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Oxidative DNA damage in retinopathy of prematurity

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PURPOSE. *This study examines the levels of oxidative damage in patients with retinopathy of prematurity (ROP).*
METHODS. *Fifty patients were recruited with a birthweight below 1500 g or gestational age below 32 weeks. The cases were classified into those who developed ROP (n=25) and those without ROP (n=25). The authors obtained blood and urine samples from each infant, for measuring 8-hydroxy 2-deoxyguanosine (8-OHdG) and malondialdehyde (MDA) levels, at the time of the first examination at 4–6 postnatal weeks.*
RESULTS. *A significant difference was observed in leukocyte and urine 8-OHdG levels in patients with ROP compared to those without ROP (p<0.001 for both). Similarly, a significant difference was observed in plasma and urine MDA levels in patients with ROP compared to those without ROP (p<0.001 for both). In addition, significant correlations were found between levels of 8-OHdG in leukocyte DNA and plasma MDA (r=0.859, p<0.001), and between levels of urine 8-OHdG excretion and urine MDA (r=0.563, p<0.001).*
CONCLUSIONS. *8-OHdG in leukocyte DNA and urine levels in premature infants can be useful as an indicator for ROP screening. (Eur J Ophthalmol 2009; 19: 80-5)*

KEY WORDS. *Oxidative damage, Retinopathy of prematurity*

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INTRODUCTION

Retinopathy of prematurity (ROP) is a disease of the incompletely vascularized immature retina, characterized by retinovitreal neovascularization, and possibly retinal detachment and blindness (1, 2). Several factors increase the risk of ROP, especially those associated with short gestation, low birthweight, frequency of blood transfusions, sepsis, intraventricular hemorrhage, exposure to light, mechanical ventilation, and oxygen therapy (3, 4). ROP was the original classical manifestation of oxygen toxicity due to excessive oxygen administration in extremely premature infants. Supplemental oxygen therapy causes toxic effects on the retina, which contributes to the development of ROP (5). Although the cellular mechanisms of oxygen toxicity are still not fully defined, oxidative damage of cellular components by increased generation of reactive oxygen species (ROS) is probably the mechanism by which high concentrations of oxygen damage cells (6, 7). Abnormally high levels of ROS, due to overproduction/inadequate removal, can damage cellular proteins, nucleic acids, and membrane lipids (6, 7). ROS-mediated oxidation of cell membrane lipids leads to the formation of lipid peroxidation products. Lipid peroxides cause a well-established mechanism of cellular injury in humans, and their presence is used as an indicator of oxidative damage in cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds. These include reactive carbonyl compounds, among which malondialdehyde (MDA) is the most abundant (8). Therefore, measurement of MDA is widely used as an indicator of lipid peroxidation (9, 10).

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Oxidative damage affects biomolecules other than lipids – notably proteins and DNA. As ROS causes strand breaks in DNA and base modifications, including the oxidation of guanine residues, its oxidation product, 8-hydroxy 2-deoxyguanosine (8-OHdG), can serve as a sensitive biomarker of oxidative DNA damage (11). Recent studies have reported significantly increased levels of 8-OHdG in a number of systemic diseases (12, 13). Levels of products of oxidative DNA lesions in biological fluids (e.g., urine, serum, cerebrospinal fluid) and tissues have been reported to be reliable biomarkers of oxidative stress (14).

In this study, we aim to investigate the levels of these oxidant damage markers in patients with ROP. For this reason, we determined the 8-OHdG levels in leukocyte DNA and in urine samples, as specific marker of oxidative damage to DNA, and plasma and urine MDA levels, as the index of lipid peroxidation, in patients with ROP.

METHODS

In this study, we included 50 premature babies admitted to the neonatology unit of our hospital. The inclusion criterion for premature neonates was gestational age (GA) under 32 weeks or weight at birth (WB) <1500 g. Patients with larger WB or GA with risk factors for ROP or when the neonatologist considered that they should be assessed were also included.

Patients who had other associated pathology, such as requiring oxygen therapy during the first days of life in order to maintain normal arterial pO_2 levels (average oxygenation time: 50 h), were excluded from the study. These patient subjects were divided into two groups: patients with ROP (n=25) and patients without ROP (n=25). This study was approved by the Ethics Committee. The parents or guardians of each patient signed a purpose-made informed consent form. For evaluation of ROP, the patients were examined in week 4–6 after birth (15). Thereafter, pupillary dilatation (phenylephrine 2.5% and cyclopentolate 0.5%) indirect ophthalmoscopy was used to evaluate the severity of retinal manifestation of ROP. For measuring 8-OHdG and MDA levels, a single sample of peripheral venous blood (1 mL) and a single sample of urine (3 mL) were collected from premature neonates at the time of the first screening for ROP, in weeks 4–6 after birth. Urine samples were frozen immediately after collection and stored at –20°C until analysis.

Isolation and hydrolysis of DNA

DNA isolation from whole blood was performed according to Miller et al and Adeli (16, 17), with some modifications. Five hundred µL of blood with ethylene diamine tetraacetic acid (EDTA) was mixed with 750 µL of erythrocyte lysis buffer and incubated for 10 min on ice, followed by centrifugation (10 min at 3500 rpm). The supernatant was decanted and the pellet was thoroughly resuspended in sodium dodecyl sulfate (10%, v/v), proteinase K (20 mg/mL), and 1.9 mL leukocyte lysis buffer. The mixture was incubated at 65 ºC for 1 h and then mixed with 0.8 mL of 9.5 M ammonium acetate. After centrifugation at 3500 rpm for 25 min, the clear supernatant (2 mL) was transferred to a new sterile tube, and DNA was precipitated by addition of 4 mL of ice-cold absolute ethanol. DNA samples were dissolved in Tris EDTA buffer (10 mM, pH 7.4), and then were hydrolyzed according to Kaur and Halliwell's method (18).

Analysis of 8-OHdG and dG by the HPLC method

The levels of 8-OHdG and dG in the hydrolyzed DNA samples were measured by HPLC with electrochemical (HPLC-ECD) and variable wavelength UV detector (HPLC-UV) systems as previously described (19). Before analysis by HPLC, the hydrolyzed DNA samples were redissolved in the HPLC eluent (final volume 1 mL). Twenty µL of final hydrolysate was analyzed by HPLC-ECD (HP, Agilent 1100 modular system with HP 1049A ECD detector, Germany): Column, reversed phase-C18 (RP-C18) analytical column (250 mm × 4.6 mm × 4.0 µm, Phenomenex, CA, USA). The mobile phase consisted of 0.05 M potassium phosphate buffer, pH 5.5 containing acetonitrile (97: 3, v/v), at a flow rate of 1 mL/min. The dG concentration was monitored based on absorbance (245 nm) and 8-OHdG based on the electrochemical reading (800 mV).

Levels of dG and 8-OHdG were quantified using the standards of dG and 8-OHdG (Sigma Chemicals, St. Louis, MO, USA). Analysis of urine for oxidative DNA damage was performed according to Kasai (20). The urine samples were separated on RP-C18 column (250 mm × 4.6 mm × 4.0 µm, Phenomenex) connected to a guard column, using 50 mM K_2HPO_4 (pH 4.0) and 10% MeOH as the mobile phase. 8-OH-dG was detected with an electrochemical detector (600 V) and dG was detected with a UV detector (254 nm). 8-OHdG level is expressed as the number of 8-OHdG molecules per 10^6 dG (21).

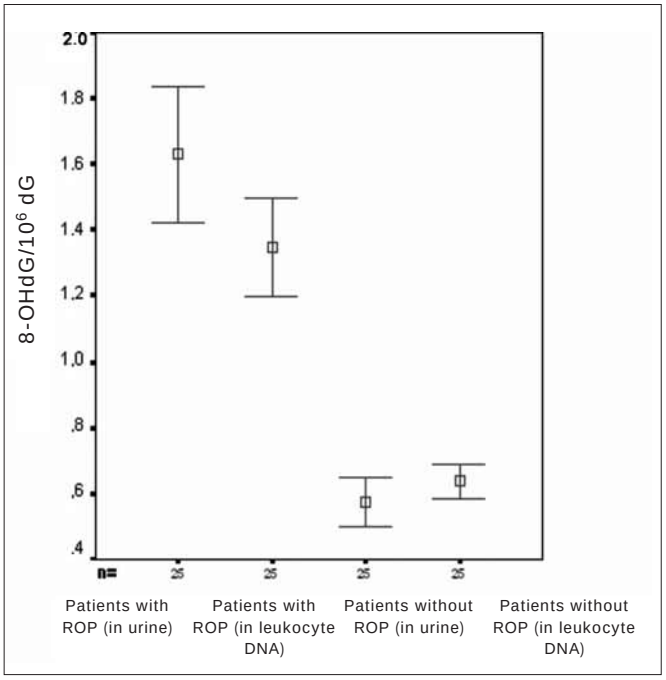

Fig. 1 - *8-OHdG/106 dG levels in leukocyte DNA and urine in patients with and without retinopathy of prematurity.*

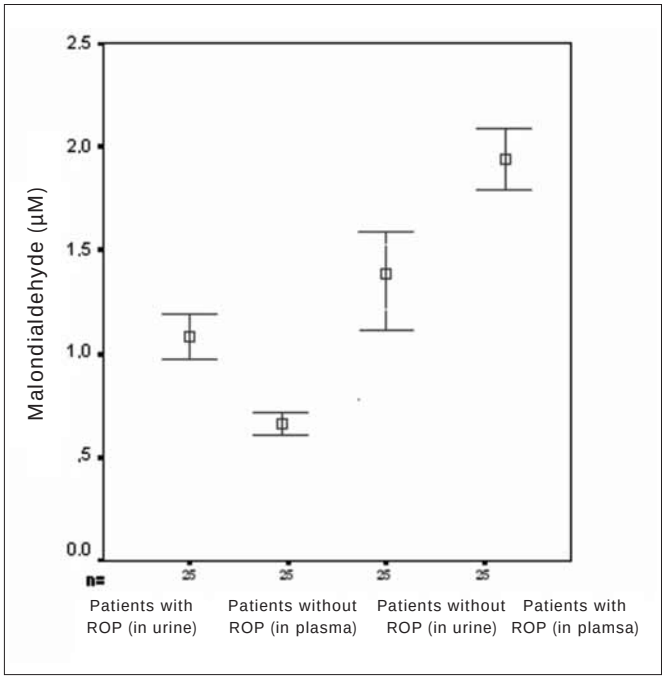

Fig. 2 - *Levels of malondialdehyde in urine and plasma of patients with and without retinopathy of prematurity.*

Analysis of plasma and urine for MDA by HPLC

MDA concentrations in blood plasma and urine samples were measured by HPLC with fluorescent detection (HPLC-FLD), as previously described (22). Briefly, 50 µL of plasma or urine sample was mixed in 0.44 M H_3PO_4 and 42 mM thiobarbituric acid (TBA), and incubated for 30 min in a boiling water bath. After rapidly cooling on ice, an equal volume alkaline methanol was added to the sample, and the sample was vortexed and centrifuged (3000 rpm for 3 min), and the aqueous layer was removed.

Next, a 20 µL aliquot of supernatant was analyzed by HPLC (HP, Agilent 1100 modular systems with FLD detector, Germany): Column, RP-C18 (5 µm, 4.6 × 150 mm, Eclipse VDB- C18, Agilent); elution, methanol (40:60, v/v) containing 50 mM KH_2PO_4 buffer (pH 6.8); flow rate, 0.8 mL/min. Fluorometric detection was performed with excitation at 527 nm and emission at 551 nm. The peak of the MDA-TBA adduct was calibrated with a 1,1,3,3-tetraethoxypropane standard solution treated in exactly the same manner as plasma and urine samples.

Statistical analysis

Statistical analyses were done using SPSS for Windows (version 11.5, SPSS Inc., Chicago, IL, USA). The statistical significance was calculated using the independent samples *t*-test. $p<0.05$ Was considered statistically significant. All the results were expressed as means with their standard deviations (mean ± SD).

RESULTS

The mean weights were 1100.45±103.97 g for patients with ROP and 1114.66±113.45 g for patients without ROP. The mean GA values were 29.21 weeks for patients with ROP and 31.32 weeks for patients without ROP. There were no significant differences in the two groups for the mean weights and GA values ($p>0.05$).

8-OHdG in leukocyte DNA and urine samples of patients with ROP ($1.38\pm0.36/10^6$ dG and $1.63\pm0.5/10^6$ dG, respectively) were higher than the patients without ROP ($0.57\pm 0.18/10^6$ dG and $0.63 \pm 0.12/10^6$ dG, respectively) ($p<0.001$) (Fig. 1). Mean levels of plasma and urine MDA

of patients with ROP (1.08±0.26 µM and 1.97±0.36 µM, respectively) were higher than the patients without ROP (00.64±0.13 µM and 1.38±0.46 µM, respectively) ($p<0.001$) (Fig. 2). We also confirmed that there was a significant correlation between levels of 8-OHdG in leukocyte DNA and plasma MDA ($r=0.859$, $p<0.001$), and between levels of urine 8-OHdG excretion and urine MDA ($r=0.563$, $p<0.001$).

DISCUSSION

The retina is one of the organs most directly exposed to high oxygen tension. Excessive retinal oxygenation may increase levels of ROS, and thus, may play a significant role in the pathogenesis of ROP (23, 24). ROS can attack double bonds in polyunsaturated fatty acids, inducing lipid peroxidation, which, in turn, results in more oxidative damage (25-27). ROS mediated oxidation of cell membrane lipids leads to the formation of lipid peroxidation products, such as MDA (28-30). In our study, we found high MDA levels in patients with ROP, compared to patients without ROP, and this is in accordance with previous studies (29, 30).

In this study, we also investigated the hypothesis that DNA damage could result from an oxidative damage inducing lipid peroxidation. DNA damage is well repaired by cellular enzymes. Thus, unlike the measurement of oxidation of other biomolecules, such as lipids or proteins, which are not repaired and/or have a slow turnover, the measurement of DNA damage clearly shows the level of oxidative damage (31). ROS cause strand breaks in DNA as well as base modifications including the oxidation of guanine residues to 8-OHdG. Therefore, 8-OHdG can be a sensitive biomarker of oxidative DNA damage (31).

Recently, significantly raised levels of 8-OHdG were reported to be associated with the lymphocytes of patients with Type I and Type II diabetes mellitus (DM), with diseases with inflammatory processes and aging (31-33), and following treatment with certain drugs and carcinogens (34, 35). We confirmed significantly higher levels of leukocyte 8-OHdG and urine 8-OHdG in patients with ROP than in patients without ROP as well as a significant positive correlation between leukocyte 8-OHdG levels and urine 8-OHdG excretion. These data suggest that 8-OHdG level is possibly a useful marker of oxidative DNA damage in patients with ROP.

Furthermore, in a recent study, Charissou et al (36) determined the relationship between cellular and genomic oxidative damage in freshwater bivalves under realistic field conditions of exposure. They showed that oxidative damage leading to lipid peroxidation can be accompanied by an increase in oxidative DNA damage. Matsubasa et al (37) measured 8-OHdG and estimated the degree of oxidative damage in very low birth weight infants (VLBW). They also examined if there was any link between administered oxygen and oxidative damage. The authors concluded that, as body weights at sampling increased, the average levels of urine 8-OHdG decreased. Also, as postconceptional age at sampling increased, the average levels of 8-OHdG decreased. The authors also implied that the more premature the infants were, the more intense was the oxidative damage; hence, it is the prematurity rather than the administered oxygen which causes oxidative damage in VLBW infants.

Oxidative DNA damage, in terms of 8-OHdG, was evaluated in the eyes of glaucoma patients. It was found that levels of 8-OHdG were significantly higher in glaucoma patients than in controls and it was concluded that oxidative damage may represent an important pathogenetic step in primary open-angle glaucoma. This was because it could induce human trabecular meshwork degeneration, favoring an intraocular pressure increase, thus priming the glaucoma pathogenetic cascade (38).

In a more recent study, oxidative damage parameters were measured in type 2 DM, with or without retinopathy, to investigate the relationship between oxidative damage and patients with type 2 diabetic retinopathy. The authors suggested that the concentrations of MDA and 8-OHdG in type 2 DM were significantly higher than in the control subjects, and the concentration of MDA and 8-OHdG in patients with retinopathy was significantly elevated in comparison with patients with diabetes without retinopathy. Their results indicated that severe lipid peroxidation, protein oxidation, and oxidative DNA damage occurred in type 2 diabetics, and that oxidative damage in diabetes may be speculated to contribute to the pathogenesis of DM. Oxidative damage may also play an important role in the development of diabetic retinopathy (39).

In our study, we found an overall elevation of leukocyte DNA and urine 8-OHdG in patients with ROP. Strikingly, it was correlated with our other marker; namely, higher MDA concentrations also were found in patients with ROP. This is the first report of a direct association between oxidative DNA damage and ROP.

Thus, it can be said that 8-OHdG in leukocyte DNA and urine serves as a useful biomarker for the evaluation of oxidative damage in patients with ROP.

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