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THE MORPHOLOGICAL AND IMMUNOCYTOCHEMICAL EVALUATION OF PRIMARY RAT HEPATOCYTES UNDERGOING SPONTANEOUS CELL DEATH: MODULATION BY THE NITRIC OXIDE DONOR *S*-NITROSO-*N*-ACETYLPENICILLAMINE

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Nitric oxide (NO) is one of the smallest molecules synthesised in the human body. It is produced by three distinct nitric oxide synthase isoenzymes (NOS) and plays a number of physiological functions in many organs and tissues. Among its numerous properties is the ability to influence programmed cell death. NO can either inhibit or induce apoptosis depending on the context of its production. In the liver, NO is produced in greater amounts especially during inflammation. The effect of NO in liver physiology and pathophysiology can be both beneficial and detrimental. Therefore, the aim of our study was to examine NO effect on cell viability and cell death in primary rat hepatocyte culture. By using NO donor, S-nitroso-N-acetylpenicillamine (SNAP), the potential of exogenously delivered NO to influence spontaneous cell death in culture was examined. The morphological approach was used in order to discriminate between apoptotic and necrotic cell death. The nitrite level, urea production and alanine aminotransferase leakage were determined in the culture medium. The immunocytochemical detection of three apoptotic markers: cleaved caspase-3, cleaved caspase-9 and lamin A, was performed. Immunocytochemical analysis of hepatocyte apoptosis revealed different labelling pattern for each method, while the detection of cleaved caspase-3 best correlated with defined phenotypical criteria. Our data showed that under present conditions NO improved the viability of primary rat hepatocytes compared to untreated cells. This was manifested by the increase of viable hepatocytes in contrast to the decrease of necrotic and apoptotic hepatocytes as assessed by the morphological examination of cell culture. The NO effect was dose-dependent in the range of SNAP concentration between 200-800 μM.

INTRODUCTION

Nitric oxide (NO) is endogenously produced and a highly reactive free radical that takes part in the regulation of many physiological and pathophysiological processes in practically every organ[1]. Its production is accomplished by three nitric oxide synthases existing in the human organism. There are two constitutive isoforms, endothelial (eNOS) and neuronal (nNOS), which are expressed mainly in endothelial and nerve cells[2,3]. These constitutive isoforms intermittently produce smaller amounts of NO. The inducible isoform (iNOS) is expressed as a response to cytokines or as a result of stimulation by bacterial products and produces great amounts of NO. NO-induced effects are studied in various physiological and pathological circumstances by exploiting a number of models. Basically, there are two possible approaches to influence the processes which can be affected by NO. One approach is based on the modulation of NOSs activity. The other methods use various NO–releasing compounds (NO donors) to increase NO levels. Similar to the situation in other organ systems, NO production in the liver can have either beneficial or detrimental consequences[4,5]. The isoform of NOS that produces NO in a given situation is of particular significance for the outcome of NO action. Hepatic blood circulation, for example, is sustained by NO constitutively produced by eNOS[6]. The stimulation of iNOS expression and the production of greater amounts of NO by this isoform can have positive effects in liver regeneration on the one hand[7] or, on the other hand, it can contribute to oxidative liver injury and circulatory failure[8]. Another important aspect of NO interaction with cellular functions is its effect on apoptosis. There are studies, which indicate that NO possesses antiapoptotic effects in the liver as NO improves hepatocyte survival after liver injury caused by apoptosis inducing agents[9,10].

In the present study we evaluated the effect of the NO donor, S-nitroso-N-acetylpenicillamine (SNAP), on the viability and several other functional parameters of primary rat hepatocytes. In an attempt to distinguish between various modes of cell death we evaluated some methods of apoptosis detection in hepatocyte culture. Several immunocytochemically detected apoptotic markers were compared with the morphological evaluation of

cell viability. The morphological criteria were also used for quantitation of apoptotic and necrotic cells.

MATERIAL AND METHODS

Experimental animals

For the present study, male rats of Wistar strain (Velaz-Lysolaje, 200–280 g of body weight) were used. The experimental procedures with rats were carried out in accordance with the general guidelines of the 1st Faculty of Medicine, Charles University in Prague, for animal care and were approved by the Faculty Ethical Committee.

Primary rat hepatocyte cultures

Hepatocytes were isolated from rats by the two-step collagenase perfusion method as previously reported[11]. As assessed by trypan blue exclusion, cell viability after the isolation was more than 90 %. Cells were plated into 35-mm collagen-coated cell culture dishes provided with sterile glass coverslips 22×22 mm and maintained at 37 °C, 95 % air and 5 % CO_2 in William's medium E, supplemented with gentamicin, L-glutamine and 5 % foetal bovine serum. After 3-h attachment period the medium was changed.

Experimental design: To evaluate SNAP-induced effects, two protocols were followed. In both protocols, SNAP was added to the medium after the attachment period to obtain desired concentration. In the first experiment, cells were incubated for different time intervals (3, 6, 9, 12 h) in the medium without (control) or with 200 µmol/l SNAP. It was followed by another experiment where cells were treated with SNAP (0, 200, 400 and 800 µmol/l) for 9 h. After the incubation, medium was collected for biochemical analysis and cultures were processed for morphological examination.

Biochemical analysis

Urea concentration and alanine aminotransferase (ALT) leakage to the culture medium were determined using commercial kits (Sigma-Aldrich, Prague, Czech Republic). Nitrite levels in the cell culture supernatant were measured colorimetrically using Griess reagent[12].

Light microscopy and quantitative evaluation

For light microscopic examination, treated and control cell cultures grown on coverslips were fixed with 4 % formaldehyde and stained by haematoxylin-eosin (H-E) method. The following types of cultivated cells were quantified according to their morphology in H-E staining as viable cells, apoptotic cells and necrotic cells. Cells on each coverslip were counted in 20 randomly chosen non-overlapping fields in 63x objective magnification.

Electron microscopy

Cells from control culture were fixed at 4 °C with Karnovsky's solution: 2 % paraformaldehyde, 2.5 % glutaraldehyde in 0.08 M Na-cacodylate buffer (pH 7.4), with the addition of 20 mg $CaCl_2$/100 ml of the fixative fluid. Thereafter, cells were harvested using a rubber scraper, centrifuged and osmicated. Cells were then mixed with liquid agarose, which formed gel after cooling. Pieces of agarose gel (approximately 1 mm^3) containing hepatocytes were then dehydrated and processed into Epon.

Immunocytochemistry

The detection of immunocytochemical apoptotic markers was performed on untreated primary rat hepatocytes cultured for approximately 12 h. After this time period, both apoptotic and necrotic cells were present in sufficient quantity. For the detection of cleaved caspase-3, cells were briefly washed with phosphate-buffered saline (PBS) and fixed with 4 % paraformaldehyde in PBS (pH 7.4) for 20 min at 4 °C. Thereafter, coverslips with cells were washed with PBS and Tris-buffered saline + 0.1 % Triton X-100 (TBS/T). Unspecific antibody binding was blocked by incubation with 5 % normal goat serum (Sigma-Aldrich, Prague, Czech Republic) in TBS/T for 60 min. After 5 min wash with TBS/T, anti-cleaved caspase-3 (Asp175) rabbit polyclonal antibody (#9661; Cell Signaling Technology, Beverly, MA, USA) diluted 1:800 in TBS/T + 5 % normal goat serum was applied for 60 min at room temperature. To block endogenous peroxidase activity coverslips were immersed in 0.6% hydrogen peroxide in TBS. After 30 min, coverslips were washed with TBS/T and incubated with secondary antibody DAKO EnVision+, peroxidase, Rabbit (DAKO, Carpinteria, CA, USA) for 30 min at room temperature. Visualization was performed using diaminobenzidine (DAB; DAKO) substrate-chromogen solution. Coverslips were washed in H_2O and mounted with DAKO Faramount aqueous mounting medium. Some coverslips were counterstained with Harris's haematoxylin.

For the detection of cleaved Lamin A, cells were briefly washed with PBS and fixed with 4 % paraformaldehyde in PBS (pH 7.4) for 10 min at 4 °C. Permeabilization was done using 0.2 % Triton X-100 in PBS for 5 min at room temperature. After washes in TBS/T coverslips were blocked with 5 % normal goat serum in TBS/T for 60 min. Cells were washed with TBS and then incubated with anti-cleaved lamin A (Asp230) rabbit polyclonal antibody (#2031; Cell Signaling Technology) diluted 1 : 100 in TBS for 12 h at 4 °C. The blockage of endogenous peroxidase activity and visualization was performed as described in cleaved caspase-3 detection protocol.

For the detection of cleaved caspase-9, cells were briefly washed with PBS and fixed with 4 % paraformaldehyde in PBS (pH 7.4) for 10 min at 4 °C. After washes in TBS/T coverslips were blocked with 5 % normal goat serum in TBS/T for 60 min. Coverslips were then incubated with anti-cleaved caspase-9 (Asp353) antibody (rat specific) rabbit polyclonal antibody (#9507; Cell Signaling Technology) diluted 1 : 400 in TBS for 12 h at 4 °C. The blockage of endogenous peroxidase activity and visualization was performed as described above.

Statistical evaluation

Data obtained from the quantitative morphological evaluation of cultures incubated with different concentrations of SNAP were analysed by a two-way analysis

of variance (ANOVA). An alpha-value equal or less than 0.05 was considered statistically significant.

RESULTS

Morphological evaluation of the effect of snap on primary rat hepatocyte viability

For the quantitation of hepatocyte viability we have evaluated the cell morphology in haematoxylin-eosin staining where the following criteria were applied. Those cells, which attached to the layer of collagen and became rather flat in comparison to liver cells in vivo, were classified as viable. The adhesion of hepatocytes to the substratum could be observable already several hours after seeding. These cells had one or two round nuclei with evenly dispersed fine chromatin in which nucleoli could be distinguished (Fig. 1A). Those cells that were rounded and apparently began to detach from the substratum were regarded as necrotic. Moreover, these cells had darkly staining nuclei and pale, grey-coloured, granular cytoplasm (Fig. 1B). Apoptotic cells had characteristic blebs, homogenously eosinophilic cytoplasm and compacted nuclear chromatin or fragmented nuclei (Fig. 1C). These criteria were previously verified by the ultrastructural examination of cultured hepatocytes. Using electron microscopy, we could observe that viable and adherent hepatocytes had preserved cellular ultrastructure (Fig. 2). Apoptotic hepatocytes had intact cytoplasmic membranes. Their organelles, though densely packed due to the shrinkage of cells, were without significant alterations (Fig. 3). Meanwhile, cells presumed to be necrotic had disrupted cell membranes as well as disintegrated mitochondria and other membranous cellular components (Fig. 4). These characteristics pointed to an uncontrolled mode of cell death - necrosis.

The viability of hepatocytes in culture gradually decreased in time (Tab. 1). At the early phase of cultivation (3h after attachment) cell death was mainly necrotic. After 6hrs of cultivation, first apoptotic hepatocytes appeared. The percentage of both necrotic and apoptotic cells then further increased. The effect of added NO on the time course of these degenerative changes was analysed in the experiment where cells were incubated with 200 μM of SNAP. After quantitative evaluation of cultures in different time periods, it was found that the number of viable cells in SNAP-treated cultures was higher in all time intervals (after 3, 6, 9 and 12 h of cultivation) compared

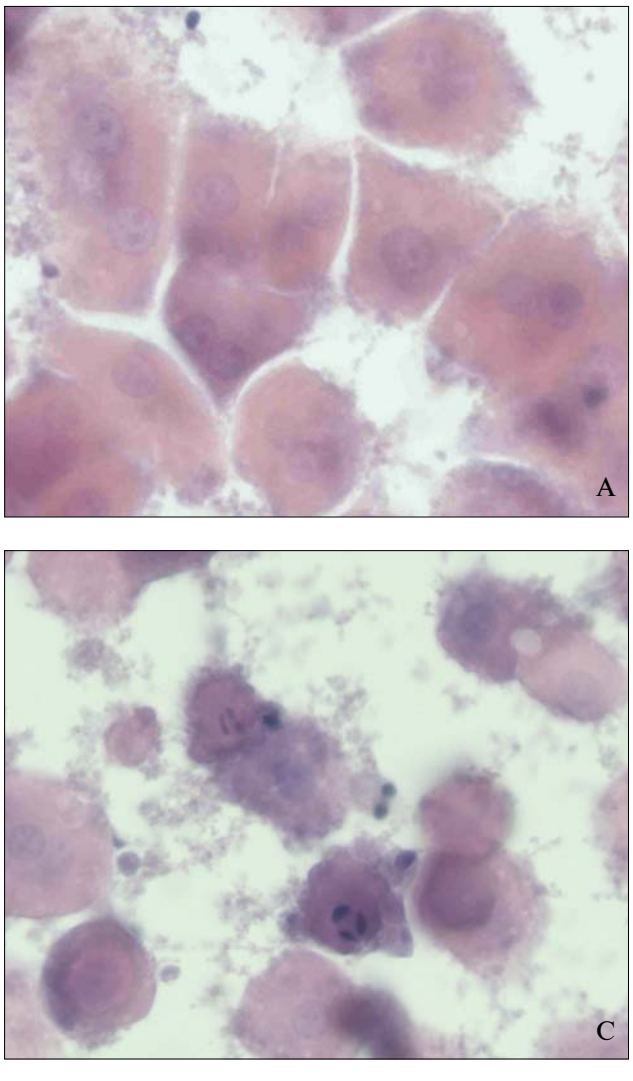

Fig. 1. (A) Viable and adherent primary rat hepatocytes. Nuclei contain finely dispersed chromatin. (B) Necrotic hepatocytes detached from the substratum display pale staining granular cytoplasm and dark (pyknotic) nuclei. (C) Two apoptotic hepatocytes. Note cytoplasmic protrusions (blebs) and nuclei with the condensed chromatin located on the nuclear periphery. (Haematoxylin-eosin staining, Obj. mag. 63X).

Table 1. Quantitative evaluation of hepatocyte viability. Cells were incubated with SNAP (0, 200μM) and counted according to their morphology at 3, 6, 9 and 12 h.

SNAP concentration	incubation time		cell type		
			viable	apoptotic	necrotic
0 mikromol/l					
	3h	average	80,9	0,3	18,8
		SEM	2,7	0,0	2,7
	6h	average	78,9	2,1	19,0
		SEM	2,2	0,3	2,4
	9h	average	65,1	10,2	24,6
		SEM	2,4	0,8	3,2
	12h	average	53,9	20,5	25,6
		SEM	2,1	1,3	3,3
200 mikromol/l					
	3h	average	87,3	0,6	12,1
		SEM	0,5	0,1	0,5
	6h	average	85,6	0,8	13,6
		SEM	0,8	0,1	0,7
	9h	average	81,1	6,0	12,9
		SEM	1,3	0,8	0,9
	12h	average	67,6	15,4	17,0
		SEM	3,8	0,6	4,2

Table 2. Quantitative evaluation of hepatocyte viability at 9h of incubation with SNAP (0, 200, 400, 800 μM). Cells were counted according to their morphology as viable, necrotic or apoptotic. *Statistically significant as compared with control culture.

SNAP concentration		percentage of examined cells		
mikromol/l		viable	apoptotic	necrotic
0	average	69,8	7,5	22,6
	SEM	1,7	0,5	2,2
200	average	74,7*	8,5	16,8
	SEM	1,1	0,1	1,0
400	average	78,1*	5,7*	16,2*
	SEM	0,9	0,6	1,5
800	average	83,7*	1,8*	14,5*
	SEM	0,7	0,3	0,8

Table 3. Biochemical analysis of culture medium after 9 h of cultivation with increasing concentrations of SNAP.

incubation time	9 hours	control	SNAP 200 μM	SNAP 400 μM	SNAP 800 μM
nitrite levels (mM)		21,08	52,43	84,12	167,33
		25,04	49,52	85,55	171,16
		20,68	52,17	94,63	171,22
	average	22,26	51,37	88,10	169,90
	SEM	1,14	0,76	2,69	1,05
urea concentration (mg/dcl)		10,00	11,12	19,28	10,80
		12,56	11,60	11,04	13,12
		11,52	11,12	10,32	15,36
	average	11,36	11,28	13,55	13,09
	SEM	0,61	0,13	2,35	1,07
ALT concentration (U/L)		31,03	37,37	50,24	32,92
		39,25	50,19	39,14	32,71
		31,50	32,34	50,03	49,09
	average	33,93	39,97	46,47	38,24
	SEM	2,18	4,34	2,99	4,43

to control. The greatest effect of SNAP on the viability of hepatocytes, as seen in Tab. 1, was observed after 9 h of cultivation.

This 9 h incubation period was then chosen for experiment to examine the effect of different SNAP concentrations. As Tab. 2 shows, the number of viable hepatocytes increased and the number of necrotic and apoptotic hepatocytes decreased dose dependently when cells were treated with SNAP (200, 400, 800 μM).

Nitrite levels in the medium after snap addition and biochemical evaluation of no effects

By determining the NO-end oxidation product, nitrite, in the culture medium, it was confirmed that NO was in fact released from SNAP. Nitrite levels increased gradually as a function of time. In the experiment where different amounts of SNAP were used, nitrite levels increased in a dose dependent manner. ALT leakage to the culture medium as well as urea production was not affected by SNAP (see Tab. 3).

Immunocytochemical evaluation of hepatocyte spontaneous apoptosis

In order to further validate our morphological criteria for apoptosis detection we tried to detect some apoptotic markers using the immunocytochemical approach. The immunocytochemical detection of three apoptotic markers: activated caspase-3, activated caspase-9 and cas-

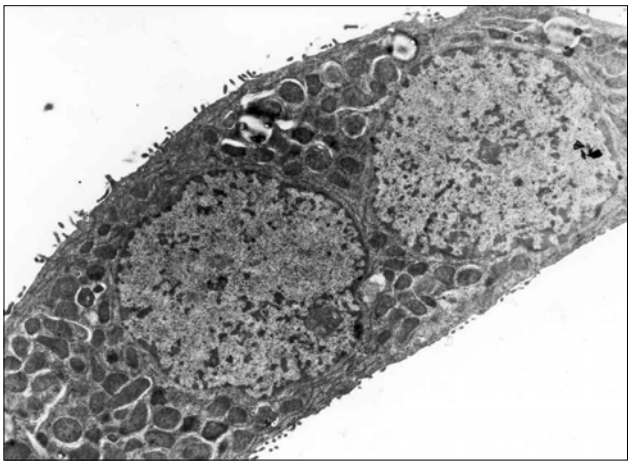

Fig. 2. Electron micrograph of a binucleate hepatocyte with the flattened basal surface reflecting its adhesion to the substratum. Ultrastructure corresponds to the viable phenotype of the primary hepatocyte in culture. (Orig. mag. 12500X).

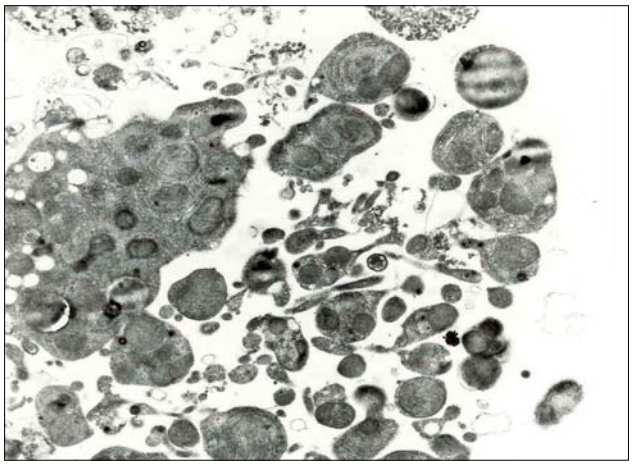

Fig. 3. Electron micrograph of an apoptotic hepatocyte after it fragmented into apoptotic bodies containing relatively preserved organelles surrounded by intact membranes. (Orig. mag. 12000X).

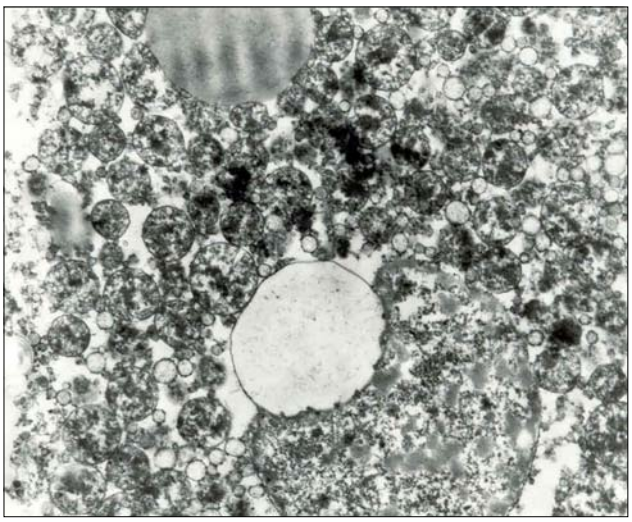

Fig. 4. Electron micrograph of a necrotic hepatocyte. Membranous structures lost their integrity as a result of uncontrolled cell lysis. (Orig. mag. 15000X).

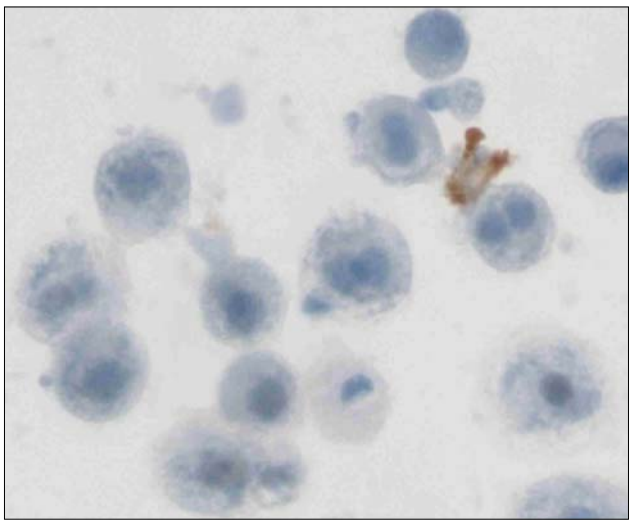

Fig. 5. Immunocytochemical detection of active caspase-3. Positively labelled cell with typical apoptotic morphology. (Immunoperoxidase reaction –DAB, haematoxylin counterstaining, Obj. mag. 63X).

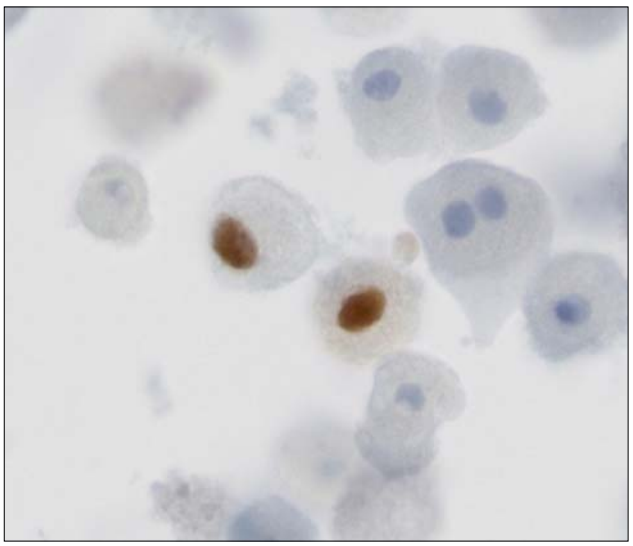

Fig. 6. Immunocytochemical detection of cleaved lamin A. The immunoreactivity is localized in the nuclei of some rounded and detached necrotic cells, meanwhile the majority of these cells, as can be noted on this photograph, is negative. (Immunoperoxidase reaction –DAB, haematoxylin counterstaining, Obj. mag. 63X).

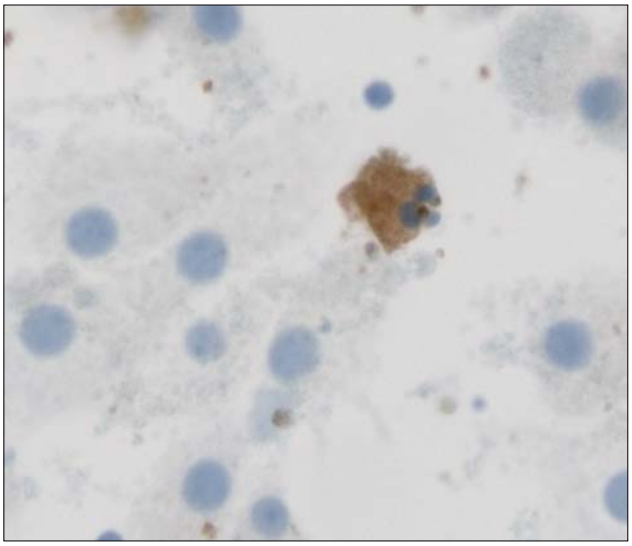

Fig. 7. Immunocytochemical detection of active caspase-9 shows positivity in the cytoplasm of an apoptotic hepatocyte containing nuclear fragments. Neighbouring adherent cells display no immunoreactivity. (Immunoperoxidase reaction –DAB, haematoxylin counterstaining, Obj. mag. 63X).

pase-cleaved lamin A, was performed. Cells labelled with the antibody against cleaved caspase-3 were the same as those classified as apoptotic in haematoxylin-eosin staining (Fig. 5).

The results of the immunocytochemical detection of cleaved lamin A showed that the cell labelling was somewhat different from that obtained using cleaved caspase-3 antibody. Cleaved lamin A immunopositivity could be located in nuclei or nuclear fragments of apoptotic cells as well as in some cells considered to be necrotic (Fig. 6). Similar results were obtained after the detection of active caspase-9 (Fig. 7). In this case, the immunoreaction was positive not only in the cytoplasm of the blebbing apoptotic hepatocytes and apoptotic bodies, but also in some cells with necrotic features.

DISCUSSION

We examined the potential of NO donor SNAP to suppress spontaneous apoptosis of primary rat hepatocytes. SNAP improved rat hepatocyte viability as manifested by an increase in viable compared to dead cells in treated cultures. NO apparently decreased both types of cell death. However, NO did not abolish spontaneous cell death in culture completely. It should be taken into the account that, to a certain extent, the isolation and processing of cells for cultivation is a kind of stress in itself. Therefore, although the initial viability of isolated hepatocytes was more than 90%, after 3 hours of cultivation there was apparent decrease in the number of viable cells. This spontaneous loss of viability is caused by the obvious difference in hepatocyte environment between in vivo and in vitro situation. The type of attachment substratum, cell density and the composition of culture media (especially the addition of some hormones) can significantly influence but not completely abrogate the rate of apoptosis in primary hepatocyte culture[13]. The importance of cell attachment for maintaining the epithelial cell viability is emphasised, for example, by the finding that already 15 min after cells had detached from the substratum proapoptotic molecule Bax was activated[14].

Within the present experimental conditions it was demonstrated that NO was beneficial, which is in agreement with the previous findings of NO antiapoptotic effects in hepatocyte cultures[15, 16], but not relevant to the report on apoptosis caused by NO (ref.[17]).

Here, exogenously delivered NO did not alter urea production – a functional parameter of cultured hepatocytes. This probably reflects NO ability to improve hepatocyte viability by preventing cell death rather then by stimulating hepatocyte metabolic activity. Moreover, the common substrate for NO and urea synthesis is L-arginine. NO can inhibit activity of arginase, which converts L-arginine to L-citruline in urea cycle, thus exogenously delivered NO can inhibit urea synthesis[18]. Furthermore, as assessed by ALT leakage from the cells to the culture media, SNAP did not affect cell membrane integrity when compared to basal conditions, which supports our morphological findings of the stabilising effect of exogenous NO against spontaneous cell death.

As a part of the present study of primary rat hepatocyte apoptosis and necrosis we evaluated various apoptotic markers that could be used to distinguish between

different modes of cell death. In our previous study of cyclosporine A effects on primary rat hepatocytes we applied Annexin V-biotin to label apoptotic cells in culture. However, our results did not allow us to discriminate between apoptotic and necrotic hepatocytes since Annexin V labelled cells undergoing both types of cell death[19].

Data presented in this study show that comparison of immunocytochemical detection of apoptotic markers with the morphological assessment of hepatocyte morphology makes it possible to determine the specificity of applied methods. The best correlation between morphological criteria and immunocytochemistry was found by using antibody against active caspase-3. When antibodies against cleaved lamin A or active caspase-9 were applied, they also labelled cells that were apparently necrotic or were perhaps in a late stage of apoptosis, so called secondary necrosis. The antibody against cleaved lamin A recognizes the epitop that appears after specific cleavage of lamin A by active caspase-6. The immunoreactivity for cleaved lamin A implies, that caspase-6 was active in these cells considering that this caspase is the only one known to act on lamin A in the execution phase of apoptosis[20]. Furthermore, caspase-6 is activated after cleavage by caspase-3 (ref.[21]). Since not all necrotic hepatocytes were positive for cleaved lamin A and the majority of apoptotic cells were labelled, we conclude that this epitop is retained in the apoptotic cells even after they proceed to secondary necrosis. This conclusion is also confirmed for the result of cleaved caspase-9 detection. The question whether this caspase is still active in the final phase of apoptosis remains unresolved. In contrast to cleaved lamin A and active caspase-9 immunoreactivity, the positive labelling of hepatocytes for active caspase-3 disappears in these late apoptotic or rather secondary necrotic cells. These findings allow immunocytochemical differentiation between various steps of the apoptotic process.

CONCLUSION

Our data support a modulatory effect of the NO donor SNAP on primary rat hepatocytes. This modulatory effect was documented by the increased number of viable cells and decreased number of apoptotic and necrotic cells in treated cultures. The criteria for the morphological evaluation of hepatocyte viability and hepatocyte cell death were confirmed using apoptotic markers: cleaved caspase-3, cleaved caspase-9 and lamin A, which allow one to detect apoptotic cells in various stages of cell death. Be applying these markers it will be possible to delineate the effect of NO on primary rat hepatocyte viability in this culture system in future studies dealing with different kinds of cell injury.

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