Mitochondrial and Nuclear DNA in Patients with Severe Polytrauma

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The components of mitochondria from the cells damaged by injury are a key component for the development of systemic inflammatory response syndrome (SIRS) under aseptic conditions. At the same time, there is a significant increase in the plasma level of mitochondrial DNA (mtDNA), which may be a prognostic marker for infectious complications in patients with severe polytrauma. *Objective:* to study the time course of changes in the serum levels of mtDNA and nuclear DNA (nDNA) in healthy individuals and patients with polytrauma and to reveal its possible association with the development of infectious pulmonary complications and with mortality. *Subjects and methods*. Seven healthy volunteers and 25 polytrauma with polytrauma of a mean injury severity score (ISS) of 40.2±9.2. Sixteen (64%) patients developed purulent tracheobronchitis and pneumonia; 5 (20%) patients died. The amount of mtDNA and nDNA was determined within the first at 12 and 24 hours, then on days 3 and 5–7 after injury by the authors' modified procedure using as the exogenous control of a circular DNA molecule. The content of mtDNA and nDNA was expressed as absolute values, by taking the arithmetic mean values as 100% for the volunteers. *Results*. There was a more than 2.5-fold increase in mtDNA levels in dead patients as compared to survivors ($p<0.05$); the differences in nDMA levels were insignificant ($p=0.1$). Within the first 12 hours, the mean mtDNA level in patients with pneumonia was 34 times greater than the reference values and continued to rise in the following 12 hours whereas in those without pneumonia, it was only 17 times higher with its further decrease in the comparable time periods. In the first 12 hours, nDNA was increased in both groups, but 24 hours after injury it was 2555 times more than the reference value only in patients with pneumonia whereas it was decreased 3-fold in those without this condition. *Conclusion*. This paper is the first to describe the time course of changes in the amount of mtDNA and nDNA with the new modification of using an external control DNA molecule in the serum of patients with polytrauma who developed infectious pulmonary complications and in patients without the latter. The findings point to the fact that it is advisable to measure mtDNA and nDNA in patients with polytrauma within the first 24 hours of hospital admission for the prediction of the development of infectious bronchopulmonary complications and for timely etiotropic therapy and that it is promising to conduct further investigations in this area. *Key words:* **polytrauma, infectious complications, pneumonia, mitochondrial DNA, nuclear DNA, PAMS, DAMPs, CCBO, predictor, mortality, injury severity score, mechanical ventilation.**

Infectious pulmonary complications that occur in polytrauma patients at early stages, are one of the major problems of modern medicine that results in the increasing the duration and cost of treatment of this category of patients in intensive care units (ICU), and are accompanied by a high mortality rate [1–3]. They take the first place among the polytrauma patients in the structure of all infectious complications they display [4] and occur in 25–65% of patients on average. Moreover, these complications are the primary cause of death in 50–70% of all deceased patients [5–7]. Despite significant progress achieved in diagnostics and treatment of polytrauma, this problem currently remains unsolved since many aspects have not been yet expanded upon: issues of prediction, early detection of the disease, pathogenesis and treatment of infectious complications in ICU.

Previously, bacterial infiltration from the ischemic intestinal tract into the blood flow considered as a cause of systemic inflammatory response syndrome (SIRS), but, later this hypothesis was reconsidered [8]. Patients normally die as a result of multiple organ failure primarily due to insufficient tissue blood supply, resultant oxidative stress and developing the inflammatory reactions after all. the inflammatory immune responses are carried out mainly by the innate immune system and are regulated by the neuroendocrine system [9, 10]. Such response starts from recognition of elements from microbes or damaged cells by some receptors presented in immunocompetent and other cells carrying structures generating a response on an antigen invasion. Consequently, specific signaling pathways are engaged to activate transcription factors and regulate gene expressionwhich invoke production of inflammatory signals. In addition, in blood plasma the complement system is activated which in concert with other systems enhance the inflammatory response. First initiating phase of inflammatory response induced by infection or tissue damage, associated with trauma is recognition of so called danger signals. There are two types of these signals – ones are those that are induced outside of the system and they include exogenous pathogens (pathogen-associated molecular patterns, PAMPs) while another are those that are represented by endogenous molecules aroused from the damaged host tissue (damage-associated molecular patterns, DAMPs) [11]. PAMPs are represented by lipopolisacharide of the outer membrane of gram-negative bacteria, their peptidoglycans and DNA, viral nucleic acids mannans of the yeast cell wall. PAPMs are specifically recognized and each binds to a proper receptor, in particular, to toll-like and nod-like receptors starting an inflammatory response. Besides these two, RAGE (receptors for advanced glyca-

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tion final products) can also bind DAMPs (especially associated with infection and oxidative stress) and regulate immune and inflammatory responses [12]. DAMPs released as a result of tissue damage include endogenous equivalents of PAMPs. They are represented by heat shock proteins, nucleosomes, interleukins, and proteins of a class S100. In addition, since recently DAMPs started to include mitochondrial elements.

Mitochondrias are essential cellular components of humans and animals, providing energy for cells and participating in numerous other processes. In terms of evolution, mitochondria has an origin of an ancient gram-negative bacteria that came into symbiosis with eukaryotes cell, that's why few mitochondrial elements including mitochondrial DNA (mtDNA) and some proteins in many aspects in many features resemble bacterial ancestor and contrary to the common rule of banning the reaction to the host organism elements activated inflammatory response In recent experiments it has been found that mitochondrial components from cells damaged in the injury were able to activate named receptors and fire an inflammatory response. The response proceeded under sterile conditions, although resembled bacterial sepsis which implied that these mitochondrial components were the key elements of SIRS under aseptic conditions, and mtDNA level significantly increases in the plasma of trauma patients [13—15].

It should be noted that the mtDNA level also increases in the blood plasma in some mitochondrial pathologies as well as in patients who take reverse transcriptase inhibitors during antiretroviral therapy [16]. Furthermore, the level of free circulating mtDNA can be increased in case of certain malignant neoplasms, e.g. solid tumors [17].

Hauser and coauthors were succeeded in identification of two mitochondrial components which could activate receptorscapable to recognize PAMP or DAMP: proteins with N-formylmethionine at the N-terminus, typical for bacterial proteins and mitochondrial DNA [13, 14]. Therefore the increase of the mtDNA concentration in the blood of patients with an injury might serve as a potential marker to predict consequences of injury. Moreover, it was shown that free circulating nucleic acids can be identified in the plasma of patients with an injury while the mtDNA level correlates with the severity of injuries and mortality [18].

For the first time, the increase in the number of extracellular DNA copies in the serum of patients with injuries of mild and moderate severity was described by Lo and coauthors in 2000 [19]. The main idea which explains the appearance of extracellular DNA in the blood of patients

Table 1

Characteristics of patients

	Patients, *n* (%)
Total	25
Deceased	5
Mortality rate	20%
Gender	
Male	75%
Female	25%
Age	42,6±17,4 years
Period of stay in Intensive Care Unit	
Average stay	8,3
Kind of injury	
Traffic accident	44%
Fall from height	36%
Train injury	8%
Home accident	4%
Work-connected injury	4%
Injury under unclear circumstances	4%
Tracheostomy	
Tracheostomy performed	11 (44%)
Without tracheostomy	14 (56%)
Mechanical ventilation (MV)	
No MV	2
1 day	5
2 days	4
3 days	3
Over 3 days	11
ISS scale, <30	2 (8%)
ISS scale, ⩾30	23 (92%)
Nosocomial pneumonia	16 (64%)
Without pneumonia	9 (36%)
Specimen collection, hrs after injury	
Up to 12 hours	16
12—24 hours	12
3rd day	15
5—7th day	12

Table 2

Sequences of primers for real-time PCR and basic characteristics of PCR products

Primer's name	Nucleotide sequence of primers, from 5'to 3'	Specificity	PCR product length, bp
hMT3459F	ACGCCATAAAACTCTTCACCAAAG	Human mtDNA	110
hMT3569R	GGGTTCATAGTAGAAGAGCGATGG		
FWD(+)	GCCAGGGTTTTCCCAGTCACGA	Control DNA	275
REV(-)	GAGCGGATAACAATTTCACACAGG		
hID1F	TCTTAACTGTTCCATTTTCCGTATCTG	Human nDNA	87
hID1R	TCTTGGCGACTGGCTGAAAC		

Table 3

Real-time PCR protocol

Period	Time	Temperature, °C	Cycles number
Initiation period	3 min	95	1
Denaturation	1 min	94	45
Annealing	20 sec	58	
Elongation	20 sec	72	
	1 min	58	
Melting profile	10 sec	Starting from 58°C every 10 sec the temperature increment by 0.5°C	74

with an injury is a release of DNA molecules from damaged tissues directly to the blood flow, although the defects of the extracellular DNA degradation processes are also possible. Typically, one cell contains 100—1000 mtDNA copies, that is why the circulated mtDNA level might possess better potential as an injury predictive marker compared to nuclear DNA (nDNA). Existing methods of quantitative analysis of mtDNA in the blood either suppose to use nDNA as a reference point to determine the normal range, or use direct methods of real-time determination of concentration by PCR. However, nDNA fundamentally differs from mtDNA in molecular weight, base composition and conformation. This means that nDNA might serve as a better endogenic control in detecting mtDNA required for accurate quantitative measurement [20]. In this paper, the extracellular mtDNA and nDNA were quantitatively determined in sera of polytrauma patients. In addition, to exclude the use of expensive fluorescent probes the PCR-conditions were optimized with the aid of internal DNA control.

The objective of the study is to study the levels of mtDNA and nDNA in serum in healthy people and in patients with severe trauma over time as well as to detect a possible association between the circulating DNA levels and infectious pulmonary complications and mortality.

Materials and methods

The blood of 25 patients with severe polytrauma who received medical treatment in the Intensive Care Unit of Sklifosovsky Research Institute for Emergency Medicine in 2012—2013 including the blood of 7 healthy volunteers among hospital physicians. Within different time interval after the injury (first 12 hours, 12—24 hours, 3rd and 5—7th day) in patients with severe polytrauma the levels of mitochondrial and nuclear DNA in the blood serum were determined.

The study exluded patients with combined injury and patients of age over 70 years. The severity of injury was evaluated under Injury Severity Score (ISS), due to localization thereof: head, chest, abdomen, back bone, pelvis and extremities [21]. The average score as per ISS was 40,2±9,2. Gender, age, aspiration of blood and gastric contents, tracheostomy, ventilation period and average permanence in the hospital were considered (Table 1).The course of injury was complicated by purulent tracheobronchitis and pneumonia in 14 patients (56%).

Two groups of patients were formed: survived (average ISS = 38±6) and deceased (average ISS = 49±12). The average values of mtDNA and nDNA were determined in each group within first 12 hours after injury.

As to the development of infectious pulmonary complications, two groups of patients were formed: group I — 9 pneumonia-free patients and group II — 16 patients with pneumonia diagnosed on days 3—7 after the admission. The diagnosis of nosocomial pneumonia was made based on occurrence of new focal infiltrative changes on the chest X-ray in combination with two patterns of following: fever, purulent sputum and (or) leukocytosis, bacteria in aspirate from tracheobronchial tree or blood as determined by generally accepted standards [22].

The results obtained were processed by methods of statistics (SPSS 11.5). To characterize random variables, averages of random variables (*M*), (standard error of the mean (*m*) and Pearson correlation coefficients were calculated When calculating the data non-parametric criterium Mann-Whitney *U*-test, Moses test for extreme reactions, and median test were used [23, 24].

Purification and quantity determination of mtDNA and nDNA.

Sample preparation. Prior to DNA purification, 10 µl of 1.7 ng/µl concentration sample DNA of pBlueScriptSKII(-) plasmid was added to each 0.4 ml sample of the patient's serum, as an internal control of efficiency of the purification and further evaluation of the mtDNA level. Purification of the total DNA from the patients' blood serum was carried out with the use of DNeasy Blood&Tissue kit (QIAGEN, USA) under the manufacturer's protocol. The volume of the DNA purified was 100 µl.

Real-time PCR. The primer pairs used in the study are listed in Table 2.

The real-time quantitative PCR was carried out in the BioRad iCycler amplifier with the mixture made up as follows: 5 µl of the DNA sample; 10 µl of the mixture B Eva Green (Syntol); 0.5 µl 10 µM of mixture of each of specific primers (sequences are given below); 9.5 µl of deionized water. Each DNA sample was used as a template in three identical PCR reactions. The conditions of PCR are given in Table 3.

Data processing. Real-time PCR allows to compare the quantities of nucleic acids provided that the efficiency of reaction and the given level of threshold fluorescence are the same for each of reactions under comparison.The threshold cycle (C(T)) means

Table 4

Levels ($M\pm m$) of mtDNA and nDNA in serum in deceased and survived patients 12 hours post-trauma

Survived patients		Deceased patients	
mtDNA (%)	**nDNA (%)**	**mtDNA (%)**	**nDNA (%)**
2060±1003*	24349±8258	4976±2271*	176611±161315

Note. * — $P<0.05$, Mann-Whitney U-test. Survived patients-ISS 38±6; deceased patients-ISS 49±12.

Table 5

Levels ($M\pm m$) of mtDNA and nDNA over time in groups with and without pneumonia

Time periods after injury	Pneumonia (n=16)		No pneumonia (n=9)	
	mtDNA (%)	**nDNA (%)**	**mtDNA (%)**	**nDNA (%)**
< 12 hours	3433±1507	88226±64298	1715±395	19395±5698
12-24 hours	4850±1952*#	255522±140035	756±213*#	4438±1084
3rd day	1028±463	7435±3000	536±151	25259±11141
5-7th day	431±140#	11290±7416**	357±166#	5664±1278**

Note. * — differences between groups, mtDNA ($P<0.05$, median test); # — intragroup differences, mtDNA ($P<0.05$, Moses test); ** — intergroup difference, nDNA ($P<0.05$, Moses test).

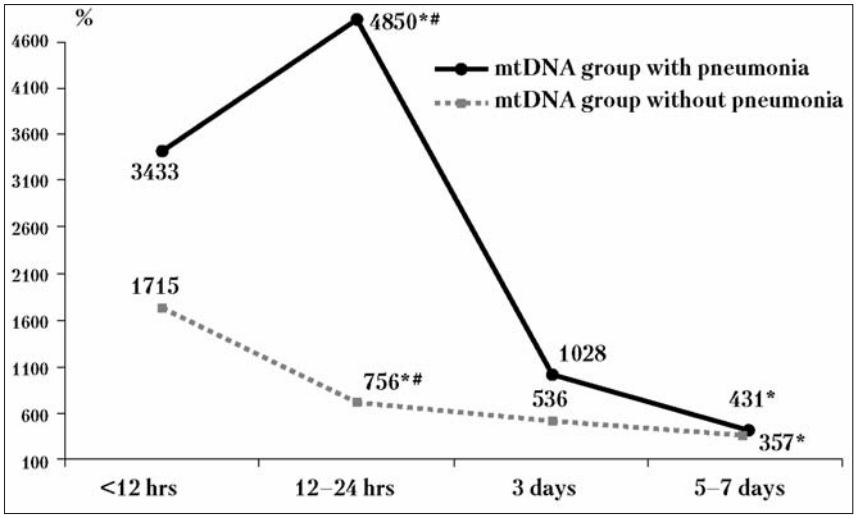

Fig. 1. Dynamics of mtDNA content in patients with and without pneumonia
Here and in Figure 2: Ordinate: mtDNA (% to the control values). Absciss: time after the injury. * — differences between groups ,mtDNA ($P<0.05$, median test); # — intragroup differences, mtDNA ($P<0.05$, Moses test).

such a cycle n, in which some given level of reporter fluorescence is achieved — threshold fluorescence $\mathbf{P_{C(T)}=const.}$

In processing the values obtained the method of direct data comparison was used: in case of equal efficiency of reaction E (E — amplification efficiency) in samples 1 and 2 under comparison, the relative concentration of substrate R will amount to: $\mathbf{R = P1/P2 = E^{-(C(T)_1-C(T)_2)} = E^{-\Delta C(T)}}$

The PCR reaction efficiency was estimated with the use of dilution of control molecules in the ranging from 1pg/ml to 10 μg/ml. The results of the amplification data were normalized using control plasmid DNA added to the probes. The reactions carried out with the primers (Table 3) proceeded with similar efficiency close to 100%. That's why the following formula was used for further calculations: $\mathbf{R = P1/P2 = 2^{-(C(T)_1-C(T)_2)}}$

The mtDNA and nuclear DNA levels were expressed in relative values taking arithmetic mean values R for healthy volunteers as 100%.

Results and discussion

The analysis of correlations between the ISS scores and mtDNA levels showed that there was a valid ISS correlation (r=0.809, $P<0.05$) with measurements performed at the end of the first day of trauma. Significant correlation between ISS and the levels of nDNA was determined after 12 hours post injury (r=0.697). Twelve hours after the injury levels of mtDNA and nDNA in both groups of deceased and survived patients with severe polytrauma were significantly increased compared to the control (Table 4).

Despite the fact that the differences in the groups between the nDNA values were not significant (P=0.1), the level of mtDNA level was higher in deceased vs. survived patients ($P<0.05$).

Further, the comparative analysis of the mtDNA and nDNA levels in polytrauma patients depending on whether the further course of injury was complicated by pneumonia or not, was performed. It showed significant variations in both quantitative indices and trends thereof within 5 days. Specifically, within first 12 hours in both groups a significant increase in mtDNA serum content was observed compared to values in control group. However, the level of mtDNA in patients with nosocomial pneumonia developed in the unit exceeded the normal value more than 30 times. Moreover, during further 12 hours mtDNA levels increased about 50 times, whereis pneumonia-free patients showed only 17-fold increase followed by a more than two-fold decrease of mtDNA content in the next 12 hours. After 3 days, the differences between the groups were minimal (Table 5 and Figure 1), though both groups exhibited elevated level of mtDNA.

Significant differences in amount and kinetics of nDNA changes were also identified in patients depending on development of nosocomial pneumonia. In patients with

pneumonia, the nDNA levels exceeded the normal range of values about 900 times and 3000 times in 12 hours and 24 hours, respectively. In patients without pneumonia level of nDNA was about 200 times higher within 12 hours after injury with the following 4-fold decrease during next 12 hours (Table 5 and Figure 2).

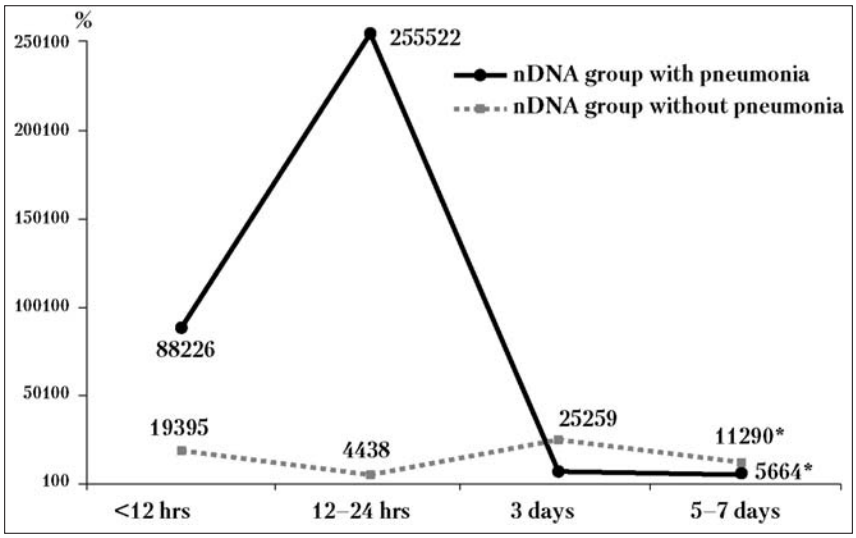

Fig. 2. Dynamics of nDNA in patients with and without pneumonia
* — $P<0.05$, Moses test

It is known that mtDNA can serve as DAMP when it is accumulated in relatively increased amount in systemic circulation in a cell-free form [13, 14]. Previously published papers, reported the increase of the levels of mtDNA in injured patienrs [18, 25]. However, the pathogenic role and clinical impact of DAMPs in severe polytrauma as well as in the development of infectious pulmonary complications and critical illness without infection still remains unclear. This study for the first time described the dynamics of the extracellular DNA levels in the serum in patients with severe polytrauma and with/without pulmonary infection.

Several studies have demonstrated accumulation of typical mitochondria-derived products, formylated proteins and mtDNA, in blood early after the injury [11, 12]. We suggest that ischemia-reperfusion injury of organs and tissues in polytrauma causes severe oxidative stress and subsequent apoptotic or necrotic cell death accompanied by the release of cellular content, including mitochondrial matrix components (formylated proteins and mtDNA) into the blood. Massive release of these molecules intocirculation activates the innate immunity system, which in turn initiates the onset of SIRS during the first hours after injury. There is evidence that endogenous danger signals (DAMP) and exogenous structures (PAMP) cause different types of immune responses [26]. Hyperactivation of immune reactions caused by DAMPs probably prevents the immune system from reacting adequately to pathogenic microorganisms, thereby contributing to further development of infectious pulmonary complications. High concentrations of mtDNA found during the first 12—24 hours after the injury in patients who further developed infectious pulmonary complications indirectly confirm this hypothesis, though does not prove it.

We applied a new modification of the method of quantitative determination of mtDNA using DNA molecule as an intrinsic control, which significantly increased the method accuracy, and also eliminated the use of expensive PCR fluorescent probes thus leading to cost reduction of the test. Compared to the previous papers, there are some differences in patients category used for the study: we analyzed patients with severe polytrauma (average ISS = 40,2±9,2) In addition, Futhermore, in this study for the first time the concentration of mtDNA was determined in serum and not in plasma from trauma patients as shown in earlier studies

Conclusion

The performed statistical analysis demonstrates significant correlation between the ISS-based severity of injury and the levels of mitochondrial DNA in patients with polytrauma within 24 hours (r=0.809, $P<0.05$).

Over two-fold increase in the mtDNA and nDNA levels was observed in the group of the deceased patients during first 12 hours after injury vs. group of the survived patients; moreover, the differences between these groups inf mtDNA level were significant (Mann-Whitney U-test, $P<0.05$).

Significant increase in the levels of mtDNA and nDNA during the first day after injury between patients who further developed nosocomial pneumonia and patients without infectious complications denotes the potential for further research in this area.

This paper demonstrates the reasons for monitoring mitochondrial and nuclear DNA content in the blood of patients with severe polytrauma early after the admission at the intensive care unit. Further study of dynamics of mtDNA and nDNA content in blood in extended groups of patients with severe polytrauma and correlations with clinical, biochemical and immune system parameters is warranted.

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