

# Nitric oxide and apoptosis in white mice heart during aging

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## ABSTRACT

The process of aging and senescence is associated with a decline in several organ functions and ultimately takes away independence and reduces quality of life. Programmed cell death or apoptosis is highly involved throughout the aging process, from early developmental changes to senescent declines in function. Multiple pathways exist for inducing apoptosis. In recent years, several studies have established that nitric oxide (NO) and its reaction products can either promote or prevent apoptosis in a multitude of settings. The ubiquitous distribution of the NO synthases and the remarkable diffusibility and diverse chemical reactivity of NO in biological systems make this molecule unique among the regulators of apoptosis. This study was designed to investigate the influence of nitric oxide (NO) on cardiomyocyte's apoptosis in white mice during aging. 30 white mice were used. The animals were distributed in three age groups: juveniles, adults and senescents. The animals were killed under ether narcosis. The heart was removed. Apoptotic nuclei were detected by immunomorphological and Flow cytometry assay. Concentration of NO in samples was measured by ESR Study. The results showed, that the increase of apoptosis in cardiomyocytes during aging is accompanied by decrease in NO production. According to our data, it can be considered that the antiapoptotic effect of nitric oxide on cardiomyocytes declines with aging.

**KEYWORDS:** *cardiomyocyte, aging, nitric oxide, apoptosis*

Programmed cell death or apoptosis is highly involved throughout the aging process, from early developmental changes to senescent declines in function [4].

It is now widely accepted that the age-related downregulation of apoptosis does occur in the postmitotic organs, such as brain and heart [1].

Apoptosis have significant effects in the age-associated decline in cardiac function. During aging, the human heart loses a significant number of myocytes. In fact, the initial ventricular myocyte population may decline by 30% as the heart ages [4].

Multiple pathways exist for inducing apoptosis. In recent years, several studies have established that nitric oxide (NO) and its reaction products can either promote or prevent apoptosis in a multitude of settings. The ubiquitous distribution of the NO synthases and the remarkable diffusibility and diverse chemical reactivity of NO in biological systems make this molecule unique among the regulators of apoptosis [7].

The aim of present work was to investigate the possible correlation between the intensity of NO production and the rate of apoptosis in cardiomyocytes during aging.

## MATERIALS AND METHODS

**Animals:** 30 white mice were used. According to the age the animals were distributed in three groups: I group - juveniles (10 mice, 2-months old,  $18,0 \pm 2,0$  g body weight), II group - adults (10 mice, 10-months old,  $25,0 \pm 2,0$  g body weight), III group - senescents (10 mice, 18-months old,  $30,0 \pm 2,0$  g body weight). The animals were maintained at 18-22°C temperature and light-controlled environment with a 12:12-h light-dark cycle and provided with food and water ad libitum.

**Methods:** The animals were anesthetized with ether narcosis and were sacrificed by the method of decapitation. The chest was opened and heart was removed.

**Immunomorphological Assay:** Apoptosis was detected in heart sections by the in situ oligo ligation (ISOL) assay, using an ApopTag ISOL assay kit (Chemicon, a Serologicals Company). This assay relies on the selective

binding of biotin-labeled hairpin oligonucleotide probes to the types of genomic DNA ends that are characteristic of the double-strand breaks in apoptotic cells. Briefly, the paraffin-embedded tissue sections were deparaffinized, rehydrated in a graded alcohol series, and incubated with proteinase K at room temperature for 15 minutes. Endogenous peroxidase in the sections was inhibited with 3% hydrogen peroxide for 20 minutes. The slides were then incubated with T4 DNA ligase at 16-20°C overnight as per the manufacturer's instructions to catalyze blunt-end ligation of biotinylated oligo A with fragmented double-strand DNA. Slides were then incubated with streptavidin peroxidase conjugate, and oligo A binding (i.e., DNA fragmentation) was detected by staining with DAB, with methyl green as a counterstain. The slides were viewed under light microscope. Numbers of apoptotic nuclei were counted in 100 visual area.

**FACScan Flow Cytometry Assay:** Samples were homogenized with a glass-Teflon Potter homogenizer in the 2,2 M succrose solution prepared on the phosphate buffer (pH=7,4). Nuclei were collected by centrifugating the homogenate at 18000 x g for 45 min. Received pure of nuclei was suspended in 3 ml TMS solution and centrifugated at 3000 x g during 10 min. 70% ethanol was added to the pellet and samples were incubated during 24-h at 4°C. Then 20 µl of RNase (10 µg/ml) was added and samples were incubated during 30 min at 37°C. After 1 ml of EB staining solution was added, samples were incubated during 30 min at 37°C. Number of apoptotic nuclei were counted by a Becton Dickinson (Mountain View, CA) FACScan flow cytometer.

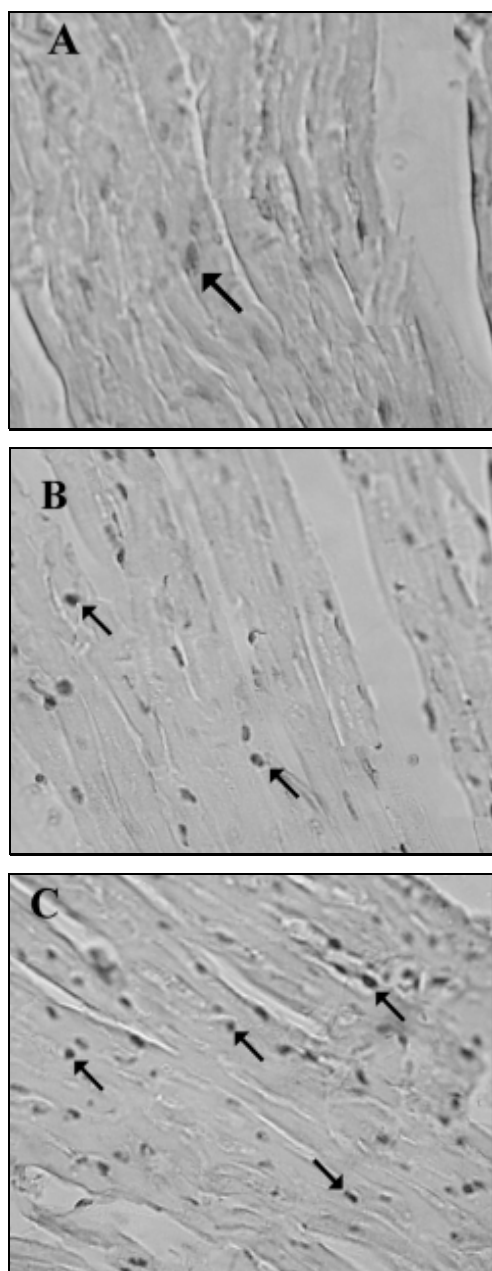
**ESR Study:** The intensity of NO production was studied by ESR method. The tissue samples were treated by the diethyldithiocarbamate-Na ( $C_5H_{10}NS_2Na$ ) with the further storage in liquid nitrogen. The amount of NO was counted by ESR spectrometer RE 1307 (Chernogolovka, Russia).

**Statistical analyses** were performed using Student t test for independent samples. Probability (P) values of <0,05 were considered to be significant. All data are expressed as mean (standard deviation).

## RESULTS AND DISCUSSION

Data obtained by Flow cytometry indicate, that the number of apoptotic cardiomyocytes increases in adult

animals ( $P < 0,05$ ) and remains at the same high level in senescent animals ( $P > 0,05$ ) (Tab.1).



**Fig.1** A. I group - juvenile mouse heart; B. II group - adult mouse heart; C. III group - senescent mouse heart. Black arrows represent ISOL-labeled apoptotic nuclei.

The immunomorphological study of paraffin-embedded tissue samples by ISOL method revealed more precise changes in the intensity of apoptosis in cardiomyocytes. The apoptotic nuclei were observed in the tissue sections of all studied age groups (Fig.1-A,B,C). The quantitative analyses of the apoptotic nuclei have shown that there is the continuous increase of this parameter during aging. The highest rate of apoptosis is characteristic for cardiomyocytes of senescent animals. The amount of apoptotic nuclei in senescent animals is significantly higher when compared with the same parameters of both previous age groups ( $P < 0,05$ ) (Tab.1).

ESR study showed, that the intensity of NO production is approximately at the same level in juvenile and adult mice ( $P > 0,05$ ) and significantly decreases in senescent animals ( $P < 0,05$ ) (Tab.1).

Thus, the increase of apoptosis in cardiomyocytes during aging is accompanied by decrease in NO production (Fig.2).

The decision for a cell to undergo apoptosis is the result of a shift in the balance between the antiapoptotic and proapoptotic forces within a cell [7].

Razavi et al. [2005] have shown, that NO is an important regulator of apoptosis within the mammalian system, capable of both inducing and preventing apoptosis, depending upon the level of NO production and environmental milieu. It is reported that high levels of NO produced by inducible nitric oxide synthase (iNOS) promote apoptosis while basal levels of NO production from endothelial nitric oxide synthase (eNOS) protect cardiomyocytes from apoptosis [5,6]. NO is believed to act as an anti-apoptotic agent by inhibiting caspase activity via S-nitrosylation in cardiomyocytes. [3]. Whereas the NO stimulated apoptosis in cardiac myocytes is associated with an altered expression pattern of apoptosis regulators of the Bcl-2 family [2].

According to our data, it can be considered that the antiapoptotic effect of nitric oxide on cardiomyocytes declines with aging.

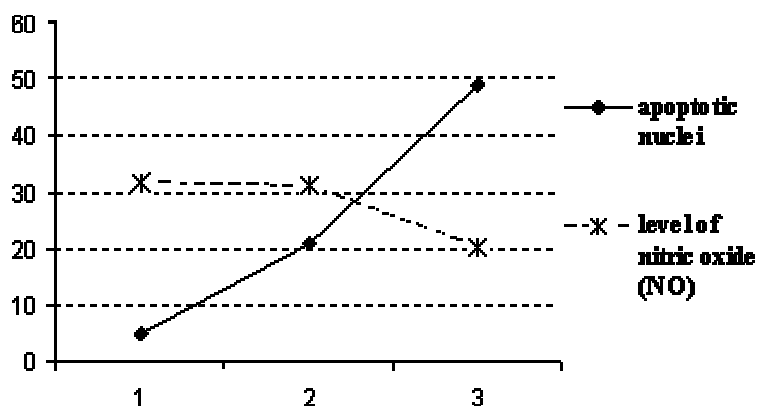
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Groups	Parameters	Apoptotic nuclei Flow cytometry	Apoptotic nuclei ISOL	Nitric oxide (NO)
I group – juvenile mice		8,27 %±0,9 %	4,77±5,14	31,67 mm/mg±1,53 mm/mg
II group – adult mice		12,19 %±2,5 % *	20,76±15,09 *	31 mm/mg±1,41 mm/mg
III group – senescent mice		13,12 %±1,26 % **	48,79±20,45 **	20,33 mm/mg±2,08 mm/mg **, ***

Note: \* I-II group -  $P < 0,05$ ; \*\* I-III group -  $P < 0,05$ ; \*\*\* II-III group -  $P < 0,05$

**Tab.1** The distribution of apoptotic nuclei and nitric oxide (NO) in white mice heart during aging.



1. I group – juvenile mice; 2. II group – adult mice; 3. III group – senescent mice.

**Fig.2** Age relation changes in white mice.

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## Возрастные особенности влияния оксида азота (NO) на апоптоз в сердце белых мышей

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### РЕЗЮМЕ

Данные литературны указывают, что оксид азота является двояким регулятором процесса апоптоза, поскольку характеризуется как про- так и антиапоптотическим эффектом. Целью нашего исследования являлось определение возрастных особенностей влияния NO на степень апоптоза кардиомиоцитов в эксперименте на белых мышах. Эксперименты проводились на ювенильных, зрелых и старых животных. Количество апоптотических ядер в кардиомиоцитах определялось с помощью иммуноморфологического метода и метода проточной цитометрии. Количество NO определяли с помощью радиоспектрометра РЭ-1307. Установлено, что в процессе старения у белых мышей увеличение числа апоптотических кардиомиоцитов находится в положительной корреляции с уменьшением интенсивности образования NO.

**Ключевые слова:** кардиомиоцит, старение, оксид азота, апопто.