

Original article:

Features of leukocytes' apoptosis and emoxypine succinate efficacy in case of combined trauma of the chest and both thighs in rats

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Abstract:

Objective: This study aims to establish features of blood leukocytes' apoptosis and substantiate the efficacy of emoxypine succinate applying in case of combined trauma of the chest and both thighs in rats.

Materials and Methods: Analysis of cell samples to determine reactive oxygen species was evaluated by the flow laser cytometry method, using 2.7-dichlorodihydrofluorescein diacetate (Sigma Aldrich, Germany). The number of leukocytes with low mitochondrial transmembrane potential was evaluated by the flow laser cytometry method, using a kit of reagents «MitoScreen» («BD Pharmingen», USA). The number of apoptotic leukocytes was evaluated by the flow laser cytometry method, using a kit of reagents «ANNEXIN V FITC» («Beckman Coulter», USA). Emoxypine succinate to animals was injected intraperitoneally 1 time per day during 14 days from the first day of experiment in the dosage of 40 mg/kg.

Results and Discussion: It was established the progressive, statistically significant increasing of Annexin V- positive cells percentage from the first day of the combined trauma of the chest and both thighs in rats with the highest values within 7-14 days of observation. On 28 day of experiment the reduction of apoptotic white blood cells percentage by 7.7% than the findings on 14 day was observed, but it remained 33.3% higher than control. The analysis of data in case of emoxypine succinate applying indicates that production of reactive oxygen species by leukocytes began to decline after 3 days of experiment and continued to decrease with maximum of action on 7 day. On 28 day of experiment the production of reactive oxygen species by leukocytes has decreased by 39.8 %; the percentage of leukocytes with low transmembrane potential has decreased by 34.6 % vs rats without medical treatment. At the same time the dynamics of FITC Annexin V- positive leukocytes changes in case of combined trauma of the chest and both thighs in rats and emoxypine succinate applying on 28 day of experiment has decreased by 16.7 % vs rats without medical treatment. **Conclusion:** One of important signaling pathways of apoptosis triggering in case of experimental combined trauma of the chest and both thighs is reactive oxygen species overproduction and disruption of the mitochondrial inner membrane due to the decreasing of transmembrane potential in 3-7 days of observation. Emoxypine succinate applying in post-traumatic period has a positive effect, characterized by decreasing of the production of reactive oxygen species, the percentage of leukocyte with low mitochondrial transmembrane potential and the percentage of FITC Annexin V- positive cells of leukocyte suspension. But the dynamics of FITC Annexin V- positive leukocytes changes leads us to believe that in the initiation and implementation of cell death in case of combined trauma apart mitochondrial, there are other mechanisms.

Keywords: combined trauma; rats; apoptosis; emoxypine succinate.

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Introduction:

Structural and functional alteration of mitochondria plays an important role in the pathogenesis of many diseases, since mitochondria, as a semiautonomous organelle in cells, apart from containing their own genetic material, play an important role in energy metabolism, oxidation-reduction balance and calcium homeostasis, pathways of mitochondria-mediated or receptor-independent apoptosis, provide mechanisms of signal transduction in the control of nuclear function¹⁻⁶.

The molecular mechanisms of apoptosis involve several pathways and activation of caspases, a family of cysteine proteases, represents a common event for several pro-apoptotic stimuli⁷. Regarding on the characterisation of the events upstream from caspase activation, mitochondrial damage has been reported to trigger this process. Consistent with this hypothesis, anti-apoptotic proteins such as Bcl-2 are located in the mitochondria, suggesting a role for this organelle in the induction of apoptotic death. Moreover, the release of mitochondrial proapoptotic factors, such as cytochrome c, is blocked by Bcl-2^{8,9}. Excessive mitochondrial Ca²⁺ uptake is of particular importance in the brain, heart and muscle, where prolonged unphysiological increases in Ca²⁺ influx, especially when combined with oxidative stress, may result in a pathological transformation – the opening of the mitochondrial permeability transition pore (mPTP) and induction of necrotic cell death^{3,4}.

Oxygen is used to support mitochondrial respiration which in turn is used to build a proton gradient across the expanded surface area of the cristae. It is the proton gradient, expressed largely as a mitochondrial transmembrane potential gradient, that then drives much of mitochondrial physiology – the synthesis of adenosine triphosphate (ATP), the transfer of calcium and other ion exchangers, and the import of proteins^{4,8}.

Studies have shown that in case of hypoxia free oxygen radicals have been accumulated and can damage polyunsaturated fatty acids of cell membranes. This process is accompanied by disruption of their bioelectrical activity^{10,11}.

The results of our previous studies point to the development of tissue hypoxia in early posttraumatic period in case of combined trauma of the chest and both thighs in rats. We also have shown the increasing of reactive oxygen species contents in the blood leukocyte suspension in 24 hours of posttraumatic

period with progressive dynamics¹². Analyzing the own results and published data we have suggested that damage of the cell plasma membrane is accompanied by their apoptotic death.

That's why we conducted this study to establish features of blood leukocytes' apoptosis and substantiate the efficacy of emoxypine succinate in case of combined trauma of the chest and both thighs in rats.

Materials and methods: Experimental studies were conducted on 104 male, nonliner, white rats of 200-210 g body weight, that were housed at 25±3 °C and humidity of 55±2 %, under a constant 12 h light and dark cycle. Water was available ad libitum.

The animals were randomly divided into 11 groups: 1 control (C) and 10 experimental (E1, E2, E3, E4, E5, E6, E7, E8, E9, E10): E1 - combined trauma of the chest and both thighs, 24 hours of observation; E2 - combined trauma of the chest and both thighs, 3 days of observation; E3 - combined trauma of the chest and both thighs, 7 days of observation; E4 - combined trauma of the chest and both thighs, 14 days of observation; E5 - combined trauma of the chest and both thighs, 28 days of observation; E6 - combined trauma of the chest and both thighs + emoxypine succinate, 24 hours of observation + emoxypine succinate; E7 - combined trauma of the chest and both thighs, 3 days of observation + emoxypine succinate; E8 - combined trauma of the chest and both thighs, 7 days of observation + emoxypine succinate; E9 - combined trauma of the chest and both thighs, 14 days of observation + emoxypine succinate; E10 - combined trauma of the chest and both thighs, 28 days of observation + emoxypine succinate.

Right sided closed pneumothorax with rib fracture, which was combined with a broken left and right femur, was modeled using a trocar to the animals of the experimental groups under the sodium thiopental anesthesia (40 mg/kg of the animal weight). Skeletal injury was modeled by applying a single dosed impact by specially designed device on each thigh, which caused a closed fracture¹³. Impact energy was 0.375 J, which corresponded to the injury of moderate severity. Combined injury was modeled by sequential application of the two injuries.

Experimental animal mortality: 11 rats.

Emoxypine succinate to animals was injected intraperitoneally 1 time per day during 14 days from the first day of experiment in the dosage of 40 mg/kg¹⁴.

In 24 hours, 3, 7, 14 and 28 days euthanasia was performed for rats by administration of sodium thiopental, 90 mg/kg of the animal weight in accordance with the requirements of the Animal Care Committee.

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

Analysis of cell samples to determine reactive oxygen species (ROS) was evaluated by the flow laser cytometry method on flow cytometer Epics XL (Beckman Coulter, USA), using 2,7-dichlorodihydrofluorescein diacetate. The value of the studied parameter was expressed as a percentage (intensity of luminescence per cell).

The number of cells with low transmembrane mitochondrial potential ($\Delta\psi$) was evaluated by the flow laser cytometry method, using a kit of reagents «MitoScreen» («BD Pharmingen», USA) on flow cytometer Epics XL (Beckman Coulter, USA).

All procedures were conducted according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986)¹⁵.

All of the data were processed using the software package Statistica 6.1 for Windows. Intergroup comparisons were performed using Mann–Whitney–

Wilcoxon U test. The median (Me) and interquartile range (IQR [Q25-Q75]) were deduced. Significance was accepted at $p < 0.05$. Correlation analysis was performed by Spearman 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 0.3-0.69 described linkage as medium strength and interval 0.7-1.0 pointed to strong correlation interaction. The correlation coefficient was significant at $p < 0.05$.

Ethical Clearance: The study has been approved by the Human Research Ethic Review Committee, I Danylo Halytskyi Lviv National Medical University, Lviv, Ukraine

Results and Discussion:

The analysis of data indicate that production of ROS by leukocytes in case of combined trauma of the chest and both thighs in rats has increased: in 24 hours - by 2.5 times, in 3 days - by 2.8 times, reaching a maximum in 7 days when this index was in 3.2 times higher vs control group (Table 1). Although production of ROS by leukocytes in subsequent periods of observation (14 and 28 days) had decreased by 11.0 % than the findings after 7 days, but remained in 2.8 times greater than control. The same trend is observed for changing the percentage of white blood cells with low mitochondrial transmembrane potential. Thus, the maximum value of the investigated index was detected after 7 days of the experiment, exceeding in 2.6 times the data of control group. In spite of the fact that after 28 days of experiment the percentage of white blood cells with low mitochondrial transmembrane potential was significantly lower comparing to data of second and third experimental groups, it exceeded twice control data.

To investigate the mechanism of apoptosis onset, the percentage of Annexin V-positive cells was determined. It was established the progressive, statistically significant increasing of Annexin V-positive cells percentage from the first day of the experiment with the highest values within 7-14 days of observation.

During this period, the investigated index was 44.4% higher than the data of the control group. On 28 day of experiment the reduction of apoptotic white blood cells percentage by 7.7% than the findings on 14 day was observed, but it remained 33.3% higher than control.

Table 1. The indices of mitochondrial apoptosis pathway in case of combined trauma of the chest and both thighs in rats (Me [Q25-Q75])

| Index | The percentage of reactive oxygen species of leukocyte suspension cells | The percentage of leukocytes with low mitochondrial transmembrane potential | The percentage of FITC Annexin V-positive leukocytes |
|------------------------------|---|---|--|
| Control group, (n=10) | 17.7 [17.4; 18.1] | 1.3 [1.2; 1.6] | 5.4 [5.1; 5.8] |
| Experimental group 1, (n=11) | 42.6* [41.9; 42.6] | 1.5* [1.4; 1.6] | 6.2* [6.1; 6.6] |
| | p≤0,001 | p≤0,001 | p=0,01 |
| Experimental group 2, (n=9) | 48.7* [46.1; 50.5] | 2.6* [2.5; 2.8] | 6.6* [6.4; 6.8] |
| | p≤0,001 | p≤0,001 | p≤0,001 |
| Experimental group 3, (n=10) | 56.5* [54.7; 57.8] | 3.5* [3.2; 3.9] | 7.8* [7.4; 8.1] |
| | p≤0,001 | p≤0,001 | p≥0,05 |
| Experimental group 4, (n=9) | 50.1* [47.5; 51.9] | 3.0* [2.8; 3.2] | 7.8* [7.5; 7.9] |
| | p≥0,05 | p≤0,01 | p≤0,001 |
| Experimental group 5, (n=10) | 50.5* [48.4; 51.8] | 2.6* [2.4; 2.7] | 7.2* [7.1; 7.2] |

Note: * - significant differences compared to control animals, p<0.05;
p - significant differences between the experimental groups.

Two known ways of apoptosis include internal or mitochondrial, involving protein family Bcl-2, cytochrome C and caspase - 9 and external with the activation of caspase - 8 linking a specific cell Fas receptor and soluble tumor necrosis factor receptors on the cell surface¹⁶, but our objective was to assess the impact of the mitochondrial pathway.

Analyzing the dynamics of the investigated parameters interdependence of changes can be seen. Thus, in 24 hours after combined trauma of the chest and both thighs in rats the production of free radicals increases, the source of which, in our opinion, is not only mitochondria. In 3 days of experiment bioelectric activity of mitochondrial membranes is disrupted under the influence of reactive oxygen species, leading to the decreasing of transmembrane potential. Accordingly, we consider that the most critical period in apoptotic destruction triggering in case of combined trauma is 7 day, when proapoptotic factors are going out into the cell and lead to its death. In 14 days of experiment the production of reactive oxygen species is stabilized, reducing negative impact on the mitochondria and the cell. Up to 28 day essential, protective functions of the organism are restored, which leads to reduction of negative

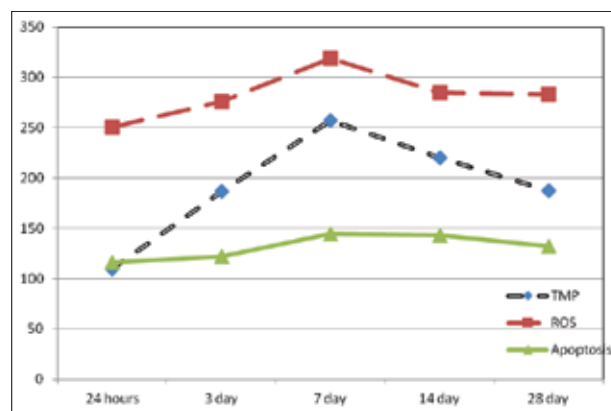


Figure 1. The dynamics of the mitochondrial apoptosis pathway in case of combined trauma of the chest and both thighs in rats.

influence of oxygen radicals on cells (Figure 1.).

Conducted correlative analysis showed statistically significant, medium strength linkage between ROS and the percentage of FITC Annexin V-positive cells in all periods of observation except 14 day (Table 2.). It should be noted that there is a significant interdependence between the percentage of cells with low transmembrane potential and apoptosis in 3 and 7 days after combined trauma of the chest and both thighs in rats.

Table 2: Correlative linkages between the percentage of FITC Annexin V- positive cells of leukocyte suspension with ROS and mitochondrial transmembrane potential in case of combined trauma of the chest and both thighs in rats (r_{xy})

| Index | The percentage of reactive oxygen species of leukocyte suspension cells | | | | |
|---|---|--------|--------|---------|---------|
| | 24 hours | 3 days | 7 days | 14 days | 28 days |
| The percentage of FITC Annexin V- positive leukocytes | 0.52* | 0.56* | 0.56* | 0.31 | 0.57* |
| | The percentage of leukocyte suspension cells with low mitochondrial transmembrane potential | | | | |
| | 24 hours | 3 days | 7 days | 14 days | 28 days |
| | 0.23 | 0.63* | 0.70* | 0.36 | 0.43 |

Note: * - significant differences of correlation coefficients, $p < 0.05$

Mitochondrial regulatory factors can be used as a specific targets for pharmacological effect. It is known that the effect of emoxypine succinate is associated with antioxidant activity and its ability to activate succinate dehydrogenase pathway of glucose oxidation, altering cellular metabolism to more oxygen saving direction of energy exchange¹⁷. Its chemical structure looks as follows: 2-ethyl-6-methyl-3-hydroxypyridine and resembles that of pyridoxine. On the other hand, it contains succinate as its component which functions in the organism as substrate for increasing the intracellular energy metabolism. Emoxypine succinate has a wide range of pharmacological effects realized on at least two levels - neuronal and vascular. It exercises anxiolytic, antistress, anialcohol, anticolvulant, neuroprotective, vegetotropic action. Besides, emoxypine succinate improves cerebral blood circulation, inhibits thrombocyte aggregation, lowers cholesterol levels, has cardioprotective and antiatherosclerotic action^{18,19}.

Zadnipyanyy I.V. and Satayeva T. P. note that excess of succinic acid causes monopolization of oxidation respiratory chain by succinate, resulting in fast resynthesis of ATP and increasing of antioxidant activity²⁰.

The analysis of data in case of combined trauma of the chest and both thighs in rats and emoxypine succinate applying indicate that production of ROS by leukocytes began to decline after 3 days of experiment and continued to decrease with maximum of action on 7 day (Table. 3). On 28 day of experiment the production of ROS by leukocytes has decreased by 39.8 % vs rats without medical treatment. The reduction of ROS production resulted in declining their negative impact on the mitochondrial membranes. Thus, the percentage of leukocytes with low transmembrane potential on 28

day of experiment has decreased by 34.6 % vs rats without medical treatment.

At the same time the dynamics of FITC Annexin V- positive leukocytes changes in case of combined trauma of the chest and both thighs in rats and emoxypine succinate applying (on 28 day of experiment has decreased by 16.7 % vs rats without medical treatment) leads us to believe that in the initiation and implementation of cell death in case of combined trauma apart mitochondrial, there are other mechanisms.

Summarizing the findings we can talk about the triggering of apoptotic cell death due to ROS overproduction and disruption of the mitochondrial inner membrane due to the decreasing of transmembrane potential in 3-7 days after combined injury of the chest and both thighs. This statement demonstrates the role of mitochondrial apoptotic death initiation pathway in case of experimental combined trauma of the chest and both thighs, but does not deny the importance of other signaling pathways of apoptosis triggering. It should be noted that in 14 days of observation mitochondrial apoptosis mechanisms are devalued, but increased percentage of FITC Annexin V- positive cells is remained.

So, in case of experimental combined trauma of the chest and thighs “vicious circle” have developed, which is associated with energy deficiency¹² and overproduction of free radicals on the background of hypoxia. Superoxide anion the most actively is produced by mitochondria. It has a very short half-life, but in presence of superoxide dismutase is transformed into oxygen and hydrogen peroxide, the level of which we have found via 2.7-dichlorodihydrofluorescein diacetate by flow cytometry. H_2O_2 itself is relatively inactive, but can lead to the formation of toxic hydroxyl radicals ($\bullet OH$). Henry J. H. Fenton showed that the formation of toxic hydroxyl radicals ($\bullet OH$) from hydrogen

Table 3. The indices of mitochondrial apoptosis pathway in case of combined trauma of the chest and both thighs in rats with emoxypine succinate applying
(Me [Q25-Q75])

| Index | The percentage of reactive oxygen species of leukocyte suspension cells | The percentage of leukocyte suspension cells with low mitochondrial transmembrane potential | The percentage of FITC Annexin V- positive cells of leukocyte suspension |
|---|---|---|--|
| Control group, (n=10) | 17.7 [17.4; 18.1] | 1.3 [1.2; 1.6] | 5.4 [5.1; 5.8] |
| Experimental group 6, (n=8) | 41.6* [41.3; 42.1] | 1,5 [1.4; 1.7] | 5,9* [5.8; 6.1] |
| | p≥0,05 | p≤0,01 | p≥0,05 |
| Experimental group 7, (n=6) | 41.9* [41.6; 42.2] | 2,0* [1.8; 2.1] | 6,1* [6.1; 6.2] |
| | p≤0,001 | p≤0,01 | p≤0,01 |
| Experimental group 8, (n=7) | 39.1* [38.8; 39.3] | 2.5* [2.3; 2.6] | 6.5* [6.3; 6.6] |
| | p≤0,001 | p≤0,01 | p≤0,05 |
| Experimental group 9, (n=7) | 35.7* [35.2; 37.4] | 2.1* [2.0; 2.2] | 6.1* [6.0; 6.2] |
| | p≤0,001 | p≤0,01 | p≥0,05 |
| Experimental group 10, (n=7) | 30.4* [30.0; 31.0] | 1.7* [1.6; 1.9] | 6.0* [5.8; 6.1] |
| Note: * - significant differences compared to control animals, p<0.05; p - significant differences between the experimental groups. | | | |

peroxide is catalyzed by iron ions, called the "Fenton reaction". Joseph Weiss and Fritz Haber discovered that $O_2\cdot$ can be converted into H_2O_2 and further to $\cdot OH$, called the Haber-Weiss reaction^{21, 22, 23}. $\cdot OH$ are strong oxidants of biopolymers and lipids, inducing processes of lipid peroxidation, disruption of membrane structures, leading to cell death and increasing of energy deficiency²⁴.

Emoxypinesuccinateduetohighpenetrativeproperties of emoxypine enters the cell cytosol and dissociates in two components, each of which acts independently. Emoxypine succinate effectively inhibits free radical oxidation of biomembrane lipids, reacts to peroxide radicals of lipids, primary and hydroxyl radical of peptides. It increases the activity of antioxidant enzymes, specifically that of superoxide dismutase, responsible for the formation and consumption of lipid peroxides and active oxygen forms; inhibits free radicals during the synthesis of prostaglandin catalyzed by cyclooxygenase and lipoxygenase, increases the correlation prostocyclin/ thromboxane A2 and blocks the leukotriene formation¹⁸. The advantage of emoxypine succinate compared to other

cytoprotectants is its ability to direct increasing of the energy production of mitochondria by improving the delivery and consumption of succinate in case of hypoxia, realization of rapid oxidation of succinic acid by succinate dehydrogenase phenomenon, activation of the mitochondrial respiratory chain, which leads to ATP resynthesis²⁵.

Conclusions: In post-traumatic period of combined trauma of the chest and both thighs in rats statistically significant overproduction of reactive oxygen species by blood leukocytes in 24 hours of experiment leads to disruption of mitochondrial membrane bioelectrical activity. This process is characterized by dynamic increasing of the percentage of leukocytes with low transmembrane potential with a maximum in 7 days (exceeding in 2.6 times the data of control group, p≤0,001). It was established the progressive, statistically significant increasing of the percentage of apoptotic leukocytes from the first day of the experiment with the highest values within 7-14 days of observation.

2. In case of experimental combined trauma of the chest and both thighs in rats we have established

statistically significant, medium strength linkage between reactive oxygen species and the percentage of FITC Annexin V- positive cells in all periods of observation except 14 day ($p \leq 0,05$); significant interdependence between the percentage of cells with low mitochondrial transmembrane potential and apoptosis in 3 days ($r_{xy} = 0,63$) and 7 days ($r_{xy} = 0,70$) of observation.

3. Emoxypine succinate applying in post-traumatic period of combined trauma of the chest and both thighs in rats has a positive effect, characterized by decreasing of the percentage of reactive oxygen species, the percentage of leukocyte with low mitochondrial transmembrane potential and the percentage of FITC Annexin V- positive cells of leukocyte suspension (effect of emoxypine succinate

appears after 3 days of observation with a maximum of action after 7 days).

Conflict of interest

All the authors declare no conflict of interest

Authors' Contribution:

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Data gathering: Marushchak M, Krynytska I, Holovatiuk L, Shkrobot L

Writing and submitting manuscript: Marushchak M, Sokhor N, Stepas J

Editing and approval of final draft: Marushchak M, Krynytska I

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