# Caspase 8 - biomarker of extrinsic pathway of apoptosis in exogenic nitric oxide (NO) – induced death of rats' hepatocytes

## Kaspaza 8 maker zewnętrznej drogi przewodzenia sygnału do apoptozy w śmierci hepatocytów szczura wywołanej egzogennym tlenkiem azotu

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**Abstract**

The aim of present study was immunhistochemical evaluation of caspase 8 expression in L-arginine – substrate of nitric oxide (NO) induced apoptosis of rats'hepatocytes

The rats used in this experiment were divided into 2 equal groups. Experimental: rats received per os L-arginine 40mg/kg body weight, every other day for 2 weeks and were decapitated after 3 weeks of the experiment. Control rats received per os 2ml of distilled water every other day for 2 weeks and were decapitated after 3 weeks of the experiment.

Specimens of the liver taken after decapitation were examined in immunohistochemical way using standard three step method to detect immunolocalization of caspase 8. The results of immunohistochemical examinations were subjected to qualitative evaluation taking into account the intensity of colour reaction at the antigen-antibody site in rat liver examined in individual groups. The quantitative evaluation was using the Analysis-pro software. The surface area of cells with positive reaction (+) was calculated.

The quantitative evaluation of caspase 8 expression showed that the area occupied by positive caspase 8 reaction in the rat liver of the experimental group (243,59 um2+/-262,43) was comparable to that in the control group (403,10 um2+/-215,91)(p=0,38). In the present study the dose of L-arginine was similar to that used in pregnant women treated for gestosis. The study shows that L-arginine as a donor of exogenous nitric oxide did not have an apoptotic effect leading by extrinsic pathway on the hepatocytes of the rats.

*Keywords:* nitric oxide, apoptosis, caspase 8, liver

**Streszczenie**

Celem pracy była immunohistochemiczna ocena eksresji kaspazy 8 podczas apoptozy hepatocytów szczura wywołanej L-argininą – substratem tlenku azotu (NO).

Szczury użyte w doświadczeniu zostały podzielone na dwie równoliczne grupy. Doświadczalne szczury dostawały per os L-argininę 40mg/kg masy ciała co 2-gi dzień przez 2 tygodnie i zostały dekapitowane po 3 tygodniach. Kontrolne szczury dostawały per os 2ml wody destylowanej co 2-gi dzień przez 2 tygodnie i zostały dekapitowane również po 3 tygodniach. Wycinki wątroby wzięte po dekapitacji zostały zbadane immunohistochemicznie przy użyciu standardowej trzystopniowej metody w celu wykrycia immunolokalizacji kaspazy 8. Wyniki badań zostały przedstawione jakościowo - opis intensywności barwnej reakcji w miejscu poszukiwanego białka w wątrobie szczurów. Ilościowej oceny wyników badań dokonano przy użyciu programu komputerowego Analysis-pro –liczono pole powierzchni zajętej przez barwną reakcję antygen-przeciwciało.

Ilościowa ocena ekspresji kaspazy 8 wskazała, że pole powierzchni zajętej przez dodatni odczyn dla tego białka w wątrobie samic z grupy doświadczalnej (243,59 um2+/-262,43) było porównywalne do obliczonego w wątrobie samic z grupy kontrolnej (403,10 um2+/-215,91)(p=0,38). W niniejszym doświadczeniu dawka L-argininy podana samicom szczura była zbliżona do dawki podawanej kobietom w ciąży powikłanej gestozą. Doświadczenie wykazało, że dawka ta nie wywołuje apoptozy hepatocytów, w której sygnał do indukcji przebiega drogą zewnętrzną przez aktywację kaspazy 8.

*Słowa kluczowe:* tlenek azotu, apoptoza, kaspaza 8,wątroba

### Introduction

Nitric oxide (NO) is produced in the organism by the endothelial cells [1], macrophages [2], hepatocytes, nerve endings and some neurons [3], neutrophils, monocytes, mastocytes, blood platelets [4].

NO is syntetized from L-arginine. This reaction is regulated by nitric oxide synthase (NOS) also called

digoxygenase [5]. This is a two-step reaction of L-arginine oxidation in which NADPH is the source of electrons while L-citruline and NO are the products [6].

NO is involved in many physiological processes, i.e. neurotransmission, regulation of smooth muscle tension, destruction of neoplasms and pathogenic organisms. The most common NO synthesized by endothelial synthase (eNOS), also called EDRF (endothelium-derived relaxing factor) [7] regulates the blood pressure by dilating blood vessels, inhibits the aggregation of thrombocytes and inhibits the adhesion of leukocytes to the endothelium.

The effects of NO on cell death are not fully known. It was demonstrated that the proapoptotic influence of NO was caused by oxidative stress induced in the cell [8]. The so-called "nitrosative stress" was described in the rat's macrophages, where NO-induced apoptosis was observed [9].

The present study examined the process of L-arginine - induced apoptosis in the hepatocytes of rats. We studied expression of caspase 8 - biomaker of extrinsic pathway of apoptosis.

## Material and methods

The study material consisted of 16 white Wistar female rats aged 2.5-3 months. The rats were divided into 2 equal groups. The rats in group I - were administered L-arginine through the stomach tube (Argininum, Curtis Healthcare, Poznań, Poland) every other day in the amount of 40mg/kg body weight (5mg of L-arginine in 1ml of distilled water) 7 x for 2 weeks and were decapitated after 3 weeks of the experiment - 8 individuals. The rats in group II - received 2 ml of distilled water through the stomach tube every other day for 2 weeks and were decapitated after 3 weeks of the experiment - 8 individuals.

The study was approved by the Local Ethics Committee in Lublin attached to the Medical University of Lublin, Al. Racławickie 1, no. 551/2005.

After decapitation the hepatic specimens collected for immunohistochemical examinations were fixed in 10% formalin, dehydrated in the alcohol series and embedded in paraffin blocks. The blocks were cut into 5μm-sections, which were placed on the silanized glasses. Two specimens from liver collected from each animal were used. Next paraffin was removed in xylene and in a graded alcohol series. The specimens were subjected to thermal preparation in the acid medium (10mM citrate buffer pH 6.0). Next endogenous peroxidase was blocked by incubation in $H_2O_2$. After that the specimens were incubated with rabbit primary antibody caspase 8 (Lab Vision Ab-4, RB-1200-PO) in 1% TBS/BSA, dilution 1/100. Then the DakoCytomation kit was used for immunohistochemical reactions, which included: biotinylated secondary antibody, streptavidin conjugated with horseradish peroxidase and AEC substrate. After chromatogen staining the specimens were placed in the haematoxylin solution and rehydrated. The photographic documentation was prepared using the computer-guided Colour Video Camera CCD-IRIS (Sony).

The results of immunohistochemical examinations were subjected to qualitative evaluation and quantitative evaluation using the Analysis-pro software, version 3 (Soft Imaging System GmbH, Germany). The microscopic images, magnification x125 were analysed assessing the protein expression in 3 randomly chosen areas, 781193.35μm$^2$, each. The surface area of cells with positive reaction (+) was calculated.

The results were presented as means and standard deviation of the mean using the MANOVA, ANOVA, post hoc Scheffe tests. 5% error risk and statistical significance at $p \leq 0.05$ were accepted.

## Results

The caspase 8 reaction was focal in all examined groups. Most intensive reaction was found in the L-ARG group and concerned the whole hepatocyte cytoplasm. It was dark pink and diffused (Fig. 1, 2).

Quantitative evaluation showed statistically insignificantly increased caspase 8 reaction in the L-ARG group compared to the control.

Tab. 1 Mean area occupied by caspase 8 reaction in the rat liver in control and experimental groups (um$^2$). Statistical significance of differences. Post hoc Scheffe and ANOVA tests

| ANOVA | Control group | | Experimental group | | F | p |
|---|---|---|---|---|---|---|
| | M (mean) | SD (standard deviation) | M (mean) | SD (standard deviation) | | |
| Caspase 8 | 262.43 | 243.59 | 403.10 | 215.91 | 0,529 | p=0,38 |

## Discussion

The scientists describe two main pathways leading to apoptosis [10]. Extrinsic pathway is induced by an external signal and is connected with the membranous death receptors which have intracellular death domain (DD) [11]. In intrinsic pathway the mitochondria and endoplasmic reticulum are involved.

Sometimes both pathways overlap. This is called the amplification of proapoptotic signalling.

The extrinsic pathway of apoptosis, which is examined in present study, is connected with membranous death receptors. The binding of the receptor with the

Tab. 1 Caspase 8. Comparison of control and experimental group

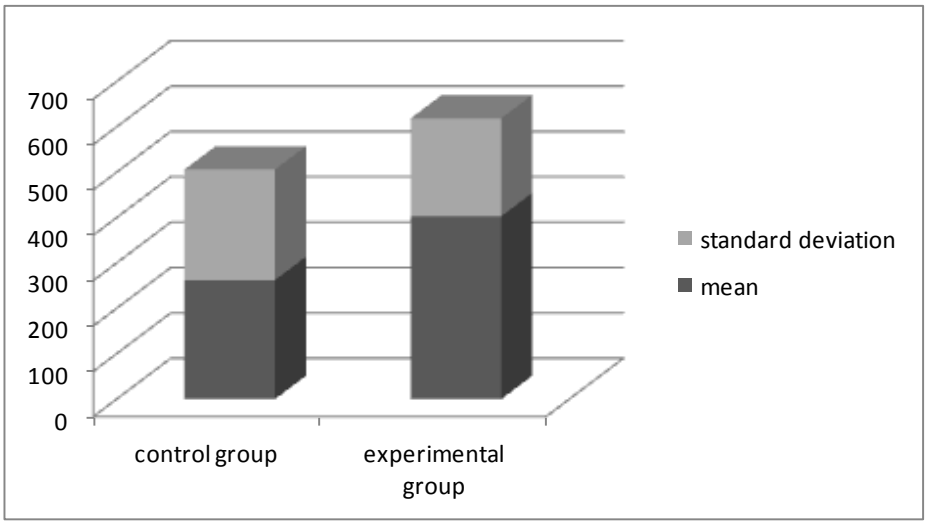



Fig.1 CONTROL GROUP. The rat liver section showing caspase 8 reaction of low intensity. AEC+H staining. Magnification about x 280.

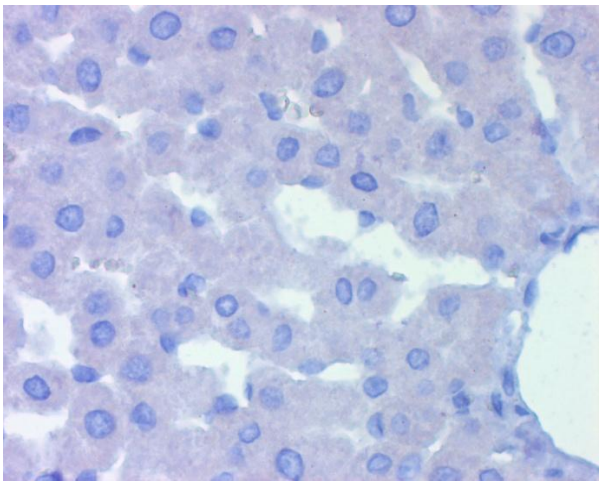
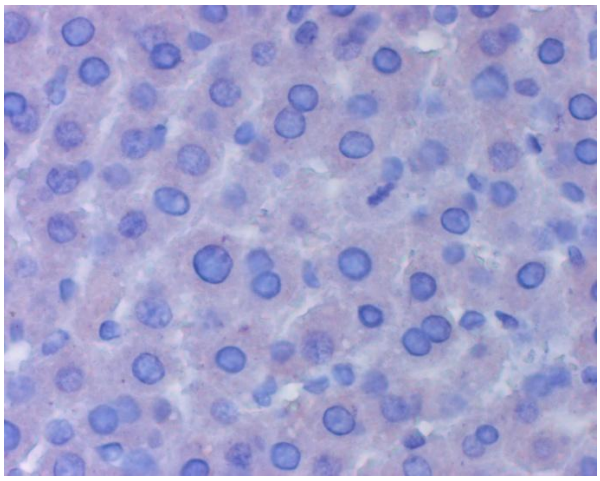


Fig.2 EXPERIMENTAL GROUP. The liver section of the L-ARG - treated rat. The rat liver section showing caspase 8 reaction of low intensity. AEC+H staining. Magnification about x 280



ligand results in the activation of caspase 8, less often of caspase 10 or 2. Active caspase forms initiate the caspase cascade [12].

The most important membranous death receptors are: tumour necrosis factor receptor 1 *(TNFR-1), CD95/Fas/Apo-1* (CD95-cell death 95), *TRAIL-R1, TRAIL-R2* (DR4, DR5 - death receptor 4,5), NGRF(nerve growth factor receptor).

In the present study the effects of exogenous NO on the hepatocytes were examined by administering L-arginine as a substrate of NO [13].

The endotheliocytes after the formation of a portion of NO are immediately capable of regenerating L-arginine from L-citrulline [4]. This is why to examine the effects of NO on various life processes in the cells, including apoptosis, exogenous nitric oxide is used.

L-arginine administered exogenously is converted into NO [14]. This drug is administered to lab animals in many different ways. Holm and colleagues [15] administered L-arginine to rats orally in the amount of 2.25% in the drinking water, Raff and colleagues [14] – intraperiteneally.

Evans and colleagues demonstrated that the amount of L-arginine sufficient to increase its concentration in the organism was 9 or 21g/day while 3g/day, even if administered for a week,(similar to the dose used in the present study) caused only a few side effects [16]. Such a dose is sufficient to decrease pregnancy-induced hypertension

In the present study the dose of L-arginine was similar to that used in pregnant women treated for gestosis. This dose should be safe for a mother and a foetus (the so-called dose scavenging free radicals) [4].

The our previous study [17] showed that L-arginine as a donor of exogenous nitric oxide induced the apoptotic signal in normal renal tubular cells of the rats.

The our present study shows that L-arginine as a donor of exogenous nitric oxide did not have an apoptotic effect leading by extrinsic pathway by caspase 8 on the hepatocytes of the rats.

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