Correction of NO-inducible apoptosis with Plaferon LB in the Jurcat cells culture

M. Khizanishvili, M. Shakarashvili
(Institute of Experimental Research of Morphology after N. Natishvili, Tbilisi.Georgia)

Apoptosis, or self-liquidation process of cell is an active organized form of a cell`s death. The process develops in physiologic, as well as in pathologic conditions.

Physiological apoptosis is detected in embriogenesis, it provides elimination of autoreactive lymphocytes from peripheral blood etc. Apoptosis is an opposite process of mitosis. The programmed process is very important in order to regulate of tissue size and maintain homeostasis.

The disturbance of physiologic apoptosis may lead to cancerogenesis, autoimmune diseases (f.e. lupus erythematosus (Mysler e. et al., 1994) and others. During brain infarction and insults, cells are dead by apoptosis (Love S., Barber R., wilcock G.K., 2000, Eskes R., et al., 1998). In some conditions the inducible stimuli of apoptosis can cause necrosis. Thus, the intensity of inducible stimuli causes apoptosis as well as necrosis.

Stemming from above mentioned, the pharmacological regulation of apoptosis is very important. The preparations, which regulate intensity of apoptosis, act on the local factors of apoptosis and on the intensity of proapoptotic stimuli - on the one hand and they directly inhibit apoptosis, on the other hand. The suppression happens due to the regulation of caspase activity (low-molecular inhibitors of c Jun-terminal and p-38 kinases (Yue T.L. et al., 1999)) and amount of reactive oxygen species. The regulation is achieved with antioxidative preparations. Antioxidants are widely used in the treatment of various diseases: atherosclerosis, heart ischemic fealure, preeclampsia, diabetes mellitus, acute pancreatitis and so on.

Plaferon LB belongs to such preparations. The oxidative homeostasis, energogenesis and apoptosis modulation ability of the preparation is proved by several researches. Its known that cells` death mechanism (apoptosis/necrosis) is considerably dependent on the intensity of energogenesis. Declined energetical resources initiate oxidative stress and apoptosis. Significant decline and exhaustion of a cell`s energetical resources leads to substitution of apoptosis by necrosis. The process of energogenesis is firmly connected with intensity of mitochondrial respiration. In the regulation of this process NO – molecule-messenger has an important role through the nitrozilation of electron transporting proteins in the respiratory chain.

Stemming from the above said, the goal of our study was to determine some mechanisms of NO-inducible apoptosis in Jurkat-cells culture and to estimate the efficiency of Plaferon LB.

The purposes of the investigation were:

1. Create a model of NO-inducible apoptosis in Jurcat-cells culture;

Under the conditions of the model:

2. To determine the changes of oxidative stress intensity, free NO, nitrated complexes (originated in the process of NO`s interaction with heme-containing and heme- noncontaining proteins of respiratory chain) and mitochondrial respiration.
3. To estimate the dynamics of changes in mitochondrial membrane potential ($\Delta\Psi$)
4. To estimate the redistributional changes of cycle phases of cells;
5. To determine the ultra-structural changes in cells.
6. To estimate several mechanisms of influence and efficiency of Plaferon LB on NO-inducible apoptosis in Jurkat cells culture.

Key words: apoptosis, Jurkat cell, NO-molecule-messenger.

Materials and methods: The research was fulfilled on human leukemia-transforming mature T-cells (Jurkat cells) (DSMZ-Deutshe Sammulung von Mikroorganismen und Zellkulturen (Germany). The cells grown by using biologically medium RPMI 1640 (GIBCO), inactivated embryonic calf serum, L-glutamine (4mM) penicillin (100U ml) at 37°C temperature, in humidified atmosphere containing 5% CO2. Experiments were carried at concentration of 0,3-0,6X10^6 cells in 1-ml area.

In a model of sodium nitroprusid (SNP) induced apoptosis SNP (Nariprus, Sopharma) was added in cells culture in dose 1 mM and cells were incubated for 24 and 48 hours. In part of cells the SNP was added simultaneously with plaferon LB (PLB) by dose 0,2μg and 0,4μg. Intact Jurkat cells were in the control group. After incubation the redistribution of phases of cell cycle were studied by flow cytometry during different times. Mitochondrial potential **Δψ** was determined. For detection of superoxiradicals (O_2^-), lipoperoxides ($LOO^\cdot$) and free nitric oxide (NO) EPR signals and the spin-traps were used.

Flow cytometry Assay, estimating of changeability of mitochondrial membrane potential - Δψ dinamics in the cells culture

The changes of mitochondrial potential Δψm in the cell culture were determined by flow cytometry with lipophilic cation assay 3,3'-dihexiloxacarbocyanine iodide – $DiOC_6$. Δψ may determinate in the heterogeneous population of cells with flow cytometry together with fluorescent assay. A little amount of cells (10^3-10^5) is sufficient for analysis. These cells are sown with low concentration (less then 1μM) (Castedo, M., et al., 2002). The cells 1X10^5 were incubated in 120 μM of 0,2μM DiOC6 solution during 15 minutes at 37°C temperature for the purpose of Δψm determining. The essay was carried out on Becton Dickinson (US) apparatus. The irritation of DiOC6 has been registered on 488 nm.

Redistribution of cell cycle phases in cell culture. Study of redistribution of cell cycle phases in cell culture was detected with flow cytometry. The method was carried out with staining of propodium iodite.

The staining with propodium iodite is widely used for receive of cells cycle picture. Propodium Iodide connects to diploid strains of DNA and gives the information about distribution of DNA double strains in the phases of cells cycle. The samples are fixed in 70% ethanol at 4°C during 12 hours. After elimination of ethanol and addition of RNM-ase (Sigma) (10μg/μL) the deposit is incubated at the room temperature during 30 minutes. Afterwards the cell suspendition takes place in the Propodium Iodide solution (30minutes) and the samples were analyzed with flow cytometry. was distinguished: 1. Haploid region- apoptotic cells; 2. – Diploid region – the cells in G0/G1 phase; 3. Transitional region between diplodal and haploidal conditions – the cells in S phase; 4. Tetraploid region – The cells in G2/M phase. Percentage contents of cells were estimated in each region.

EPR spectra of cell culture suspendium were detected on the EPR-radio-spectrometer PE-1307 with modulation frequency 9,77GHz at the temperature of liquid nitrogen (-196°). EPR spectra of mitochondrial respiratory chain free radicals, hemic (HbNO) and nonhemic (FeSNO) complexes, superoxideradicals, Mn2+ ions, NO were determined. For detection of free NO spin-trap – Na diethilthiocarbamate (DETC) (SIGMA) was used. DETC (500mg/kg) and iron citrate (50mg $FeSO4 \cdot 6H_2O$+ 250mg. sodium citrate). EPR spectra of NO-Fe^{2+}-$(DETC)_2$ complexes were determined at liquid nitrogen temperature on 20mvt. For detection of peroxyl-radicals (LOO.) EPR signals the spin trap α- phenil-tert butilnitron (PBN) (SIGMA) was used. After 5 minutes incubation in PBN the EPR spectra of LOO were determined at the room temperature on the microwave 20mvt.

For detection of reactive oxygen species (superoxid-radicals) 5,5 dimethil-1-pyrolin-IV oxide (DMPO) (SIGMA) is used. The incubation in DMPO (50mM suspensium per mL) takes place at room's temperature with 3 minute's duration. The EPR spectra of superoxiradicals were determined at the room temperature on the microwave - 20mvt.

Electron microscopy. Tye received objectives were fixed in 2,5% solution of glutaraldehyde (pH=7,4). The samples received from apone calibres were strained with teluidine. The research of ultra-thin samples were carried with electron microscopy tesla BS 500.

The results and their discussion. Data obtained from our research indicates to the time-dependent decline in mitochondrial potential after 24, 48 and 72-hour incubation of Jurkat cells with SNP (egzergonic donor of NO) (table 1. diagram 1).

As it is known, molecular oxygen reduction and water formation take place in the respiratory chain of mitochondria by transportation of electrons. In parallel to it, protons (H+) are pumped out in the intermembrane space of mitochondria. It is accompanied by formation of electrochemical gradient – referred to as proton-moving force. Once a substantial electrochemical gradient is established, the accumulated energy may be used either for synthesis of ATP by ATP-syntase, or spent in various energy-dependent processes (Boyer P.D. 1975). The proton-pumping force causes formation of mitochondrial membrane potential ($\Delta\psi$). $\Delta\psi$ is an important indicator of mitochondrial energetical status and homeostasis of a cell.

Table 1

Redistribution of cells in Jurkat cells culture according to changes of mitochondrial membrane potential (Δψ) after incubation of Jurkat cells with sodium nitropruside

Jurkat cells	control	Na-nitroprusid 24 h	Na-nitroprusid 48 h	Na-nitroprusid 72 h
All	100%	100%	100%	100%
M1	66,7%	51,0%	33,9%	13,2%
M2	33,3%	49.0%	66,1%	86,8%

M1 – the cells with high mitochondrial membrane potential (%)

M2- the cells with low mitochondrial potential (%)

It's known that intensity of energogenesis is considerably dependent on the intensity of electron-transport chain and mitochondrial respiration. NO is an important regulator of intensity of mitochondrial respiration. The very function of NO is achieved through nitrozilation of electron transport proteins. Revealed EPR signals of Fe-containing complexes in Jurkat cells incubated with SNP, indicate to the nitrozilation and consequently, inhibition of I and IV complexes of respiratory chain (table 2).

Table 2

Changes of EPR-signals in Jurkat cells culture after influence of Sodium nitropruside on the

cells Significant difference compare to control

Jurkat cells		O_2^-	NO	$LOO^\cdot$	Free radicals	FeSNO	HbNO
control	24 h	-	10,0±0,5	-	10,0±0,5	-	-
	48 h	-	10,0±0,6	-	12,0±0,6	-	-
	72 h	-	10,0±0,6	-	11,0±0,6	-	-
Na-nitroprusid	24 h	3,0±0,6	10,0±0,5	-	11,0±0,6	5,0±0,6	10,0±0,6
	48 h	5,0±0,6	10,0±0,7	10,0±0,5	6,0±0,4*	6,0±0,5	12,0±0,4
	72 h	9,0±0,5	9,0±0,8	12,0±0,9	3,0±0,4*	6,0±0,6	11,6±0,6

After the influence of NO on the I complex of mitochondrial respiratory chain (FeSNO is formed) ROS are formed and oxidative stress is initiated. This process is revealed with the signals of superoxide-radicals (O_2^-) in the EPR- specter of Jurkat cells (table 2). SNP is the source of NO. Enhanced O_2^- provides transformation of NO to peroxinitrite (NOO^-). Peroxinitrite is very cytotoxic- it initiates peroxidative processes,

inhibits mitochondrial electron transport and underpins respiratory chain. These changes after 48-hour incubation of SNP in the EPR specter of Jurkat cells are revealed with intensive EPR signals of lipoperoxides ($LOO^{\cdot}$) and sharply declined EPR-signal of free radicals.

The decrease of mitochondrial membrane potential ($\Delta\varphi$) indicates to declined intensity of respiration and energogenesis. Decreasing of energetical resources initiate cells` death mechanisms (apoptosis and necrosis). According to some scientists, inhibition of I complex (revealing by FeS-NO complexes) activates proapoptotic program (Seaton T.A. et al., 1998, Barrientos A., Moreas C.T., 1999).

Nitrozilation of heme-containing groups (HbNO complex) of mitochondrial respiratory chain proteins (cytochrom c, cytochromoxidase-IV complex) regulates their activity and function (Schounhoff C.M. et al., 2003). This regulation is performed by the conformational changes of proteins- on the one hand, and by the influence of interaction of phospholipids-rich mitochondrial membranes with anionic phospholipids vesicles - on the other hand.

It is known, that activity of IV complex of the mitochondrial respiratory chain and the interaction of cytochrom c with mitochondrial inner membrane depends on the content of cardiolipin. It consider, that NO-inducible or oxidative degradation (peroxidation) of cardiolipin causes release of cytochrom c from mitochondria and further activation of caspases (Kluck R.M. et al., 1997; Ushmorov A. et al., 1999,). Thus, the nitrozylation of mitochondrial transport proteins (NADH-dehydrogenase, cytochrom c) favors apoptosis development. It is notable, that the decline in $\Delta\psi$ increases permeability of mitochondrial outer membrane and leads to release of cytochrom c. It was shown, that apoptosis develops during several hours after reducing of membrane potential (Fall C.P. Bannet J.P., 1999). So, the disturbance of mitochondrial function (membrane potential) is the earlier indicator of a cell`s apoptotic death.

Table 3

Percentage redistribition of cells in Jurkat cell-culture according to cell-cycle phases after incubation with SNP(Na-nitroprusid)

Jurkat cells	SubG0(Apopt.)	G0/G1	S	G2/M
control	16,8%	55,2%	13,0%	15,0%
SNP 48 h	43,6%	37,6%	11,5%	7,5%
SNP 72 h	55,9%	27,0%	11,2%	5,9%

On the basis of redistribution of cells cycle phases after incubation of Jurkat cells with SNP it has found that rise in incubation time is accompanied with increased intensity of apoptosis, also, the content of diploidal and mitotic cells is decreased, which indicates to declined proliferative processes (table 3).

On the basis of electron-microscopic research of Jurkat cells incubated with SNP during 72 hours, the reaction develops towards three directions. One group of cells

undergoes apoptosis: the plasma membrane is disrupted; Condensation, hyperagregation - the fragments of chromatin condensation are marked; There is the complete lysis in the cytoplasm, chromatin is coalesces, the center of the cell is also rounded. There are a lot of apoptotic fragments in the nuclei of the lymphocytes. In some cases the membrane of the nucleus seems to be normal, but chromatin is coalesced and rounded into vesicles; cytoplasm is also dense and homogenic. Plasma membrane is smooth. Disrupted membrane and nucleosomal autolysis had been revealed in some cells and in their nucleus.

Some cells undergo lysis, destruction: there are many large vacuoles in the cytoplasm, nucleoplasma is dispersed, nucleosomal chromatin is marginated, and it seems destruction of cytoplasm. Some cells are oval-shaped. They have smooth plasma membrane, dispersed cytoplasm, little; swelled nucleus with simple contours, nucleoplasma becomes rare. The crysts of mitochondrion have unusual form, they look like as honeycomb, and crysts' hyperplasia is characterized (Photo 1).

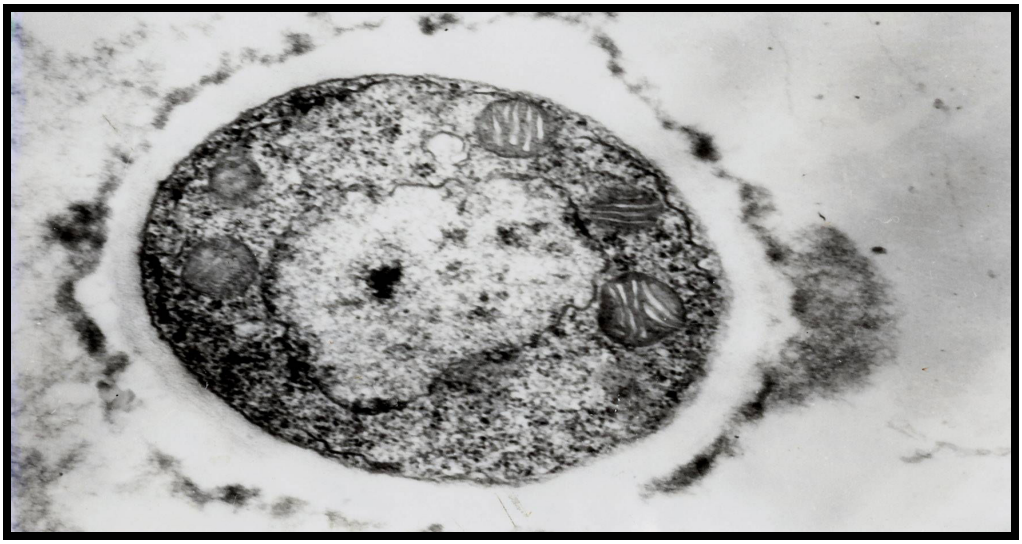

Photo 1. Jurkat cell.Mitochondrion with hyperplastic crysts

According to several authors, the changes, like mitochondrion were revealed in lymphocytes after incubation with LPS (endergonic stimulator of NO).

Its well known, that despite of outer membrane disturbances, mitochondrial inner membrane preserves the ability of oxidative phosphorylation and for a long time supplies dying cells with energy, inevitable for apoptosis. Disturbances of homeostasis in apoptosis have been identified in various changes: the disrupter of outer membrane leads to swelling of mitochondrion. The osmotic pressure in normal mitochondria is higher, then in cytoplasm. It is presumable the increase in number crysts under the influence of NO (endogenic and egzogenic) is the compensation mechanism for activation of energogenesis. Because of inner membrane large area the swelling of mitochondria continues until physical disrupter of outer membrane. The disturbances like that was revealed in second type cell in our experiment. The cell defense from apoptosis is possible with influence on regulatory factors (Bxl-x_L) of mitochondrial electric and osmotic homeostasis (Heiden M.G.V.et al., 1997).

Thus, we can consider, that in the Jurkat cells after prolonged incubation with SNP the following changes take place: nitrozilation of mitochondrial membrane electron transport proteins, activated formation of reactive oxygen species, activation of lipoperoxidative processes, decrease of mitochondrial membrane potential and energogenesis, intensification of apoptosis and necrosis. We think, the increased amount of crysts during stress is the compensatory mechanism for cells survive. This process expressed with intensification of energogenesis and is the regulatory mechanism of oxidative homeostasis.

Time–dependent elevation of mitochondrial membrane potential revealed by us after 24, 48,and 72 hours incubation of Jurkat cells simultaneously with SNP and plaferon LB (table4).

Table 4

Percentage redistribution of cells according to mitochondrial membrane potential (Δψ) changes after incubation of Jurkat cells with SNP (Na-nitroprusid) and plaferon LB.

Jurkat cells	Control	SNP 24 h	SNP 48 h	SNP 72 h	SNP+ PLB 24 h 0.2 μg/ml	SNP+ PLB 48 h 0.2 μg/ml	SNP+ PLB 72 h 0.2 μg/ml	SNP+ PLB 24 h 0.4 μg/ml	SNP+ PLB 48 h 0.4 μg/ml	SNP+ PLB 72 h 0.4 μg/ml
All	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
M1	66,7%	51,0%	33,9%	13,2%	76,6%	53,0%	37,0%	76,0%	71,0%	69,9%
M2	33,3%	49,0%	66,1%	86,8%	23,4%	47,0%	63,0%	24,0%	29,0%	30,1%

M1- cells with high mitochondrial-membrane potential (%)
M2- cells with low mitochondrial membrane potential (%)

Jurkat cells' EPR-signals were changed under influence of SNP and plaferon LB. The content of reactive oxygen and lipid species, as well as nitrozylation of mitochondrial electron transport proteins (FeSNO, HBNO) are markedly decreased, the content of free NO is elevated (because of decreased intensity it's transformation to peroxititrite), and mitochondrial respiration intensity is increased (free radicals EPR signals are intensified) (table 5).

Table 5
Changes of EPR-signals in Jurkat cells culture after influence of SNP and plaferon LB on the cells

		O_2^-	NO	$LOO^·$	***Free radicals***	FeSNO	HbNO
control	24 h	-	10,0±0,5	-	10,0±0,5	-	-
	48 h	-	10,0±0,6	-	12,0±0,6	-	-
	72 h	-	10,0±0,6	-	11,0±0,6	-	-
SNP	24 h	3,0±0,6	10,0±0,5	-	11,0±0,6	5,0±0,6	10,0±0,6
	48 h	5,0±0,6	10,0±0,7	10,0±0,5	5,0±0,4*	6,0±0,5	12,0±0,4
	72 h	9,0±0,5	9,0±0,8	12,0±0,9	3,0±0,4*	6,0±0,6	11,6±0,6
SNP+PLB 0,2 μg/ml	24 h	-	10,0±0,5	-	13,0±0,5**	5,0±0,6	13,0±0,8**
	48 h	3,1±0,6**	16,0±0,5**	6,0±0,7**	12,0±0,5**	4,0±0,6**	8,0±0,6**
	72 h	3,0±0,6**	16,0±0,6**	4,7±0,6**	12,0±0,6**	4,0±0,6**	7,0±0,6**
SNP+PLB 0,4 μg/ml	24 h	-	15,0±0,8**	-	12,0±0,5	3,0±0,6**	-
	48 h	-	20,0±0,9**	-	10,5±0,6**	2,0±0,5**	-
	72 h	-	19,0±0,8**	-	10,0±0,6**	1,5±0,6**	-

*** Significant difference compares to control group**
**** Significant difference compare to SNP group**

Intensification of mitochondrial respiration causes energogenesis amplification revealed with increased mitochondrial membrane potential (table 4), this process leads to decreased intensity of apoptosis (table 6). This revealed with decreased amount of apoptotic cells. From the results of our research, under the influence of plaferon LB the mitosis is intensified, which indicates to intensification of proliferation processes.

Table 6
Percentage redistribution of cells according cell cycle phases in Jurkat cells after their incubation with SNP and plaferon LB.

Jurkat cell	SubG0 (Apopt.)	G0/G1	S	G2/M
control group	16,8%	55,2%	13,0%	15,0%
SNP 48 h	43,6%	37,6%	11,5%	7,5%
SNP 72 h	55,9%	27,0%	11,2%	5,9%
SNP+PLB 48 h	29.5%	39.5%	16,5%	14,5%
SNP+PLB 72 h	21,0%	39,0%	21,0%	19,0%

As a result of electron microscopy of Jurkat cells after incubation of the cells with sodium nitropruside (SNP) and plaferon LB various changes were revealed. The changes were liked to previous experimental series and they were particularly expressed after 72 hour-incubation with plaferon LB with dose of 0, 4 μg.

Plasmatic membrane of some cells are smooth and double-contoured; also, in these cells lysis of separated parts take place (often after incubation of the cells with SNP) photo 2; cytoplasm is light, there are a lot of vacuoles, number of ribosomes and polysomes are decreased, mitochondrion are oval-shaped; the form of nucleus looks like to the cell's one; in some nuclei margination and condensation of chromatin are revealed near membrane and nucleoli.

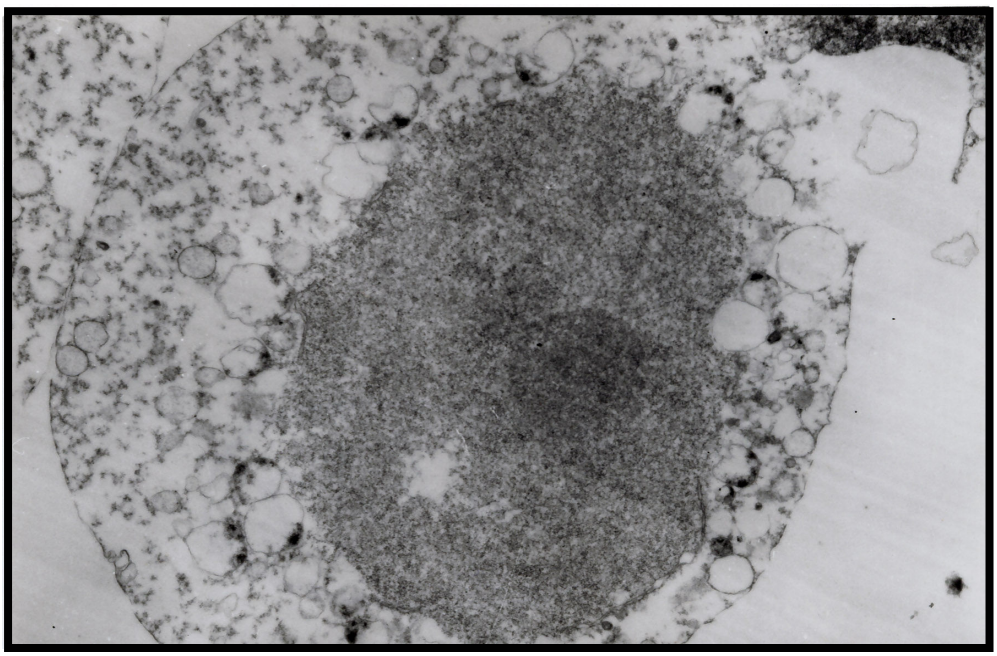

Photo 2. Jurkat cell. Plasmatic membrane is smooth, double-contoured; cytoplasm is light; there are a lot of vacuoli and small amount of ribosomes and polysomes in the photo.

Homogenic chromatin is even distributed in whole nucleoplasm of other cells. Nucleoli are bulky and dense; binuclearity, characteristic for SNP, is not revealed in this case. Sometimes swelling of perinuclear space is detected, nucleosoma of such nuclei is characterized with sharply margination of chromatin close to the wall. Apoptosis particularly is not characteristic. Apoptotic particles are revealed only in some cases. The layar of homogenic nonstructural cells and osmophilic matrics are not detected in this experimental series (which is frequent under the influence of SNP).

Thus, based on our research we found, that in the Jurkat cells culture plaferon LB decreases intensity of NO-inducible oxidative stress, declines apoptosis intensity, increases mitochondrial membrane potential, and intensifies mitosis.

Conclusions:

1. Nitrozilation of mitochondrial electron transport proteins, intensification of ROS formation, initiation of lipoperoxidational processes, and development of oxidative stress has been found In the Jurkat cells culture after prolonged incubation of the cells with SNP (egzergonic donor of NO).
2. Intensification of NO-inducible oxidative stress and decline of mitochondrial respiration lead to time–dependent reduction of mitochondrial membrane potential.
3. Intensification of time-dependent apoptosis, decreased diploidal quality of cells and mitosis intensity were determined in the Jurkat cells after prolonged incubation of the cells with NO-donor. This indicates to diminished intensity of cells' proliferation.
4. Origination of mitochondrial multiple cysts after 72-hour incubation of Jurkat cells with SNP is the compensatory process resulted in reduction of NO-inducible enegrogenesis.
5. Innermitochondial osmotic swelling is detected in the energetically active mitochondria (honeycomb-like crysts-containing) after reduction of mitochondrial membrane potential and outer membrane disturbances. This leads to curving and further lysis of mitochondrion.
6. Under influence of plaferon LB in the Jurkat cells intensity of NO-inducible oxidative stress declines, mitochondrial membrane potential increases. These processes lead to decrease of apoptosis intensity, elevation of mitosis and intensification of cells proliferation.

Practical recomendacions

1. Taking into consideration the important role of the prooxident processes in the mechanism of apoptosis, we recommend the use of antioxidiative preparations in order to oppress apoptosis.
2. The antiapoptetic activity of Plaferon LB on Jurkat cell culture relieve as result of the research gives us the opportunity to recommend the use of this preparations for the prevention of apoptosis of T lymphocytes at different infectious diseases, neurodegenerative, ischemic diseases, AIDS, as well as for the treatment of gastritis and gastric ulcer caused by the Helicobacter pylory.

,,Jurkat,, უჯრედების კულტურაში NO-ინდუცირებული აპოპტოზის კორექცია პლაფერონ ლბ-ს მეშვეობით

მ. ხიზანიშვილი, მ. შაქარაშვილი
(ნ.ნათიშვილის სახელობის ექსპერიმენტული მორფოლოგიის ინსტიტუტი. თბილისი.საქართველო)

რეზიუმე:

განხილულია „Jurkat" უჯრედების კულტურაში (ლეიკემიით ტრანსფორმირებული მომწიფებული T-უჯრედები) NO-ინდუცირებული აპოპტოზის განვითარების მექანიზმები და მათი კორექციის გზები. დაგენილია, რომ „Jurkat" უჯრედების ნატრიუმის ნიტროპრუსიდთან (NO-ს ეგზოგენურ დონორთან) ხანგრძლივი ინკუბაციის პირობებში NO-ინდუცირებული აპოპტოზის განვითარებისას დაქვეითებული ენერგოგენეზის აღდგენისათვის შესაძლებელია დაცვითი–კომპენსაციური რეაქციის ინიცირება. ეს ვლინდება მიტოქონდრიებში კრისტების წარმოქმნითა და უჯრედების ფუნქციის გაზრდით. „Jurkat" უჯრედების კულტურაში NO-ინდუცირებული აპოპტოზის დროს პლაფერონ ლბ განაპირობებს ოქსიდაციური სტრესის ინტენსივობის შემცირებას, მიტოქონდრიული სუნთქვისა და ენერგოგენეზის ინტენსიფიკაციას, მიტოქონდრიული მემბრანული პოტენციალის მატებას და უჯრედების აპოპტოზის ინტენსივობის დაკნინებას..

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