The Effects of Two Nitric Oxide Donors in Acute Inflammation in Rats

Experimental data

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We aimed to investigate the effects of two nitric oxide donors in acute inflammation in rats. The experiment was carried out on white Wistar rats, randomly distributed in 4 groups of 5 animals each; the substances were administered intraperitoneally as follows: Group 1 (SS): saline solution 0.1mL/100 g body weight (control); Group 2 (IND): indometacin 150 mg/kg body weight; Group 3 (NEB): nebivolol 1 mg/kg body weight; Group 4 (GSNO): S-nitroso-glutathione 1 mg/kg body weight. An experimental model of acute hind paw inflammation with carrageenan was used for the researches. The influence of the nitric oxide donors on blood parameters, specific inflammatory and immune markers was evaluated 24 h, respectively 72 hours after the injection of irritant agent. The experimental protocol was implemented according to the recommendations of our University Committee for Research and Ethical Issues. The administration of nitric oxide donors nebivolol and S-nitroso-glutathione was accompanied by a substantial diminution of paw edema, as well as by an important decrease in the percent of lymphocytes, a reduction of interleukin 6 and tumor necrosis factor alpha values. The effects of nebivolol were more accentuated than of S-nitroso-glutathione, but less intense than of indomethacin in the experiment. The treatment with nebivolol and S-nitroso-glutathione produced anti-inflammatory effects on local acute inflammation in the carrageenan-induced paw edema test in rats.

Keywords: Nebivolol, S-Nitroso-glutathione, inflammation, nitric oxide, rats

Nitric oxide (NO) is a signal molecule that plays a key role in the pathogenesis of inflammation, exhibiting an anti-inflammatory effect under normal physiological conditions [1]. It is also considered to be a pro-inflammatory mediator, that induces inflammation due to its over-production under pathological conditions. It is a strong neurotransmitter in neuronal synapses and contributes to the regulation of apoptosis [2] and is also involved in the pathogenesis of inflammatory disorders of the joint, intestine and lungs [3-5]. Therefore, the use of NO inhibitors represents an important therapeutic progress in the management of inflammatory diseases. The selective inhibitors of NO biosynthesis and the synthetic analogs of arginine have been proved to be useful in the treatment of NO-induced inflammation [6,7].

NO is synthesized by many cell types that participate in the immune processes and inflammation [8]. The main enzyme involved is the inductive isoform nitric oxide synthase type 2 (NOS-2), which produces a sustained NO synthesis [3,10]. The expression NOS-1 and NOS-3 is constitutive, calcium/calmodulin-dependent and generates NO in the picomolar-nanomolar range. NOS-2 in macrophages is induced by the stimulatory action of tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), interferon gamma (IFNγ), endotoxin or lipopolysaccharide (LPS), and generates large amounts of NO in the micromolar range for a prolonged period. The level of NOS-2 may reflect the state of inflammation [3]. However, the role of NO in non-specific and specific immunity *in vivo,* in immunologically mediated diseases and in inflammations is poorly understood. NO does not act via a receptor, the specificity of the target cell depends on its concentration, chemical reactivity, proximity to target cells and the way target cells are programmed to respond [8].

The aim of this study was to evaluate the effects of two nitric oxide donors in experimental-induced acute paw inflammation in rats.

Experimental part

Material and method

Adult male Wistar rats (weighting 200-250 g), from our University bio-base, were used in the research. The animals were brought one day prior to the study for accommodation, being kept under standard laboratory conditions (a constant temperature of 21 ± 2°C, relative humidity of 50-70% and alternating illumination mode - light/dark ratio = 12 h/12 h)[9]. In order to avoid the chrono-biological influences, the tests were performed between 8.00-12.00 am. The animals were fed with standardized pellets, except for the period of the experiments. Drinking water was provided *ad libitum* with special devices. The following drugs were used: nebivolol, S-nitroso-glutathione (Sigma Chemical Aldrich Co.), which were dissolved in saline, and the solutions were extemporaneously prepared.

The animals were randomly distributed in 4 groups of 5 rats each, treated intraperitoneally as follows: Group 1 (coded SS): saline solution 0.1mL/100 g body weight - control; Group 2 (coded IND): 150 mg/kg body weight indomethacin; Group 3 (coded NEB): 1 mg/kg body weight nebivolol; Group 4 (coded GSNO): 1 mg/kg body weight S-

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nitroso-glutathione. The effects of nitric oxide donors were investigated using the experimental model of acute hind paw inflammation, induced after intraplantar injection of carrageenan in rats. The subcutaneous administration of 0.2 mL 1% carrageenan was accompanied by swelling, with a maximum intensity after 3-5 h and maintained for about 24 hours after the irritant agent administration. Indomethacin was used as a positive control drug in the experiment, having known anti-inflammatory effects in various acute and subacute inflammatory model in rodents [11,12]. The degree of local inflammatory edema and its duration was assessed by using a plethysmograph (PanLab Apparatus). We measured the posterior paw volume according to the following scheme: before the induction of edema (initial volume at moment zero), at 24 h and 3 days after the inflammation was developed [13,14]. The results were expressed as percentage of reduction in inflammation, compared to initial volume in control animals. The level of edema evolution was calculated by determining the percentage of rat paw volume increase (%PVI) using the following formula:

%PVI = (determined paw volume - initial volume) x 100 / initial volume

The anti-inflammatory activity was evaluated by calculating the percent inhibition of paw edema (%PIE) according to the equation:

%PIE = (%PVI control -%CVL treated) x 100 / %CVL control

The influence of nitric oxide donors on blood parameters, specific inflammatory and immune markers was evaluated prior to the induction of inflammation, at 24 hours and 72 h in the experiment. To assess the blood count, 2 mL of venous blood were taken from the retroorbital plexus of the animals, under general anesthesia with enflurane. The HEMAVET 950, an automatic analyzer, operating on the principle of fluorescence flow cytometry, was used for hematological investigations. The evaluation of the complement fractions C3 and C4 activity was based by Hartmann-Brecy technique (consisting of *hemolysis* with serum *complement* of sensitized *erythrocytes)*. The blood levels of interleukin IL-6 and tumor necrosis factor alpha (TNF-α) were also assessed.

The experimental protocol was implemented according to the recommendations of the Committee for Research and Ethical Issues of Grigore T. Popa University, in agreement with the international ethical regulations and the EU Directive 2010/63/EU regarding the investigations performed on laboratory animals [15,16]. The data were presented as the mean of values ± the standard deviation (SD) of mean and statistically processed using the ANOVA method, implemented in the SPSS 17.0 software for Windows. The values of the coefficient *p* below 0.05 were considered to be statistically significant compared to control group.

Results and discussions

We assessed the effects of two nitric oxide donors, NEB and GSNO on the acute inflammatory process in rats with experimental-induced paw edema after local administration of carrageenan. Nebivolol is a beta 1 adrenergic receptor blocker and a NO releaser by acting on the inducible isoform of the NOS [15]. GSNO is an S-nitrosothiol NO donor with an essential role in transduction NO signaling, being a source of bioavailable NO in the body [18,19].

Subcutaneous injection of 1% carrageenan solution on the plantar surface of the hind paw induced an acute inflammatory reaction associated with visible changes such as enlargement, redness and local pain, suggested by animals licking and biting the paw. The inflammatory process was developed and progressed, reaching the highest intensity after 6 hours, thereafter gradually slowed over time, maintaining clear manifestations for about 72 hours after chemical irritation of the paw [20,21]. In our laboratory conditions, in the group treated with saline, the volume of paw injected with carrageenan progressively increased, reaching a peak between 1 hour and 6 hours, then gradually diminished, maintaining elevated values at 72 hours in the experiment (fig. 1).

In the IND group a rapid and progressive decrease in paw volume was observed even after one hour (**p<0.01), much more pronounced in the range of 3 to 6 h (**p<0.01), but statistically significant also at 24 and 72 h (**p<0.01) in the carrageenan-induced paw inflammation test in rats (fig. 1). The administration of NO donors NEB and GSNO produced a significant decrease in the volume of the inflamed paw, statistically significant (**p<0.01) compared to control in the experiment (fig. 1).

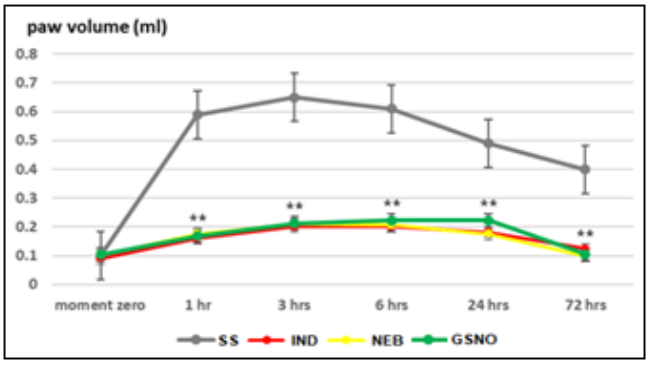

Fig. 1. Effects of NEB and GSNO on the carrageenan-induced paw inflammation in rats. Each point represents the mean ± standard deviation (SD) of the average paw volume, for 5 animals in a group. **p<0.01 statistically significant compared to control group.

IND manifested the most pronounced %PIE after one hour (84.13 ± 3.25) and at 72 h (87.41 ± 4.55). NEB and GSNO produced the highest %PIE at 1 h (85.19 ± 3.10 and 87.44 ± 5.28) respectively and at 72 h (100.65 ± 7.62 and 99.36 ± 6.23), respectively, in the rat carrageenan paw inflammation test (fig. 2).

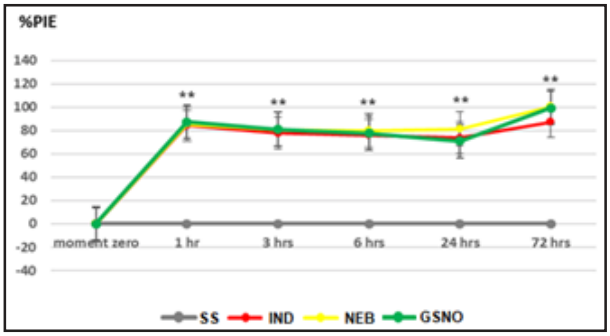

Fig. 2. %PIE determined by NEB and GSNO in the carrageenan-induced paw inflammation in rats. **p<0.01 statistically significant compared to control group.

During the experiment the gradual increase of the polymorphonuclear neutrophils (PMN) percent and the progressive reduction of the percentage of Ly was revealed in control group. The treatment with IND, NEB and GSNO induced a continuous increase in the percentage of PMN and a decrease in the percentage of lymphocytes (Ly) in carrageenan-induced paw inflammation test in rats (table 1). The treatment with IND and with NEB resulted in an increase of PMN, eosinophils (E) and monocytes (M)

		Leukocyte formula (%)				
		PMN	Ly	E	M	B
SS	M0	17.62±1.70	75.12±1.47	2.40±0.27	4.84±1.02	0.10±0.07
	24 h.	32.64±2.36	63.76±3.40	1.32±0.27	2.42±0.41	0.00±0.00
	72 h.	33.70±1.85	61.92±1.87	1.20±0.16	3.20±0.54	0.00±0.00
IND	M0	13.40±1.30*	78.20±0.20*	1.80±0.80	6.55±0.65	0.05±0.05
	24 h.	28.40±3.10*	61.20±4.50*	4.70±0.60*	5.30±0.80*	0.00±0.00
	72 h.	33.55±6.25	61.05±5.15*	2.20±0.50	3.10±1.60	0.10±0.00
NEB	M0	17.20±2.50	75.30±2.10	1.45±0.65	6.05±1.05*	0.00±0.00
	24 h.	28.60±5.20*	61.70±3.00*	5.00±2.30*	4.60±0.10*	0.10±0.00
	72 h.	28.70±0.10*	64.25±0.05*	2.05±0.55	4.95±0.75*	0.05±0.05
GSNO	M0	17.10±1.90	75.60±3.00	1.00±0.40*	6.25±0.65*	0.05±0.05
	24 h.	23.40±0.90*	68.05±0.25*	1.75±0.05	6.75±1.15*	0.05±0.05
	72 h.	29.70±1.60*	63.90±1.40*	1.75±0.45	4.55±0.65*	0.10±0.00

Table 1
THE EFFECTS OF NEB AND GSNO ON THE LEUKOCYTE FORMULA ELEMENTS IN THE CARRAGEENAN-INDUCED PAW INFLAMMATION IN RATS. VALUES ARE PRESENTED AS MEAN ± SD OF THE LEUCOCYTE FORMULA ELEMENTS PERCENTAGE FOR 5 ANIMALS IN A GROUP. *p<0.05 STATISTICALLY SIGNIFICANT COMPARED TO CONTROL GROUP

percent, and a decrease in the percentage of Ly, statistically significant (*p<0.05) compared to control group at 24 h. The percentage of PMN decreased and the percentage of Ly increased, statistically significant compared to SS group after 72 h. Intraperitoneal injection of GSNO was associated by a decrease in PMN percent (*p<0.05) and an increase in Ly and M percent (*p<0.05), statistically significant compared to control at 24 and 72 h in the experiment (table 1).

No significant variation of the reactive C protein values between IND, NEB, GSNO and control groups at 24 hours and 72 h was noted. The treatment with IND and GSNO was associated with an increase in the serum C3 fraction level, statistically significant (*p<0.05) compared to the control group at 24 and 72 h in the experiment. The administration of NEB induced the accentuation of C3 fraction activity only 24 h after the induction of local paw inflammation in rats (fig. 3). No statistically significant variations in C4 activity between the groups receiving IND, NEB, GSNO and the SS were observed during the experiment (fig. 4).

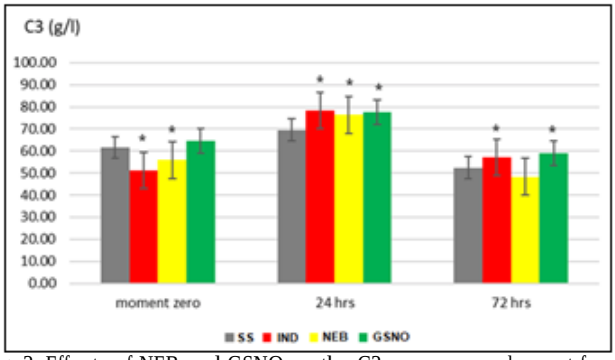

Fig. 3. Effects of NEB and GSNO on the C3 serum complement fraction values in the carrageenan-induced paw inflammation in rats. Each point represents the mean ± SD of the C3 fraction values for 5 animals in a group. *p<0.05 statistically significant compared to control

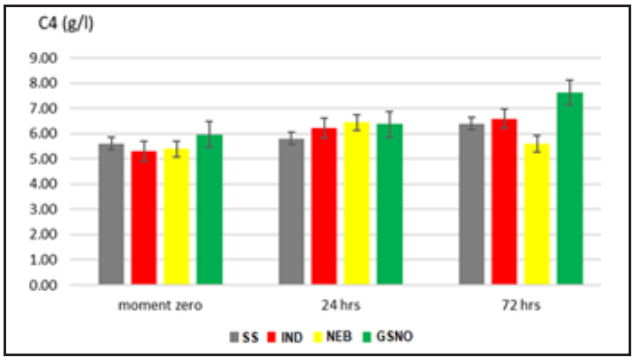

Fig. 4. Effects of NEB and GSNO on the C4 serum complement fraction values in the carrageenan-induced paw inflammation in rats. Each point represents the mean ± SD of the C3 fraction values for 5 animals in a group

The use of IND and NEB was accompanied by a decrease in serum IL-6 level, statistically significant at 24 h (**p<0.01), respectively at 72 h (*p<0.05) compared to SS group. GSNO administration determined an important reduction of IL-6 values, statistically significant at 24 hours (**p<0.01) in the experiment (fig. 5).

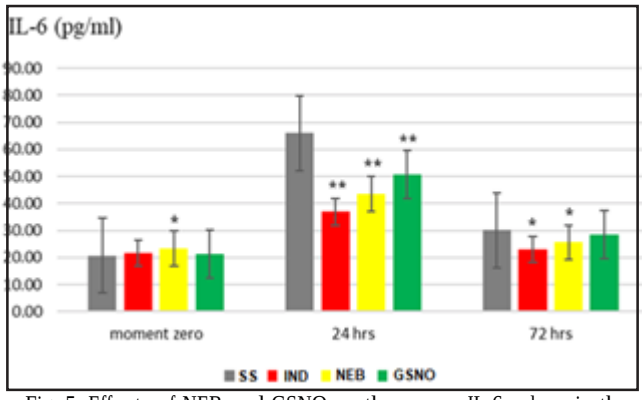

Fig. 5. Effects of NEB and GSNO on the serum IL-6 values in the carrageenan-induced paw inflammation in rats. Each point represents the mean ± SD of the IL-6 values for 5 animals in a group.

The treatment with IND, NEB and GSNO resulted in a decrease in serum TNF-α level, statistically significant at 24 h (**p<0.01) compared to SS group. The use of IND and NEB induced an important diminution of TNF-αvalues, statistically significant at 72 h (*p<0.05) in the experiment (fig. 6).

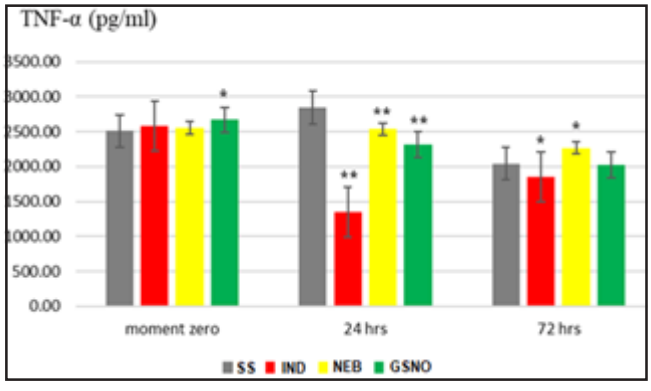

Fig. 6. Effects of NEB and GSNO on the serum TNF-α values in the carrageenan-induced paw inflammation in rats. Each point represents the mean ± SD of the TNF-α values for 5 animals in a group.

Due to its particular properties, which allow it to be very soluble and diffuse easily through biological membranes, nitric oxide exerts important actions influencing multiple intracellular processes [22]. Being an intercellular signaling

molecule, it plays a determining role in the immune system, also contributing to the generation of the peroxinitrites, free oxygen radicals, which have cytotoxic effects, causing tissue damage and apoptosis [23,24].

NO exerts a functional role in different pathological processes in the body, such as: leukocyte adhesion, transmigration, proliferation, expression of cytokines [6]. The generation of NO in inflammatory states and during the infective processes is due to inducible NOS intervention [8]. Various responses to NO are accountable for its pro-inflammatory and anti-inflammatory effects under certain pathological conditions [26]. A wide variety of factors contributes to these different effects of NO, of which a determinant role is represented by tissue concentration, the rate of reactive nitrogen species generation and the internal body environment [27]. In the inflammatory processes, the pro-inflammatory cytokines mediate the NO formation in macrophages, neutrophils and monocytes. When released to tissue macrophages, during phagocytosis, NO interferes with the modulation of this cellular process, resulting in extra phagocyte recruitment. On the other hand, the excess of NO release exerts destructive effects on the tissues, a phenomenon that occurs especially in autoimmune diseases [8,28]. The rate of NO generation in the tissues is regulated by the transcriptional mechanisms, depending on the type of stimulation produced and the cellular particularities. Moreover, NO is involved in regulating its cellular levels through positive feedback mechanisms based on increasing cAMP levels; it accelerates the iNOS formation and the subsequent stimulation of NO production and also through negative feedback mechanisms that modulate NO production by inhibiting the nuclear factor NFκβ [6,29].

The formation of NO under the action of iNOS in inflammatory conditions results in the stimulation of release of a larger amount of mediator over a longer period of time, which allows NO to participate in all three phases of this process: inflammatory, proliferative and remodeling [30]. Macrophages, efficiently participating at the onset of the healing period, provide for the release of an increased amount of NO, which subsequently generates reactive oxygen species to neutralize pathogenic agents [31]. In addition to its defensive activity against microorganisms, NO mediates functional activity, growth and destruction of various types of inflammatory and immune cells, including: macrophages, mast cells, neutrophils, T lymphocytes, natural killer and antigen presenting cells. Because of this, NO can induce or regulate the function and death of the host immune cells, thus contributing to modulation of specific immunity [32].

In our experimental conditions the intraplantar injection of 1% carrageenan solution developed a biphasic response: an early inflammatory reaction and neurogenic pain, lasting for 6 h, and a second late phase (inflammatory hyperalgesia), with a peak intensity at 72 h and which progressively diminished after approximately 96 h [33,34]. The positive control drug IND determined a significant reduction in local inflammatory process after intraperitoneal administration, its effects being concordant with the reported literature data regarding the effects of this non-steroidal anti-inflammatory drug in this experimental model in rodents [12,13,35,36].

The effects of nitric oxide donors NEB and GSNO on experimentally induced paw inflammation were objected by the significant decrease in local edema, as well as by the influence exerted on blood elements, and on the specific serum inflammatory and immune markers. The use of NEB and GSNO induced substantial anti-inflammatory effects especially at 24 h in this model of experimental-induced hind paw inflammation. Nebivolol exhibited more pronounced anti-inflammatory effects than GSNO, but less intense than indomethacin at certain times during the experiment.

Conclusions

We can appreciate that in our laboratory conditions the treatment with nebivolol and S-nitroso-glutathione produced anti-inflammatory effects on local acute inflammation in the carrageenan-induced paw edema experimental model in rats.

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Manuscript received:19.01.2018