

THE ROLE OF FREE-RADICAL OXIDATION IN THE INITIATION OF APOPTOTIC BLOOD LEUKOCYTES DEATH IN THE DYNAMICS OF EXPERIMENTAL MECHANICAL NON-PENETRATING CORNEAL INJURY

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Received 27 Dec 2018, Accepted 05 Febr 2019

<https://doi.org/10.31688/ABMU.2019.54.1.02>

ABSTRACT

The objective of the study was to investigate the features of free-radical oxidation and apoptotic leukocyte death in the dynamics of mechanical non-penetrating corneal injury in rabbits.

Material and methods. The peculiar features of free-radical oxidation (the content of TBA-reactants and Schiff bases) in blood and aqueous humor were determined using biochemical methods. The analysis of blood leukocytes samples to determine reactive oxygen species was evaluated by the flow laser cytometry method, using 2,7-dichlorodihydrofluorescein diacetate. The percentage of apoptotic blood leukocytes was evaluated by the flow laser cytometry method, using specific kit.

Results. In case of experimental non-penetrating mechanical corneal injury, the percentages of reactive oxygen species and spontaneous apoptosis of blood leukocytes increased significantly from the 3rd day of

RÉSUMÉ

Le rôle de l'oxydation radicale libre dans l'initiation de la mort de leucocytes sanguins apoptotiques dans la dynamique de la blessure cornéenne mécanique expérimentale non pénétrante chez le lapin

L'objectif de l'étude. Cette étude vise à déterminer l'importance des processus d'oxydation radicalaire dans l'initiation de la mort des leucocytes apoptotiques dans la dynamique des lésions cornéennes mécaniques expérimentales non pénétrantes chez le lapin.

Méthodes. Pour étudier les caractéristiques particulières de l'oxydation radicalaire, nous avons déterminé la teneur en réactifs TBA et en bases de Schiff dans le sang et l'humeur aqueuse à l'aide de méthodes biochimiques. L'analyse des échantillons des cellules pour déterminer les espèces d'oxygène réactives a été évaluée par la méthode de la cytométrie au laser en flux, en utilisant du diacétate de 2,7-dichlorodihydrofluorescéine.

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the experiment, with the highest values within 14th and 21st days of observation. Also, the intensification of lipid peroxidation (increased content of TBA-reactants and the Schiff bases) in blood and aqueous humor, with a maximal increase on the 14th day of observation ($p < 0.05$), has been observed. The strongest correlations between the percentage of apoptotic blood leukocytes, the content of TBA-reactants and the percentage of ROS were observed within the 7th-14th days of the experiment.

Conclusions. One of the important signaling pathway of blood leukocytes apoptosis triggering in case of mechanical non-penetrating corneal injury in rabbits is reactive oxygen species overproduction and possible disruption of the mitochondrial inner membrane (direct relationship between the increase of TBA-reactants, reactive oxygen species and percentage of annexin-positive blood leukocytes ($r=0.99$; $p < 0.05$)).

Keywords: corneal injury, rabbits, leukocytes, reactive oxygen species, apoptosis.

INTRODUCTION

Vision is one of the most valued and powerful senses. Intact binocular vision plays an important role in development, independence, quality of life, and personal safety. Ocular injury is a frequent and preventable cause of visual impairment all over the world, with significant socio-economic consequences^{1,2}. It has been shown that, globally, approximately 1.6 million people are blind from ocular trauma, with 2.3 million having binocular visual impairment, while 19 million have unocular visual loss, thus making ocular trauma the most common cause of unilateral blindness, globally³. The lifetime prevalence of any type of ocular trauma approaches 19.8%¹. Males are more often affected than females, at a rate of approx. 4:1, and suffer injury at a younger age¹. About 2 million cases of eye injuries occur annually in the United States, resulting in 40,000 patients suffering from permanent vision impairment⁴.

According to Krasnovyd, the socio-economic importance of this problem has been increasing due to the fact that the main contingent of patients with

Le pourcentage de leucocytes apoptotiques a été évalué par la méthode de la cytométrie au laser en flux, en utilisant un kit spécifique.

Résultats. En cas de lésion cornéenne mécanique expérimentale non pénétrante, les pourcents d'espèces réactives de l'oxygène et l'apoptose spontanée des leucocytes sanguins ont augmenté de manière significative à partir du troisième jour de l'expérience, les valeurs les plus élevées ayant été observées 14 et 21 jours après l'observation. L'intensification de la peroxydation lipidique (teneur accrue en réactifs TBA et bases de Schiff) dans le sang et l'humeur aqueuse avec une augmentation maximale le 14^{ème} jour d'observation ($p < 0.05$) a été établie. Les corrélations les plus fortes entre le pourcentage de leucocytes sanguins apoptotiques, la teneur en réactifs TBA et le pourcentage de ROS ont été observés de 7 à 14 jours après l'expérience.

Conclusion: l'une des voies de signalisation importantes de l'apoptose des leucocytes sanguins se déclenchant en cas de lésion cornéenne non pénétrante chez le lapin est la surproduction de l'oxygène réactif et la perturbation possible de la membrane interne mitochondriale (relation directe entre l'augmentation de TBA-réactifs, des espèces réactives de l'oxygène et le pourcentage de leucocytes sanguins positifs à l'annexine ($r=0,99$; $p < 0.05$)).

Mots-clés: lésion de la cornée, lapins, leucocytes, espèces réactives de l'oxygène, apoptose.

traumatic eye injury are mostly young men of the best working age⁵. About half a million people in the world are blind and in 30-40% of them monocular vision is associated with mechanical eye injury^{6,7}.

Among eye injuries, corneal damage is one of the leading causes of blindness worldwide⁸. The corneal endothelial monolayer of cells is the primary contributor to the maintenance of corneal transparency. When the barrier functions of the endothelium are compromised, it results a loss of visual acuity. Unfortunately, once there is damage to the cornea, the reverse of this process is complicated⁹.

The researchers have studied the prevalence of eye injury in the United States, as well as in the UK, Sweden, Greece, Russia and other countries, but we have not found in the literature any statistical data on the prevalence of non-penetrating mechanical eye injury in Ukraine. From the pathophysiological point of view, such a high incidence of eye injury necessitates the study of disease development and progression mechanisms, to work out the effective methods of conservative and surgical treatment.

Reactive oxygen (superoxide anion, hydrogen peroxide and hydroxyl radicals) or nitrogen (nitric oxide) species are products of aerobic metabolism, which in low concentrations act as signaling molecules that maintain physiological functions, such as: enzyme activity, protein kinase activity, activation of transcription factors, immune defense, inflammation-related factor production or apoptosis¹⁰⁻¹³. An overproduction of intracellular reactive oxygen species (ROS) damages DNA, lipids and proteins and is involved in disease development¹⁴. It is established that development of many eye diseases, in particular cataracts, glaucoma, retina degeneration, is associated with formation of ROS by mitochondria¹⁵. In this case, the cornea, being a main barrier between the environment and eye tissues, is very sensitive to free-radical oxidation¹⁶.

THE OBJECTIVE OF THE STUDY was to find out the significance of free-radical oxidation processes in the initiation of apoptotic leukocyte death in dynamics of experimental mechanical non-penetrating corneal injury.

MATERIALS AND METHODS

Experimental studies were carried out using 50 mature rabbits of the Chinchilla breed, of 2.5-3.0 kg body weight, that were housed at 25 ± 3 °C and humidity of $55 \pm 2\%$, under a constant 12 h light and dark cycle. Water was available ad libitum.

The animals were randomly divided into five groups: the control group – intact animals (n=10); the first experimental group – the follow-up period in 3 days after the injury (n=10); the second experimental group – in 7 days after the injury (n=10); third experimental group – in 14 days after the injury (n=10); the fourth experimental group – the follow-up period in 21 days after the injury (n=10).

The experimental model of corneal injury was simulated on both eyes of the animals under local epibulbar anesthesia with 0.5% proxymetacaine hydrochloride and retrobulbar anesthesia with 2.0% lidocaine hydrochloride. A concentric epithelial incision was performed in the upper half of cornea with a 7 mm corneal trephine; within the incision, the epithelium, as well as the anterior layer of corneal stroma, were removed with a disposable ophthalmologic scalpel (a flap was made of up to 0.2 mm in width). The control of cornea ulcer simulation was performed by the method of cornea coloring with 0.5% solution of fluorescein.

In the presence of thiopental-sodium anesthesia (25 mg/kg), blood samples were drawn from the rabbits' ear marginal vein. The aqueous humor of eyeball

was taken under aseptic conditions by puncture of limbal part of cornea with a sterile needle, attached to an insulin syringe, in the volume of 0.25-0.3 ml from each eye¹⁷. Each animal experienced single humor drawing. In the 3rd, 7th, 14th and 21th days, euthanasia was performed for rabbits by administration of sodium thiopental, 90 mg/kg of the animal weight, in accordance with the requirements of the Animal Care Committee.

Blood serum, erythrocytes and leukocytes suspensions, aqueous humor were investigated.

To study the peculiar features of free-radical oxidation in dynamics of experimental mechanical non-penetrating eye injury, the content of TBA-reactants (TBA-reactive substances) was determined spectrophotometrically at 532 nm, by the reaction between malonic dialdehyde and thiobarbituric acid, with forming a colored trimethine complex¹⁸; the Schiff bases (SB) were extracted with a mixture of chloroform-methanol, followed by spectrofluorometric determination at wavelength of 360 nm and 430 nm¹⁹.

Analysis of cell samples, to determine ROS (hydrogen peroxide) of leukocytes, was performed by the flow laser cytometry method, by means of flow cytometer Epics XL (Beckman Coulter, USA), using 2.7-dichlorodihydrofluorescein diacetate. The studied parameters were presented in percentages (the ratio of cells with ROS overproduction to general cell count $\times 100\%$).

To determine the level of apoptosis, the blood leukocyte suspension was re-suspended in a pre-diluted (1:10) binding buffer, by the kit of reagents ANNEXIN V FITC (Beckman Coulter, USA). The number of cells was counted by Goryaev chamber, under the microscope, with regard to the number of $1:10^6$ cells/mL. Then, 100 μ L of cell suspension was taken to a test tube and 1 μ L of annexin V-FITC solution, as well as 5 μ L of dissolved PI, were added. Cells were mixed and incubated in binding buffer at room temperature. We used unlabeled cells as a negative control. The determination of apoptosis was conducted using the flow cytometer Epics XL (Beckman Coulter, USA), by means of a software system. Results are expressed as the percentage of cells which have attached FITC Annexin V or PI. The cells were alive when they were FITC Annexin V and PI negative. The cells were apoptotic when they were FITC Annexin V positive and PI negative. The cells were in a stage of irreversible apoptosis (necrosis) when they were FITC Annexin V positive and PI positive.

Statistical processing of digital data was conducted using additional software Excel (Microsoft, USA) and STATISTICA 6.0. (Statsoft, USA), by parametric methods of attained data evaluation. The sample means (M), its dispersion and average error (m) were

Table 1. The percentage of reactive oxygen species of rabbit blood leukocytes in the dynamics of mechanical non-penetrating corneal injury (M±m).

Index	Control group	1 st experimental group	2 nd experimental group	3 rd experimental group	4 th experimental group
ROS, %	22.07±0.90	41.59±0.72 p ₁ <0.05 p ₂ <0.05	40.51±1.18 p ₁ <0.05 p ₂ >0.05	70.86±1.44 p ₁ <0.05 p ₂ <0.05	71.62±1.06 p ₁ <0.05 p ₂ >0.05

Notes:

p₁ - significant difference compared to the control animals;p₂ - significant difference compared to the injured animals.

assessed for all parameters. The statistically significant difference of independent quantitative values was evaluated by Student criterion.

Correlation analysis was performed by Pearson's method. Coefficient of linear correlation (r) and its reliability (p) were calculated; that was accordingly denoted in the tables (correlation matrices). If the index r=0 the linkage was considered as absent, in the range 0-0.29 - the linkage was considered as weak correlation, interval of index 0.30-0.69 described linkage as medium strength and interval 0.70-1.00 pointed to strong correlation interaction. The correlation coefficient was significant at p<0.05.

RESULTS

Our study proved that in the 1st experimental group there was a significant increase in the percentage of ROS of blood leukocytes by 88.45%, compared to the control data; such a high level of oxygen radicals was evidenced during 21 days of the follow-up (p<0.05) (Table 1). Thus, in the 2nd experimental group, the percentage of ROS increased by 83.55% compared with the norm, but was nearly the same vs data of the previous group; though in the 3rd group, the oxygen radicals increased by 74.9% compared to the results of the 2nd group. In 20 days after the modelling of a mechanical non-penetrating corneal injury, the ROS level increased significantly 3.25 times compared to the control group data (p<0.05), however, no dynamic changes were evidenced on the 14th day.

At each stage of free-radical peroxidation the products were formed, according to their level, the intensity of the development of this process in tissues of the body and the degree of their possible damage can be determined. As can be seen in Table 2, the content of TBA-reactive substances in the supernatant of erythrocyte hemolysates increased significantly during all follow-up period, in the presence of experimental mechanical non-penetrating eye injury compared to the control: in the 1st experimental group - by 36.14%, in the 2nd - by 26.78%, in the 3rd - by 46.32% and in the 4th - by 39.14%. Analyzing

the alterations of this index in the blood during 21 days, its wave-like changes were established. Thus, on the 7th day, the content of TBA-reactive substances decreased by 6.87% compared to that on the 3rd day, followed by an increase by 15.42% on the 14th day, compared to the 2nd experimental group, and a further decrease by 4.91% in the 4th group, compared with the previous data (p<0.05). The same changes of this index were revealed in the aqueous humor of anterior chamber of the eye: the level of TBA-reactive substances was higher in all experimental groups compared to the control (p<0.05), with a wave-like increase on the 3rd and 14th days and decrease on the 7th and 21st days of the experiment (Table 2).

The intensification of the processes of free-radical oxidation was proved by the increase in the content of the Schiff bases (SB) in biological fluids compared to the data of the control (Table 2). Thus, in blood serum of the animals with simulated corneal injury in the 1st experimental group, the SB content increased by 98.31%, in the 2nd - by 65.73%, in the 3rd - by 175.84%, and in the 4th - by 107.30%, compared with the intact animals (p<0.05). Analyzing the alterations of this index in blood during 21 days, its wave-like changes were established. Thus, on the 7th day, the TBA-reactive substances content decreased by 16.43% compared with that on the 3rd day, followed by the increase on the 14th day and the decrease by 24.85% in 20 days compared with the previous data (p<0.05). The same changes of this index were revealed in the aqueous humor of anterior chamber of the eye: the level of SB was higher in all experimental groups compared to the control (p<0.05) with a wave-like increase on the 3rd and 14th days and decrease on the 7th and 21st days of the experiment (Table 2).

The next stage of our study was the investigation of spontaneous apoptosis of leukocytes in cases of mechanical corneal injury. It was established that the percentage of cells with signs of apoptotic death wave-like was increasing during 20 days of the follow-up. Thus, in the 1st research group, the percentage of cells with apoptosis increased 1.59 times, in

Table 2. The indices of peroxidation in the dynamics of mechanical non-penetrating corneal injury (M±m).

Index	Control group	1 st experimental group	2 nd experimental group	3 rd experimental group	4 th experimental group
Erythrocytes hemolysate					
TBA-reactive substances, μmol/dm ³	5.79±0.03	7.89±0.01 p ₁ <0.05	7.34±0.03 p ₁ <0.05 p ₂ <0.05	8.48±0.02 p ₁ <0.05 p ₂ <0.05	8.06±0.03 p ₁ <0.05 p ₂ <0.05
Blood serum					
Schiff bases, U/mL	0.83±0.02	1.12±0.01 p ₁ <0.05	0.92±0.01 p ₁ <0.05 p ₂ <0.05	1.48±0.02 p ₁ <0.05 p ₂ <0.05	1.13±0.01 p ₁ <0.05 p ₂ <0.05
Aqueous humor					
TBA-reactive substances, μmol/dm ³	0.61±0.01	0.90±0.01 p ₁ <0.05	0.81±0.01 p ₁ <0.05 p ₂ <0.05	1.01±0.01 p ₁ <0.05 p ₂ <0.05	0.95±0.01 p ₁ <0.05 p ₂ <0.05
Schiff bases, U/mL	0.18±0.01	0.35±0.01 p ₁ <0.05	0.30±0.01 p ₁ <0.05 p ₂ <0.05	0.49±0.02 p ₁ <0.05 p ₂ <0.05	0.37±0.01 p ₁ <0.05 p ₂ <0.05

Notes:

p₁ - significant difference compared to the control animals;

p₂ - significant difference compared to the injured animals.

Table 3. The indices of spontaneous leukocyte apoptosis in rabbits in the dynamics of mechanical non-penetrating corneal injury (M±2).

Index	Control group (n=10)	1 st experimental group (n=10)	2 nd experimental group (n=10)	3 rd experimental group (n=10)	4 th experimental group (n=10)
Spontaneous apoptosis, %	2.50±0.28	3.99±0.25 p ₁ <0.05 p ₂ <0.05	4.60±0.22 p ₁ <0.05 p ₂ >0.05	6.26±0.25 p ₁ <0.05 p ₂ <0.05	6.72±0.24 p ₁ <0.05 p ₂ >0.05

Notes:

p₁ - significant difference compared to the control animals;

p₂ - significant difference compared to the injured animals.

the 2nd - 1.84 times, in the 3rd - 2.50 times and in the 4th - 2.68 times compared to the control (Table 3). Comparing the changes of the studied parameters, their semi-directional changes with the peak on the 3rd and the 14th days should be noted. Analysing these changes, we can confirm the significance of oxidative stress in apoptotic cell death.

Taking into account that the intensification of free-radical oxidation reactions was one of the manifestations of toxic action of oxygen metabolites, that manifested as the increase of TBA-reactive substances level, we compared the attained results of TBA-reactants and ROS and established a significant correlation between these indices (Table 4). It is worth noting that the strongest correlations between the percentage of early apoptosis and the indices of TBA-reactants and ROS were observed within 7-14 days of the experiment. This proved that during this period, the apoptotic changes in blood leukocytes initiated free-radical processes that were significantly activated.

DISCUSSION

It is established that physiologically ROS are formed constantly in a small amount and are inactivated by the cell using the antioxidant system. Therefore, these oxygen radicals do not cause cell damage. In case of experimental mechanical non-penetrating corneal injury, the generation of ROS in blood leukocytes exceeds the protective capabilities of the cell that, according to the literature, causes damage of mitochondria, depletion of ATP and activation of lysosomes enzymes, which leads to the destruction of the cell²⁰. The results of our study proved a slow-down in the formation of ROS on the 7th and 21st days that may take place due to the activation of antioxidant defense enzymes (catalase, peroxidase, superoxide dismutase), resulting in a slow-down in the production of ROS in the cell, as well as due to disorder of esterase activity inside the cell. In addition, the increase of recycling of NADPH/NADP together

Table 4. The correlations between the spontaneous apoptosis, TBA-reactants and ROS in dynamics of experimental non-penetrating mechanical corneal injury.

Pairs of correlation relationships		Experimental groups	Correlation coefficient, r_{xy}	Probability of correlation, p
Content of TBA-reactants, $\mu\text{mol}/\text{dm}^3$	Annexin-positive blood leukocytes, %	1	0.76	<0.05
		2	0.99	<0.05
		3	0.80	<0.05
		4	0.77	<0.05
ROS, %	Annexin-positive leukocytes, %	1	0.79	<0.05
		2	0.99	<0.05
		3	0.97	<0.05
		4	0.76	<0.05
ROS, %	Content of TBA-reactants, $\mu\text{mol}/\text{dm}^3$	1	0.68	<0.05
		2	0.99	<0.05
		3	0.74	<0.05
		4	0.92	<0.05

with NADP-oxidase in the presented experimental settings reduces cytosolic concentration of ROS²¹.

The ROS initiate the chain reaction of lipid peroxidation, that causes disturbances of microcirculation and metabolic processes in the tissues of the affected organ, which can eventually lead to tissue degradation^{22,23}. In the enhancement of free-radical oxidation processes, in case of experimental mechanical non-penetrating corneal injury, the activation of leukocytes capable of producing the excess of superoxide anion, which is the main precursor of free-radical compounds, can be crucial. At the same time, the propensity to generate free radicals by blood leukocytes causes the increase of lipid peroxidation processes in serum and erythrocytes, which contact with the activated leukocytes. The source of these cells in cases of experimental mechanical non-penetrating corneal injury can be a recirculating pool of leukocytes activated in the tissues of eye due to antigenic, stress and mechanical loading. Fixing and binding on the plasma membrane of target cells, immunoreactive complexes provoke restructuring of membrane phospholipids, generation of oxygen radicals, and enhancement of lipid peroxidation processes.

It has been established that in cases of mechanical non-penetrating corneal injury, the damage of blood-ocular barrier contributes to changes of aqueous humor and blood properties. By comparing the alterations in the dynamics of changes of lipid peroxidation products in blood and aqueous humor, we have established their semi-directional changes with significantly higher rates in aqueous humor. Our research has proved that, in the presence of the modeled injury, aqueous humor is a source of oxidants that activate the processes of lipid peroxidation.

TBA-reactants and Schiff bases are integral indices, which depend on the severity of injury and systemic reaction of the body to it.

There are data that in cases of experimental non-penetrating mechanical corneal injury, mitochondria are crucial in implementation of apoptotic program in the cell, by reducing the transmembrane potential and hyperproduction of reactive oxygen species^{24,25}. Thereby, we suggest that signaling pathway of apoptosis initiation in blood leukocytes in case of experimental non-penetrating mechanical corneal injury is mitochondria-mediated (direct correlation between the increase of TBA-reactants ($r=0.99$; $p<0.05$), ROS ($r=0.99$; $p<0.05$) and percentage of annexin-positive blood leukocytes).

CONCLUSIONS

In case of experimental non-penetrating mechanical corneal injury, the percentages of reactive oxygen species and spontaneous apoptosis of blood leukocytes increased significantly in all experimental groups to the utmost on the 3rd and 14th days.

In the dynamics of experimental non-penetrating corneal injury modeling, intensification of lipid peroxidation (increased levels of TBA-reactants and the Schiff bases) in blood and aqueous humor, with a maximal increase on the 14th day ($p<0.05$), has been established.

The main signaling pathway of blood leukocytes apoptosis initiation in case of experimental mechanical non-penetrating corneal injury is reactive oxygen species overproduction and possible disruption of the mitochondrial inner membrane (direct relationship between the increase of TBA-reactants, reactive

oxygen species and percentage of annexin-positive blood leukocytes ($r=0.99$; $p<0.05$)).

Compliance with Ethics Requirements:

„The authors declare no conflict of interest regarding this article“

„The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law.“

„All institutional and national guidelines for the care and use of laboratory animals were followed“

„No funding for this study“

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