

**L-ARGININE DECREASES HEAT SHOCK PROTEIN 70  
(MARKER OF ENVIRONMENTAL STRESS) EXPRESSION IN KIDNEY CELLS  
OF RAT FETUSES DURING APOPTOSIS - LATE EFFECT OF ADRIAMYCIN ACTION**

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**Abstract:** Both Adriamycin and nitric oxide (NO) cause apoptosis acting through or as free radicals inciting oxidative stress in the cell. However, in some tissues the anti-apoptotic action of NO was described, thereby the impact of NO on cell apoptosis is not finally recognized. In this study, a trial of the evaluation of exogenous NO (L-arginine) impact on apoptosis induced by Adriamycin in fetal kidney cells was undertaken. For this reason, the expression of Heat Shock Protein 70 (HSP 70), environmental stress marker, as a sensitive biomarker of oxidative stress induced in fetal kidney cells with Adriamycin given to mothers prior to pregnancy was studied using immunohistochemical method. The expression of HSP 70 in fetal kidney cells, whose mothers received apart from Adriamycin, L-arginine (as NO substrate) was also evaluated. The results of the study pointed to the fact that the exogenous NO (L-arginine) could be helpful in inhibition of intensified apoptosis in fetal cells as a late effect of Adriamycin action.

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## INTRODUCTION

The main task of HSP 70 (heat shock protein 70) belonging to the family of proteins called chaperones is the reversible binding to various proteins and their protection against disfiguration, which may be the effect of a cell shock [18, 19]. Expression of HSP 70 in the cell increases during many environmental stresses, the cells are susceptible to [16, 19]. Mukhopadhyay *et al.*, while studying *Drosophila*, noticed that HSP 70 might be used as a sensitive biomarker evaluating the risk of organism exposure to environmental pollution [11].

Adriamycin, an anti-neoplastic antibiotic, causes apoptosis in cells of numerous cancers, but also in normal

cells of the body which divide vividly [4, 15]. These include also the embryo cells studied in this paper [13]. Pro-apoptotic activity of Adriamycin is ascribed among others to the action of free radicals developed in the process of bio-transformation [1, 2, 14].

L-arginine is a substrate, from which nitric oxide (NO) arises in an organism with the help of enzyme - nitric oxide synthase. NO as a free radical could develop, similarly as Adriamycin, an oxidation stress and could also exhibit pro-apoptotic activities [5]. On the other hand, NO was also described as an apoptosis inhibitor in some tissues [10]. So far, it is not known in what kinds of cells NO acts anti-apoptotically and what is its mechanism of action and eventual side effects.

The aim of the present study was to evaluate the impact of exogenous NO (L-arginine) on apoptosis (induced by Adriamycin) in fetal kidney cells. For this reason, the expression of HSP 70 as a tender biomarker of oxidation stress was immunohistochemically evaluated in fetal kidney cells in cases when Adriamycin was administered to mothers prior the pregnancy. The expression of HSP 70 in fetal kidney cells, whose mothers received apart from Adriamycin, L-arginine (as NO substrate) was also evaluated.

## MATERIALS AND METHODS

Twenty-four female Wistar rats were used in the experiment. Rats were divided into 3 groups: 2 experimental (Ex1, Ex2) and 1 control (C), 8 rats in each group.

a) Female rats from experimental group Ex1 were administered intraperitoneally Adriamycin (Adriablastin, Farmitalia, Carlo Erba, Milan, Italy) in dose 5 mg/kg of body weight. After 4 weeks female rats were mated with males. At the end of pregnancy (on 20th day) female rats were decapitated.

b) Female rats from experimental group Ex2 were administered intraperitoneally Adriamycin in dose of 5 mg/kg of body weight. After 4 weeks female rats were mated with males. Pregnant rats were administered L-arginine (Argininum, Curtis Healthcare, Poznań, Poland) (5 mg in 1 ml water) by gastric tube, every two days in dose 40 mg/kg of body weight 7 times, starting from the end of 4th week from Adriamycin administration (till about 14th day of pregnancy). At the end of pregnancy (on 20th day) female rats were decapitated.

c) Female rats from control group (C) were given 0.5 ml of 0.9% NaCl intraperitoneally. After 4 weeks female rats were mated with males. At the end of pregnancy (on 20th day) female rats were decapitated.

Two randomly chosen fetuses from each pregnant rat were taken for experiment. Fetuses were decapitated and the kidneys collected for immunohistochemical investigation.

Sections taken for immunohistochemical studies were fixed in 10% formalin, and then after dehydration and embedding in paraffin cut into 5  $\mu\text{m}$  slides. The expression of HSP 70 was evaluated in preparations originating from 16 fetuses from control group, 14 fetuses from Ex1 group (1 female was not fertilized) and 16 fetuses from Ex2 group (2 preparations from every individual, total 32 control slides, 28 Ex1 slides and 32 Ex2 slides).

The protein expression level was evaluated with a standard 3-step immunohistochemical procedure.

For antigen retrieval, preparations after deparaffinating were heat-processed in acidic environment (10 mM citrate buffer of pH=6.0 at 100°C). Consecutively, in the preparations endogenous peroxidase was blocked by incubation in 0.3% solution of  $\text{H}_2\text{O}_2$ .

• The preparations were incubated with the rabbit primary antibody against HSP 70 (Lab Vision Ab-3; RB-080-A0) in dilution of 1/100. For each preparation a

negative control was performed (a slide without primary antibody).

• The preparations were incubated with biotinylated secondary antibody against mouse and rabbit antibody (Biotinylated Link Universal, DakoCytomation, USA), and

• next with streptavidine conjugated with horse-radish peroxidase (Streptavidin-HRP, DakoCytomation, USA).

Finally, preparations were stained with AEC (DakoCytomation, USA) and hematoxylin.

Photographic documentation was undertaken using Colour Video Camera CCD-IRIS (Sony) compatible with a computer. The results of immunohistochemical research were evaluated quantitatively by the use of Analysis-pro, version 3 software (Soft Imaging System GmbH, Germany).

The analysis of microscopic picture was carried out by at 125  $\times$  magnification by evaluating HSP 70 expression. Of each preparation, 3 random fields were chosen, each of the surface of 781,193.35  $\mu\text{m}^2$ . The surface area showing the positive reaction (intensive red) was counted at each field.

Results were statistically analyzed using an ANOVA test and a Student's t-test. Average, standard deviations and percent of positive reaction in examined tissue field were determined. Differences were considered statistically significant when  $p < 0.05$ .

## RESULTS

Immunohistochemical investigation of kidney's cells with positive HSP 70 reaction showed mainly diffused, focally granular cytoplasmatic reaction (Fig. 1, 2, 3).

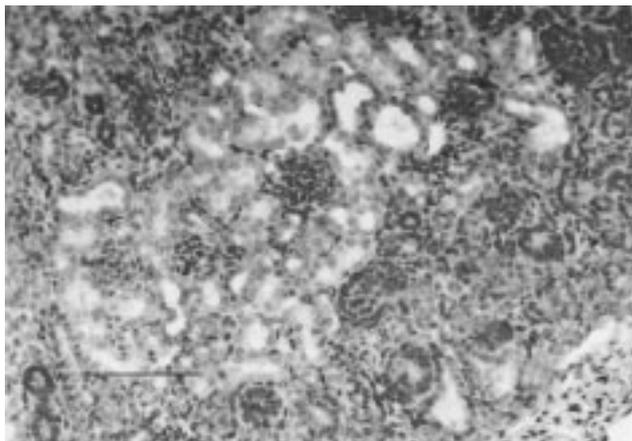
The cytoplasm staining was from bright pink to red. Intensity of HSP 70 positive cytoplasm staining of cells from experimental group Ex1 (Fig. 2) was much stronger than staining of HSP 70 positive cytoplasm cells from experimental group Ex2 (Fig. 3) where staining was of similar, or lower intensity compared to control group (Fig. 1).

Positive HSP 70 reaction appeared mainly in the cytoplasm of epithelial cells of renal tubules. In the glomeruli reaction was present rarely and usually it was seen in single endothelial cells and/or epithelial cells of the visceral layer of the glomerular capsule.

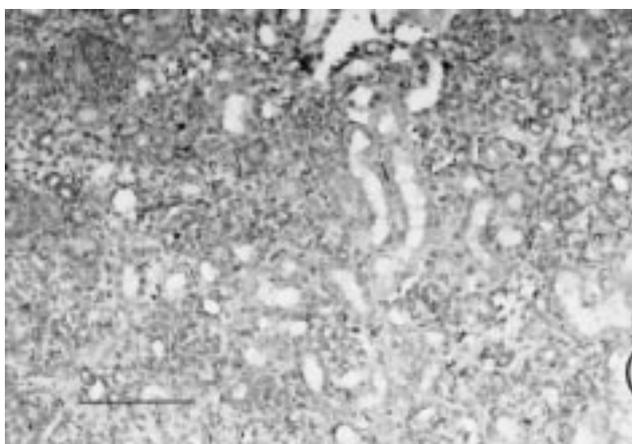
The range of colours assessed by the computer as a HSP 70 positive was set on intensive red colour, red-pink or pink colour were therefore not assumed to be positive. Because fetal tissues generally stain poorly, in preparations prevailed pink colour, but not red.

Out of 32 slides from control group, in 28 (87.5%) HSP 70 positive reactions were observed. In experimental group Ex1, in 26 slides out of 28 (92.9%) HSP 70 positive reactions were observed, and in experimental group Ex2, they were noted in 30 out of 32 (93.8%) slides.

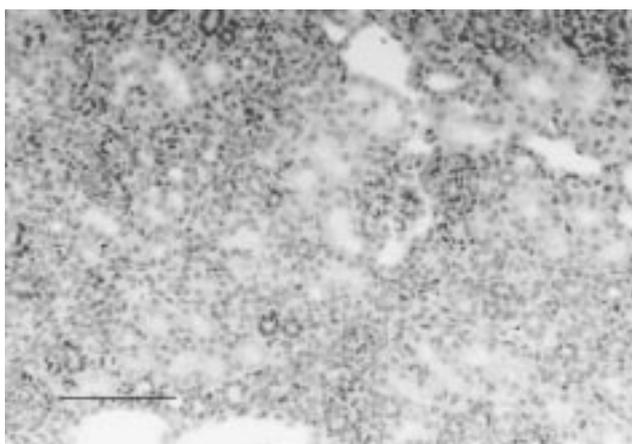
The average field covered by the HSP 70 positive reaction in experimental group Ex1 (874.4  $\mu\text{m}^2$ ) was statistically significantly greater than the average field of HSP 70 positive reaction in control group (191.3  $\mu\text{m}^2$ ) ( $p = 0.010$ ), and statistically significantly greater than the



**Figure 1.** Section of fetal rat kidney from control group (C). Pink, medium intensive, focal HSP 70 positive reaction visible in cytoplasm of tubular epithelial cells. AEC and hematoxylin staining. Bar, 100  $\mu\text{m}$ .



**Figure 2.** Section of fetal rat kidney from experimental group Ex1. Very intensive red HSP 70 positive reaction present in cytoplasm of the majority of visible tubular epithelial cells. AEC and hematoxylin staining. Bar, 100  $\mu\text{m}$ .



**Figure 3.** Section of fetal rat kidney from experimental group Ex2. Immunohistochemical HSP 70 positive reaction with low intensity. AEC and hematoxylin staining. Bar, 100  $\mu\text{m}$ .

average field of HSP 70 positive reaction in experimental group Ex2 (34.04  $\mu\text{m}^2$ ) ( $p=0.004$ ) (Tab. 1). The average field covered by the HSP 70 positive reaction in control group was statistically significantly greater compared to

**Table 1.** Comparison of the average fields with HSP 70 positive reaction in fetal kidney and percentages of fields affected with HSP 70 positive reaction in three groups of rat fetuses. Total surface of each examined field was 781,193.35  $\mu\text{m}^2$ .

	Control group C n=96	Experimental group Ex1, n=84	Experimental group Ex2, n=96
Average HSP 70 positive field ( $\mu\text{m}^2$ ) $\pm$ standard deviation	191.32 $\pm$ 8.53	874.4 $\pm$ 418.72	34.04 $\pm$ 6.8
Percentage of HSP 70 positive field in relation to examined field	0.024%	0.112%	0.004%
Statistical significance of differences*			
Control versus Ex1	Control versus Ex2	Ex1 versus Ex2	
p=0.01	p=0.0001	p=0.004	

n – number of measurements (examined fields).

\* Statistical significance of differences was calculated by ANOVA test.

experimental group Ex2 ( $p=0.0001$ ) (Tab. 1). The positive reaction in control group covered 0.024% of examined field, in experimental group Ex1 0.112% and in experimental group Ex2 0.004% (Tab. 1).

The results of histochemical investigations show that expression of HSP 70 in the kidney cells of fetuses whose mothers were given Adriamycin before pregnancy substantially increased as compared to the control. The increase in reaction to HSP 70 provides an evidence that Adriamycin induced oxidative stress in the fetal kidney cells causing damage to these cells. L-arginine, a nitric oxide substrate, given to pregnant females treated with Adriamycin significantly lowered HSP 70 expression. Thus, L-arginine decreased the oxidative stress induced by Adriamycin.

## DISCUSSION

During fetal life the dividing cells are protected against adverse effects of the environment by, among others, the heat shock proteins. This fact was noticed by Evans *et al.* [6]. The most probable reason for this phenomenon is the inability of eliminating all environmental stressors to which the healthy rat fetuses are susceptible. Neuhofer *et al.* studied the concentration of heat shock proteins in kidney papilla and noticed that in physiological conditions these cells are adjusted to their hyperosmotic environment through overproduction of HSP 70 [12]. Investigations conducted in this study confirm these dependences, as in the control group of fetuses the positive reaction to HSP 70 was noticed.

Also the apoptosis (intensified in fetuses as compared to adult individuals) of the lesion cells prevents improper development of the organism [9].

The free radicals produced by Adriamycin have great affinity to oxygen, causing that mitochondria, main cell energy producer become damaged. In the cell there occurs the so-called oxidative stress, which on the one hand is

stimulating for expression of HSP 70, but on the other hand is a cause of cell death by apoptosis [3, 7, 8]. From damaged mitochondria cytochrome C is released, which binds with other proteins and activates caspase-9. Caspase-9 starts caspase cascade, stimulating effector caspases, which in consequences leads to cutting off nuclear DNA and to apoptosis [17].

Increased expression of HSP 70 in fetal renal cells after Adriamycin administered to mothers in the present study confirms that renal cells were stressed, in that condition upon oxidative stress [19]. Damage to mitochondria and increased apoptosis of fetal renal cells after Adriamycin administered to mothers were already reported [13].

In the group of fetuses whose mothers apart from Adriamycin administered intraperitoneally before pregnancy had received L-arginine during 2 weeks of pregnancy, the substrate for NO, decreased HSP 70 expression was noted. This decreased even comparatively to control fetuses. This could be the evidence that NO had decreased cellular shock in that group. In case of the present study it decreased the oxidative stress induced by Adriamycin and leading to the unwanted apoptosis.

It should be stressed that, to the best of our knowledge, there are no reports published in the hitherto literature concerning changes in HSP 70 expression induced by NO in fetuses.

The ability of inciting and inhibiting apoptosis is extremely necessary in today's medicine as it appears to be invaluable in treating neoplasms and other difficult ailments. The results of the present study demonstrate that exogenous NO (L-arginine) could be helpful in inhibiting intensified apoptosis in fetuses as a late action of Adriamycin.

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