1201; Shimadzu Corporation, Kyoto, Japan).

The SOD activity was determined in red blood cells. A total of 0.1 ml of whole blood was haemolysed by the addition of 0.9 ml of ice-cold water (0 – 4 °C). The haemoglobin was removed by adding 0.25 ml of chloroform and 0.5 ml of ethanol, followed by vigorous mixing and centrifugation at 18 000 ***g*** for 60 min. The clear supernatant was used for the SOD assay, as described previously.[25,26] The rate of inhibition of the superoxide reaction by SOD was calculated according to the definition of McCord and Fridovich.[27]

Activity of G-6-PD was measured by the Beutler method, using the erythrocytes that were obtained from whole blood.[20] Absorbance at 340 nm was measured at 37 °C, in the presence of 1 M Tris–HCl, 5 mM EDTA (pH 8.0), 0.1 M $MgCl_2$, 2 mM nicotinamide adenine dinucleotide phosphate and 6 mM glucose-6-phosphate.

STATISTICAL ANALYSES

Statistical analyses were performed using SPSS® version 13.0 (SPSS Inc., Chicago, IL, USA) for Windows®. Descriptive statistics for biochemical variables are expressed as mean ± SD. Differences between patients and controls, and between newborn and maternal data, were assessed using two-factor variance analysis with repeated measurements. Spearman's rank correlation coefficient was calculated in both groups, in order to determine the relationship between different biochemical variables. A *P*-value of < 0.05 was considered statistically significant.

Results

The study included 40 participants: 10 newborns with oesophageal atresia and their mothers (patient group), and 10 healthy newborns and their mothers (healthy group). MDA level and CA activity were

significantly higher, and catalase, SOD and G-6-PD activities were significantly lower, in the newborns with oesophageal atresia and their mothers than in the healthy newborns and their mothers ($P < 0.05$ for all comparisons; Table 1). MDA levels, catalase activity and SOD activity were also significantly lower in newborns in both groups compared with their mothers ($P < 0.05$ for all comparisons). There was no significant difference in G-6-PD activity between newborns and their mothers in either the healthy or patient group. Although CA activity was not significantly different between the newborns and their mothers in the patient group, it was significantly higher in the newborns than their mothers in the healthy group ($P < 0.05$).

There was a negative correlation between maternal G-6-PD and catalase activities, and maternal MDA level and newborn G-6-PD activity, in the patient group ($P < 0.05$ for both comparisons; Table 2). There was a negative correlation between newborn SOD and CA activities in the healthy group ($P < 0.05$; Table 3). There was also a positive correlation between maternal SOD and newborn MDA level, and maternal SOD and newborn catalase activity, in the healthy group ($P < 0.05$ for all comparisons).

Discussion

Tracheo-oesophageal fistula with oesophageal atresia is a rare developmental abnormality, affecting 1 in 2500 to 4000 newborns.[28] It is a congenital abnormality of the oesophagus that occurs early in pregnancy, and there are no known preventive measures.[29]

Oxidative stress – a condition caused by increased ROS levels – is recognized to be a prominent feature of the development and progression of various diseases. ROS-scavenging enzymes play important roles in the protection of cells from oxidative damage.[11] The relationship between antioxidant status and the levels of well-known markers of oxidative stress, such as lipid peroxides and oxidized proteins, reflect better health indices and status.[30] There is evidence that ROS play important roles in the pathophysiology of many paediatric disorders,[26] but it is not clear how the

TABLE 1:
Malondialdehyde (MDA) level and catalase, superoxide dismutase (SOD), glucose-6-phosphate dehydrogenase (G-6-PD) and carbonic anhydrase (CA) activities in newborn babies with oesophageal atresia and their mothers, compared with a healthy control group of newborn babies and their mothers

	Patient group: newborn babies with oesophageal atresia		Healthy group: newborn babies without oesophageal atresia	
Parameter	Newborns $n = 10$	Mothers $n = 10$	Newborns $n = 10$	Mothers $n = 10$
MDA (nmol/ml plasma)	$2.38 \pm 0.29^{a,c}$	2.90 ± 0.08^{b}	0.98 ± 0.08^{c}	1.91 ± 0.24
Catalase (U/gHb)	$5.39 \pm 2.65^{a,c}$	12.16 ± 9.15^{b}	32.11 ± 8.11^{c}	93.22 ± 48.08
SOD (U/ml blood)	$10.52 \pm 5.60^{a,c}$	489.64 ± 51.70^{b}	28.56 ± 5.72^{c}	690.75 ± 74.62
G-6-PD (U/gHb)	0.16 ± 0.09^{a}	0.21 ± 0.10^{b}	2.19 ± 1.10	1.39 ± 1.29
CA (U/gHb)	4.18 ± 0.12^{a}	3.99 ± 0.50^{b}	1.75 ± 0.44^{c}	1.06 ± 0.61

Data presented as mean ± SD.
$^{a}P < 0.05$ compared with healthy newborns, $^{b}P < 0.05$ compared with healthy mothers, $^{c}P < 0.05$ compared with mothers in same group; two-factor variance analysis.
gHb, grams of haemoglobin.

TABLE 2:
Spearman's rank correlation coefficients between the five biochemical variables in newborn babies with oesophageal atresia (n = 10) and their mothers (n = 10)

	N G-6-PD	M G-6-PD	N CAT	M CAT	N CA	M CA	N SOD	M SOD	N MDA	M MDA
N G-6-PD	1.000									
M G-6-PD	–0.199	1.000								
N CAT	–0.016	–0.444	1.000							
M CAT	0.388	–0.635[a]	0.294	1.000						
N CA	–0.003	–0.167	–0.358	–0.331	1.000					
M CA	–0.154	–0.191	0.310	–0.207	–0.122	1.000				
N SOD	0.458	–0.208	0.037	0.202	–0.118	0.522	1.000			
M SOD	–0.149	–0.359	0.012	0.343	0.403	–0.491	–0.479	1.000		
N MDA	0.230	0.341	0.075	–0.245	0.370	–0.345	–0.116	0.073	1.000	
M MDA	–0.678[a]	0.432	0.269	–0.253	–0.328	0.173	–0.349	–0.183	0.123	1.000

[a]$P < 0.05$ (Spearman's rank correlation coefficient).
N, newborn; M, mother; G-6-PD, glucose-6-phosphate dehydrogenase; CAT, catalase; CA, carbonic anhydrase; SOD, superoxide dismutase; MDA, malondialdehyde.

TABLE 3:
Spearman's rank correlation coefficients between the five biochemical variables in healthy newborns (n = 10) and their mothers (n = 10)

	N G-6-PD	M G-6-PD	N CAT	M CAT	N CA	M CA	N SOD	M SOD	N MDA	M MDA
N G-6-PD	1.000									
M G-6-PD	0.029	1.000								
N CAT	–0.198	0.313	1.000							
M CAT	–0.103	–0.505	0.166	1.000						
N CA	0.029	0.000	–0.440	–0.269	1.000					
M CA	–0.053	–0.093	0.407	–0.305	–0.048	1.000				
N SOD	0.150	0.142	0.354	0.142	–0.766[a]	0.136	1.000			
M SOD	–0.414	0.349	0.668[a]	0.248	–0.278	0.391	0.340	1.000		
N MDA	–0.221	0.615	0.284	–0.006	–0.112	0.129	0.234	0.752[a]	1.000	
M MDA	0.421	0.585	0.470	0.067	–0.420	–0.330	0.497	0.134	0.176	1.000

[a]$P < 0.05$ (Spearman's rank correlation coefficient).
N, newborn; M, mother; G-6-PD, glucose-6-phosphate dehydrogenase; CAT, catalase; CA, carbonic anhydrase; SOD, superoxide dismutase; MDA, malondialdehyde.

newborn infant copes with possible excess exposure to ROS. It is possible that the still-developing antioxidant defence mechanisms of the infant may be overcome by the generation of excessive ROS during the neonatal period. Abundant evidence suggests that ROS play an important role in the pathogenesis of many paediatric neurological diseases, including retinopathy of prematurity, Down syndrome, epilepsy and mitochondrial encephalopathies.[26,31 – 33] The MDA level was significantly higher in the newborns and mothers in the patient group than the healthy group, in the present study. In addition, MDA levels were significantly lower in newborns of both

groups than in their corresponding mothers.

Cells possess an efficient antioxidant defence system, mainly composed of enzymes such as SOD, glutathione peroxidase and catalase, which can scavenge and stabilize excess ROS.[34] Any increase in SOD catalytic activity produces an excess of H_2O_2 that must be efficiently neutralized by catalase or glutathione peroxidase.[26] Newborn and maternal catalase, SOD and G-6-PD activities were lower in the patient group than the healthy group in the present study. The catalase and SOD enzyme activities were also lower in newborns in both groups than in their mothers. In accordance with these results, newborns with congenital anomalies have been shown to have decreased catalase activity.[35] There are reports regarding SOD activity in human erythrocytes in healthy newborns and young people,[26,36,37] and SOD activity has been found to be lower in neonates than in adults.[38,39] These findings indicated that the free radical/antioxidant balance might be impaired, leading to the increased free radical and decreased antioxidant levels in newborns with oesophageal atresia.

Neonatal and maternal G-6-PD activity was lower in the patient group than the healthy group in the present study. G-6-PD has a vital function in many tissue types and plays an important role in metabolism. Deficiency of G-6-PD is one of the most common genetic abnormalities: approximately 400 million people are affected worldwide.[40,41] It has long been known that term neonates have higher G-6-PD activity than adults,[42 – 44] and a few studies have suggested that this trend may extend to premature neonates.[45 – 47]

Carbonic anhydrase plays different roles in various tissues. Species can produce many different CA isoenzymes, some of which act in the cytosol, while others are membrane-bound. For example, in humans there are four cytosolic isoenzymes, four membrane-bound isoenzymes, a mitochondrial isoenzyme and a secreted salivary isoenzyme, as well as several related proteins that lack catalytic activity.[48] CA isoenzymes are key regulators of acid–base homeostasis in both normal and pathological conditions.[49 – 52] It was found in the present study that the level of CA activity was higher in newborns with oesophageal atresia and their mothers, than in healthy newborns and their mothers. The increased CA activity in the patient group may provide evidence of an acid–base imbalance in these patients, and may also indicate an attempt by the body to compensate for the low levels of the other antioxidant enzymes, G-6-PD, catalase and SOD, by increasing the CA enzyme activity.

The MDA level of newborns in both groups was lower than that of their respective mothers. This may be due to maternal oxidative stress affecting the fetus and potentially could be reduced, even partially, by antioxidant treatment methods in the mother. The higher MDA levels in newborns with oesophageal atresia compared with healthy newborns might also be indicative of inadequate antioxidant mechanisms.

The negative correlation between G-6-PD activity of the newborns and the MDA levels of the mothers in the patient group suggests an interaction between the oxidation/antioxidation processes of the babies and their mothers. The lower G-6-PD activity of the mothers and babies in the patient group, compared with the healthy group, might indicate that the newborns with oesophageal atresia and their mothers were exposed to increased oxidative stress. The negative correlation between catalase and G-6-PD activities of the mothers in the patient group suggested an attempt by the body to compensate for the low antioxidant effect

(caused by low G-6-PD activity) by increasing catalase activity. When these two negative correlations are evaluated together, it could be concluded that low G-6-PD activity in the mothers in the patient group had an impact on their newborn babies, and that this played a role in the insufficiency of the oxidative stress-coping mechanisms of the newborns.

The activity of SOD was significantly lower in newborns in the patient and healthy groups than in their mothers in the present study, and CA activity was significantly higher in healthy newborns than in their mothers. In addition, a negative correlation was found between the SOD and CA enzyme activities of the healthy newborns, indicating that low SOD activity may result in oxidative stress in healthy newborns, and that increased CA activity may be a possible compensatory mechanism.

The positive correlation between SOD activity in healthy mothers and MDA levels in their newborn babies suggests that maternal SOD may be affected by elevated newborn MDA levels. These findings might be evidence of the interaction between the oxidative stress load in babies and the antioxidant mechanisms of their mothers.

The results of some biochemical parameters suggested that the oxidative status of the mothers and newborns in the patient group was abnormal. There may be a placental interaction between the mother and the baby in terms of the functioning of the oxidation/antioxidation system, as has been shown in other cases of teratogenicity.[53] The present study is the first to determine the oxidative status of newborns with oesophageal atresia and their mothers by measuring MDA levels and antioxidant enzyme activities. The data outline some of the pathological changes in the oxidation/antioxidation mechanism of newborns with oesophageal atresia and their mothers. This maternal oxidative stress might be explained by the effect of stress factors experienced by mothers during their pregnancy, or it could be the result of the mothers' responses to the pathological oxidation status of their abnormal babies. There is a need for further research to determine the source of the oxidation/antioxidation system dysfunction in the mother and/or newborn baby with oesophageal atresia.

There were several limitations to the present study. The sample size was limited due to this being a rare congenital abnormality. The premise of the study was based on studying how the balance of oxidative stress/antioxidants affected the development of this abnormality. The results showed a significant correlation between oxidative stress and antioxidant balance and the development of oesophageal atresia, supporting our hypothesis that changes in the oxidative/antioxidant stress balance may play a role in the development of congenital abnormalities. Further studies, involving larger numbers of patients and more detailed bimolecular experimental and clinical studies, are needed to examine this association and the mechanism of action.

In conclusion, the findings of the present study indicate that increased lipid peroxidation might play an important role in the pathogenesis of oesophageal atresia. Impairment of the free radical/antioxidant balance may lead to increased free radical and decreased antioxidant levels in oesophageal atresia. In addition, the increased CA activity in newborns with oesophageal atresia and their mothers might be evidence of an acid–base imbalance in these patients.

Conflicts of interest

The authors had no conflicts of interest to declare in relation to this article.

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Author's address for correspondence
Dr Mehmet Melek
Yuzuncu Yil Universitesi, Tip Fakultesi, Cocuk Cerrahisi Anabilim Dali, 65200 Merkez, Van, Turkey.
E-mail: mmelek44@yahoo.com