Pharmacological Reports
2011, 63, 501–517
ISSN 1734-1140

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Estimation of the action of three different mechlorethamine doses on biochemical parameters during experimentally induced pleuritis in rats

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Abstract:
Nitrogranulogen (NTG) may modify the character of inflammatory reactions. These modifications are a result of cytotoxic and mutagenic effects. NTG has high affinity to DNA and causes disorders in the synthesis of acute phase proteins (e.g., haptoglobin, transferrin, fibrinogen, and complement protein C3).
Our previous studies have shown that small doses of NTG can enhance immunological defense reactions in the organism. The aim of the current studies was to determine how different NTG doses cause changes in the values of biochemical parameters in pleuritis-induced rats. The animals were randomized into five groups: Group I – control group; Group II – IP (induced pleuritis) group; Group III – NTG5 group; Group IV – NTG50 group; Group V – NTG600 group. Blood was collected from all groups of animals at 24, 48, and 72 h after the initiation of the carrageenin-induced inflammatory reaction.
These investigations revealed that a dose of 5 µg NTG/kg b.w. (body weight) can change the character of the inflammation. Our studies also show that a dose of 600 µg NTG/kg b.w. causes a rapid decrease in the level of C3 at the 72 h of the experiment (after 3 applications every 24 h), which indicates a cytotoxic action of such a large NTG dose. NTG used at doses of 50 and 600 µg/kg b.w. causes the opposite metabolism of albumins and other serum proteins. Our studies show that the different doses of NTG have distinct effects on the inflammatory reaction.

Key words:
mechlorethamine, nitrogranulogen, NTG, inflammatory reaction, pleuritis, biochemical parameters

Abbreviations: ALB – albumins, ALT – alanine aminotransferase, AST – aspartate aminotransferase, CREAT – creatinine, FBR – fibrinogen, HAPT – haptoglobin, IP – induced pleuritis, NTG – nitrogranulogen (mechlorethamine), TP – total protein, TRF – transferrin

Introduction

Inflammation is usually a result of a defense reaction proceeding in different tissues or organs. These biochemical, hematological, and immunological responses (on the local or systemic level) have specific, directed and intensified character [8]. An experimentally induced inflammatory reaction allows the determination of the dynamics of biochemical and morphological changes and the evaluation of the efficacy of anti-inflammatory or anti-exudate drugs. The dynamics of the inflammatory reaction can be analyzed by measuring the inhibition of inflammatory mediators, assaying leukocyte migration and estimating the concentrations of acute phase proteins. Experimentally induced inflammatory reactions are usually elicited by physical and chemical factors, such as carrageenin [8, 12]. Carrageenin pleural edema is caused by the release of inflammatory mediators, such as histamine, serotonin, kinins, prostaglandins and interleukins [13]. This edema syndrome is divided into three phases: phase I lasts up to 0.5 h after carrageenin injection, phase II appears between approximately 0.5–2.5 h and phase III occurs between 2.5–6 h after pleuritis induction [12]. The early carrageenin phase is composed of histamine and serotonin reactions. These amines are released simultaneously and cause a significant rise in vessel permeability. Pharmacological elimination of these amines in experimental animals causes a decrease in the edema after carrageenin injection [15, 21]. In contrast, kinins are responsible for carrageenin pleural edema propagation in the second inflammation phase. The elimination of kinins in rats with pleuritis inhibits the appearance of edema and grades the vessel permeability [7, 36, 40]. Inhibition of prostaglandin synthesis also limits the inflammatory reaction in phase III of the carrageenin pleural edema [18, 24]. The maximum blood vessel reaction occurs in the prostaglandin phase [10, 11, 15]. At the end of inflammation, carrageenin induces fibroblast proliferation and stimulates the cellular elements of connective tissue [12].

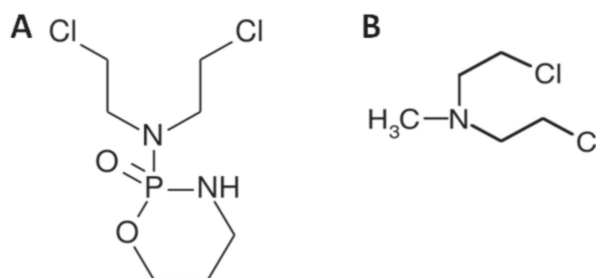

Fig. 1. The chemical formulas of cyclophosphamide (**A**) and mechlorethamine (**B**) [19]

Chemical substances or drug actions may significantly modify the character and the course of inflammatory reactions. For example, cyclophosphamide has immunosuppressive properties [19, 23, 46]. Cyclophosphamide is a nitrogen mustard alkylating agent (the oxazophorine derivative) [19, 23] (Fig. 1A) and is a cytotoxic substance which causes DNA alkylation [7, 19, 23, 24]. This modification leads to the generation of incorrect and non-coding DNA sequences and nitrogen base impairments in the DNA [19, 41]. This substance undergoes metabolic reactions in the liver mediated by the hepatic cytochrome P-450 microsomal enzymes [19, 23]. The result of these transformations is the production of an active mechlorethamine (nitrogen mustard) [2, 19, 23, 38]. The additional metabolic forms of the cyclophosphamide are 4-hydroxycyclophosphamide, carboxyphosphamide, and acrolein. Cyclophosphamide interacts with the genetic material, influences the cell cycle phases and leads to disorders in cellular function [16, 19, 23]. Cyclophosphamide metabolites are responsible for the immunomodulating and toxic actions of this drug [3, 34, 43]. Cyclophosphamide contributes to immunosuppressive effects through a direct cytotoxic action on lymphocytes [1]. The drug inhibits T lymphocyte activation and immunoglobulin production by B lymphocytes [4, 27]. An immunostimulative action (and a deficiency in the cytotoxic properties) is observed after the administration of small doses of cyclophosphamide [4, 19, 23, 27, 43]. Cyclophosphamide and its derivatives have an anti-proliferative action that is significantly stronger in the primary immunological response compared to the secondary response [19, 23, 27].

Nitrogranulogen (N-methyl-2,2'-dichlorodiethylamine; Mechlorethamine, Chlormethine; NTG) is the cyclophosphamide derivative that is associated with the im-

munosuppressive and cytotoxic drugs [6, 31, 43, 47] (Fig. 1B). NTG has quite toxic, mutagenic, carcinogenic, and teratogenic properties because of its ability to crosslink DNA [35, 41, 48] and causes DNA alkylation [6, 7, 22]. NTG is a bifunctional compound that introduces an alkyl group into the nucleophilic center of another molecule, creating the nucleophilic nucleus [25]. The consequence of alkylation is the inhibition of DNA synthesis. However, this substance is not able to inhibit RNA or protein synthesis [6]. The cytotoxic action of NTG relates to the inhibition of the mitotic divisions in the somatic cells, in which rapid growth is observed [6, 7]. Ethylamine ion, an active NTG form, is capable of forming bonds with most nucleophilic groups [21]. Ethylamine ion creates DNA crosslinks between (interstrand crosslinkages) and within (intrastrand crosslinkages) DNA strands [21, 22, 37]. Crosslinking agents, such as nitrogen mustard derivatives, inhibit DNA replication and are cytotoxic to cancer cells [48]. The ability to crosslink DNA is responsible for the high carcinogenic and genotoxic potency of this bifunctional agent [48]. NTG is unstable in water and is removed rapidly from blood. Almost 90% of this drug is eliminated from an organism in just one minute. NTG applied in doses of 400–600 μg/kg b.w. (body weight) causes cytotoxic and immunosuppressive effects [26, 43, 45]. An NTG dose of 1,000 μg/kg b.w. inhibits immunoglobulin synthesis and T and B lymphocyte proliferation [46]. On the other hand, stimulation of IL-1 synthesis contributes to blocking the interaction of this interleukin with the target cells [6, 7, 42]. However, an NTG application at a dose of 10 μg/kg b.w. once a day for 7 days decreases the amount of natural killer lymphocytes (NK), which are responsible for the natural cytotoxicity action [17, 20]. In some cases, a decrease in the number of lymphocytes has also been observed [32]. Other studies have shown that NTG significantly influences the membrane ATPase activity in B and T lymphocytes isolated from the spleen compared to the control group, in which pleuritis was induced experimentally using carrageenin [28–30]. NTG action is characterized by several undesirable side-effects, including lymphopenia syndrome (occurring 24 h after its administration), granulocytopenia, thrombopenia and a decrease in erythrocyte number after a few days. NTG therapy is a cost-effective and easily-administered treatment for cutaneous T-cell lymphomas, but the major adverse reaction is allergic dermatitis or irritant dermatitis [14].

The aim of this work was to study the influence of three different NTG doses (5, 50, 600 μg/kg b.w.), given in three repetitions, within 3 days during induced pleuritis in experimental animals [9]. Monitoring of the biochemical parameter values allowed an examination of the influence of NTG on liver metabolism, which may contribute to the changes in inflammation character. These studies facilitate the analysis of other cytostatic drugs and their hepatogenic actions. Furthermore, NTG application at doses of 5 or 50 μg/kg b.w. has immunomodulating or anti-inflammatory properties, respectively.

Materials and Methods

Experimental animals

These studies were performed on female rats from the Buffalo inbreeding strain aged 8–10 weeks, with a body weight of 120–140 g. Because some diagnostic parameters are dependent on individual features such as age, sex or strain (under invariable environmental factors), only Buffalo females (with similar mass and age) were used in these experiments [9]. These animals were also related in order to show the same reactivity on the inflammatory factor and to obtain results with similar standard deviations. Rats were bred in the Department of Pathomorphology in Wroclaw Medical University. The rats were kept under the same conditions: they were in polystyrene cages with metal lids (6 animals to each cage). The experiments were carried out in air-conditioned rooms, in which the temperature oscillated between 21 and 22°C and the humidity of the air was 62–63%. The rats were fed the standard “Murigran” diet and they received water *ad libitum*. The experiments in which animals were involved were permitted by The Local Bioethics Council for Animal Experiments.

The animals were randomized into five groups:
1) **CONTROL** – control group of 24 female rats without inflammation (intact); This is the physiological group without carrageenin and NTG applications. The blood was collected at the 72 h of the experiment;
2) **IP group** – group of 60 female rats with induced pleuritis caused by a single intrapleural injection of 0.15 ml of 1% carrageenin solution (Sigma-Aldrich), given at the 1 min of the experiment;

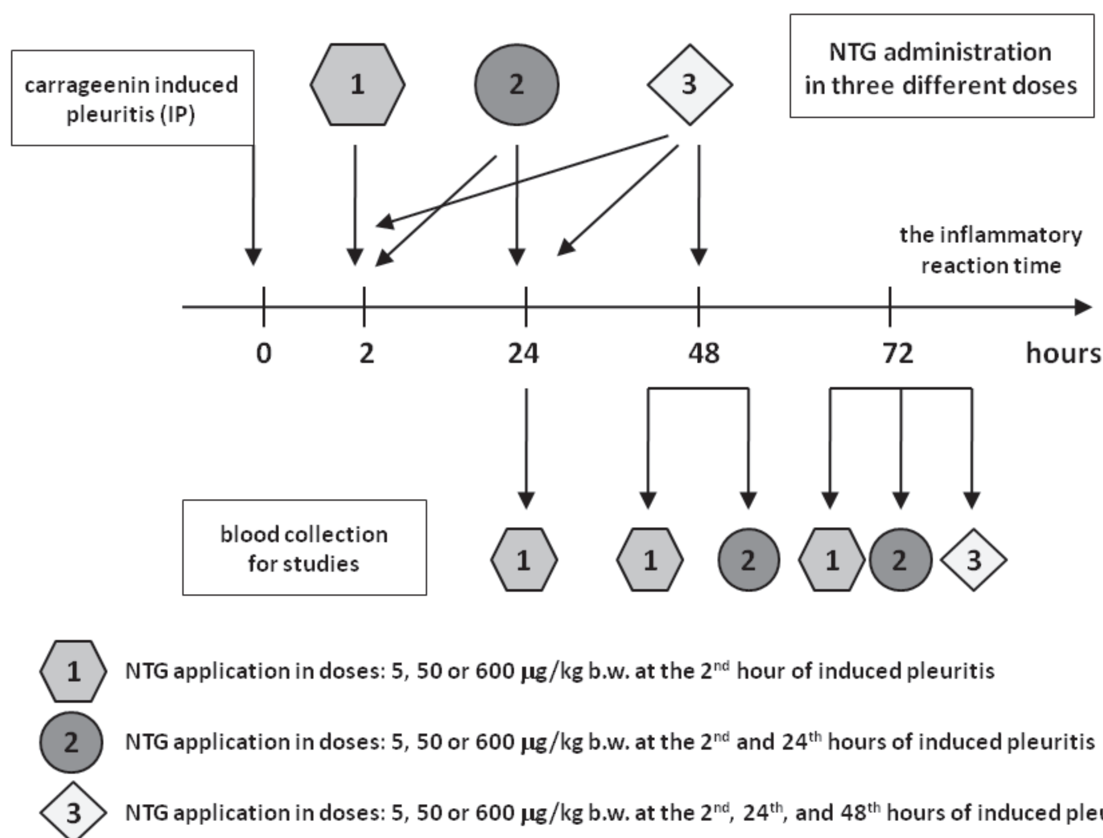

Fig. 2. The scheme of the pleuritis induction in rats with temporal monitoring of the biochemical parameters during the inflammatory reaction after NTG administration at three different doses

3) **NTG5 group** – group of 60 female rats injected intravenously with NTG (Polfa Warszawa SA) at a dose of 5 µg/kg b.w. with a 1% carrageenin solution at the 2, 24 and 48 h after the pleuritis induction. The blood was collected after a single NTG application (at the 24 h), after a double NTG application (at the 24 and 48 h) and after a threefold NTG application (at the 24, 48 and 72 h) after the inflammation initiation (Fig. 2);
4) **NTG50 group** – group of 60 female rats injected intravenously with NTG (Polfa Warsaw SA) at a dose of 50 µg/kg b.w. at the 2, 24 and 48 h after the pleuritis induction using a 1% carrageenin solution. The blood was collected after a single NTG application (at the 24 h), after a double NTG application (at the 24 and 48 h) and after a threefold NTG application (at the 24, 48 h and 72 h) after the inflammation initiation (Fig. 2);
5) **NTG600 group** – group of 60 female rats injected intravenously with NTG at a dose of 600 µg/kg b.w. at the 2, 24 and 48 h after the pleuritis induction using a 1% carrageenin solution. The blood was collected after a single NTG application (at the 24 h), after a double NTG application (at the 24 and 48 h) and after a threefold NTG application (at the 24, 48 h and 72 h) after the inflammation initiation (Fig. 2).

Materials

Carrageenin (Sigma) extracted from *Chondrus chrispus* algae was dissolved before experiments in 0.9% NaCl solution (Polfa). Next, this solution was injected into the 4–5 intercostal space on the right site.

Blood for studies was collected from all groups of experimental animals at 24, 48 and 72 h after the induction of the experimental pleuritis. The biological material was collected from rats under pentobarbital (Biochemie GmbH)-induced anesthesia, which was administrated by intraperitoneal injection at a dose of 30 mg/kg b.w. Next, the abdominal cavity was opened and catheters and needles with 2 mm diameters were inserted into the aorta for collecting blood into standardized polypropylene test-tubes produced by Sarstedt Ltd. The basic biochemical parameters were measured

using standard diagnostic tests and a Konelab 60i biochemical analyzer (ThermoFisher Scientific, Rochester, NY) at the Diagnostic Laboratory of the 4th Military Academic Hospital in Wrocław, Poland.

Statistical analysis

The values of the biochemical parameters in the rat blood were analyzed using Statistica 7.0 (StatSoft Ltd.). The arithmetic means of the parameters (X) for the determined number of animals taking part in the experiment (N), the standard deviation (SD) and the minimal (MIN) and maximum (MAX) value ranges of the parameters were computed. The mean values of these parameters are collected in Table 1. After checking whether the data conformed to a normal distribution (comparing the variables' histograms with a Gaussian distribution chart), particular groups were compared using the Student's *t*-test with Bonferroni correction under consideration to determine levels of significance (T – statistical significance towards control group (intact group); p – statistical significance towards IP group – data are shown in Table 2, column 1). The indications: *: $0.05 \geq p, T > 0.01$; **: $0.01 \geq p, T > 0.001$; ***: $0.001 \geq p, T$; NS – not significant.

Additionally, we carried out analysis of variance (ANOVA). The ANOVA test allowed us to effectively evaluate the influence of NTG doses on various biochemical parameters in different experimental time intervals. All assays had the steady probability of incurring a type I error: $\alpha = 0.05$. The statistical procedure was preceded by checking the assumption of variation homogeneity (test for equality in variances of time and dose – Levene's assay (data are shown in Tab. 2, column 2)). This assay is especially useful because it allows for an analysis of variation, even in the case of abnormal distributions. The variation is designated as homogeneity if the p-value of the assay is higher than the steady probability of a type I error. ANOVA was performed after Levene's assay. The means equality of different groups (features observed in experimental rats) was the assay hypothesis. The Tukey assay was also used to distinguish groups that demonstrate changeability (data are shown in Tab. 2, column 3). Similar to the variation assay, the variation mean is found equality if the p-value of the assay is lower than the steady probability of a type I error. In another case, we observed a statistically significant difference. In the end, we compared essentially different groups using the Tukey assay (HSD) and Fisher (LSD). We admitted as a rule of thumb:
– Statistically essential difference according to HSD are essential
– Lack of difference according to LSD means lack of difference
– Lack of difference according to HSD and difference according to LSD suggests carrying out additional analyses

Results

Every dose of NTG administered contributes to a change in the inflammation character. The monitored biochemical parameter values differed for all NTG groups relative to the IP and control groups and depended on the amount of the NTG dose in the following way:

Albumins (ALB) and total protein (TP)

Analysis of the TP parameter allows the determination of proper liver function. Albumins have significant participation in TP parameter and they both react negatively in inflammation [8].

The ALB level for the IP group relative to the control group was lower during the total experimental time, and the ALB value for the IP group still drops within 72 h of the experiment (Fig. 3a). The decrease in the ALB level compared to the IP group is observed in the NTG5, NTG50, and NTG600 groups at the 24 h after pleuritis initiation in the following order: NTG5 > NTG50 > NTG600. Although the second NTG administration at the doses of 5 µg/kg, 50 µg/kg and 600 µg/kg b.w. causes a rise in the ALB concentrations between the 24 and 48 h relative to the IP group, these values are mainly statistically insignificant (Tab. 2). The third injection of NTG into the NTG50 and NTG600 group essentially does not change the ALB values between the 48 and 72 h. The increase in the albumin level is observed in the NTG50 group in every blood analysis compared to the control and IP groups. These data probably point to anti-inflammatory properties of NTG at a dose of 50 µg/kg b.w. (Fig. 3a). The statistically significant rise in the concentration of albumins relative to the IP group at the 72 h of pleuritis is noticed only for the NTG5 group

Tab. 1. The mean values of selected biochemical parameters between individual groups of examined rats during carrageenin-induced inflammatory reaction after triple application of NTG at three different doses

Biochemical parameter	Control group	IP group	NTG5 group	NTG50 group	NTG600 group
ALB (g/dl)	524	24 h: 4.24	24 h: 3.75	24 h: 3.91	24 h: 3.95
		48 h: 4.45	48 h: 4.95	48 h: 4.00	48 h: 3.97
		72 h: 3.99	72 h: 4.93	72 h: 4.09	72 h: 4.11
TP (g/dl)	6.29	24 h: 5.44	24 h: 6.48	24 h: 6.12	24 h: 5.83
		48 h: 6.01	48 h: 6.73	48 h: 5.66	48 h: 5.79
		72 h: 5.13	72 h: 6.80	72 h: 5.11	72 h: 5.39
C3 (mg/dl)	1.79	24 h: 53.38	24 h: 73.05	24 h: 65.13	24 h: 64.78
		48 h: 68.45	48 h: 82.00	48 h: 62.29	48 h: 60.90
		72 h: 47.97	72 h: 109.7	72 h: 57.49	72 h: 1.43
C4 (mg/dl)	8.43	24 h: 5.41	24 h: 14.30	24 h: 8.93	24 h: 14.11
		48 h: 8.85	48 h: 9.53	48 h: 9.53	48 h: 8.07
		72 h: 6.00	72 h: 10.85	72 h: 7.51	72 h: 8.29
TRF (mg/dl)	106.74	24 h: 149.03	24 h: 150.8	24 h: 174.7	24 h: 173.4
		48 h: 145.23	48 h: 89.42	48 h: 125.9	48 h: 117.3
		72 h: 132.26	72 h: 116.0	72 h: 127.6	72 h: 139.5
HAPT (mg/dl)	4.91	24 h: 80.36	24 h: 77.22	24 h: 91.55	24 h: 92.29
		48 h: 91.56	48 h: 82.48	48 h: 99.06	48 h: 111.3
		72 h: 89.22	72 h: 95.79	72 h: 76.19	72 h: 100.3
FBR (g/l)	1.09	24 h: 1.99	24 h: 1.58	24 h: 1.43	24 h: 1.73
		48 h: 2.18	48 h: 1.78	48 h: 2.01	48 h: 1.83
		72 h: 1.64	72 h: 1.68	72 h: 2.38	72 h: 1.77
UREA (mg/dl)	42.80	24 h: 46.28	24 h: 70.00	24 h: 73.93	24 h: 53.14
		48 h: 37.65	48 h: 39.56	48 h: 49.79	48 h: 54.42
		72 h: 44.71	72 h: 46.33	72 h: 55.88	72 h: 68.26
CREAT (mg/dl)	0.772	24 h: 0.587	24 h: 0.645	24 h: 0.457	24 h: 0.417
		48 h: 0.484	48 h: 0.661	48 h: 0.616	48 h: 0.930
		72 h: 0.450	72 h: 0.738	72 h: 0.629	72 h: 0.413
AST (IU/l)	172.28	24 h: 156.22	24 h: 316.2	24 h: 383.1	24 h: 410.6
		48 h: 172.32	48 h: 130.0	48 h: 417.0	48 h: 282.5
		72 h: 394.44	72 h: 119.0	72 h: 254.1	72 h: 489.0
ALT (IU/l)	42.80	24 h: 46.28	24 h: 70.00	24 h: 73.93	24 h: 53.14
		48 h: 37.65	48 h: 39.56	48 h: 49.79	48 h: 54.42
		72 h: 44.71	72 h: 46.33	72 h: 55.88	72 h: 68.26

(Tab. 2). Levene's tests for equality of variances (the mean *vs.* time and NTG dose) prove that an equal variances assumption is executed because the p-values are higher than 0.05 (Tab. 2). Two-way ANOVA (the mean *vs.* time and NTG dose) shows an essential difference in the variation of NTG doses. Tukey's tests demonstrate the statistically essential differences in the control group compared to the IP, NTG50 and NTG600 groups. A lack of difference in the NTG5 group relative to the control group was not proved.

Although the TP drop in the IP group relative to the control group is also observed during the total experimental time, only decreases at the 24 and 72 h of the experiment are statistically significant (Tabs. 1, 2). Each of the NTG doses causes the TP to rise relative to the IP group at the 24 h of pleuritis initiation. This increase occurs in the following order: NTG5 > NTG50 > NTG600. The second NTG application at the 48 h causes a further TP rise only in the NTG5 group. A contrary effect is observed in the NTG50

Tab. 2. The summary of different types of statistical data analysis performed using (1) normal distribution test (comparing the variables' histograms with a Gaussian distribution chart) where particular groups were compared using Student's *t*-test to determine levels of significance (the indications: *: $0.05 \geq p > 0.01$; **: $0.01 \geq p > 0.001$; ***: $0.001 \geq p$; NS – not significant); (2) test for equal variances (Levene's test assesses the equality of variances in different samples; if the resulting p-value of Levene's test is less than some critical value (typically 0.05), the obtained differences in sample variances are unlikely to have occurred based on random sampling; the indication of $p > 0.05$ – homogeneity of variance is noticed; (3) two-way ANOVA variance analysis which is partitioned into components due to different sources of variation. In its simplest form, ANOVA provides a statistical test of whether or not the means of several groups are all equal and therefore generalizes Student's two-sample *t*-test to more than two groups (the indication: *: $p < 0.05$; NS – not significant)

	Compared group using Student's *t*-test (1)				Test for equal variances (p > 0.05) (2)		ANOVA analysis (p < 0.05) (3)	
Biochemical parameter	Control group *vs.* IP group	IP group *vs.* NTG5 group	IP group *vs.* NTG50 group	IP group *vs.* NTG600 group	mean *vs.* time	mean *vs.* NTG dose	mean *vs.* time	mean *vs.* NTG dose
ALB (g/dl)	24 h: *** 48 h: *** 72 h: ***	24 h: NS 48 h: NS 72 h: *	24 h: * 48 h: NS 72 h: NS	24 h: NS 48 h: NS 72 h: NS	0.962	0.560	0.324 (NS)	0.007 (*)
TP (g/dl)	24 h: *** 48 h: NS 72 h: ***	24 h: * 48 h: ** 72 h: ***	24 h: * 48 h: NS 72 h: NS	24 h: *** 48 h: *** 72 h: NS	0.493	0.361	0.209 (NS)	0.006 (*)
C3 (mg/dl)	24 h: *** 48 h: *** 72 h: *	24 h: * 48 h: *** 72 h: ***	24 h: NS 48 h: NS 72 h: *	24 h: NS 48 h: NS 72 h: ***	0.561	0.566	0.663 (NS)	0.008 (*)
C4 (mg/dl)	24 h: *** 48 h: NS 72 h: ***	24 h: *** 48 h: NS 72 h: ***	24 h: ** 48 h: NS 72 h: *	24 h: *** 48 h: NS 72 h: *	0.094	0.701	0.337 (NS)	0.141 (NS)
TRF (mg/dl)	24 h: *** 48 h: *** 72 h: ***	24 h: NS 48 h: *** 72 h: NS	24 h: *** 48 h: *** 72 h: NS	24 h: *** 48 h: *** 72 h: NS	0.608	0.517	0.022 (*)	0.062 (NS)
HAPT (mg/dl)	24 h: *** 48 h: *** 72 h: ***	24 h: NS 48 h: NS 72 h: NS	24 h: NS 48 h: NS 72 h: NS	24 h: * 48 h: * 72 h: NS	0.980	0.515	0.297 (NS)	0.000 (*)
FBR (g/l)	24 h: *** 48 h: *** 72 h: ***	24 h: NS 48 h: NS 72 h: NS	24 h: * 48 h: NS 72 h: NS	24 h: NS 48 h: NS 72 h: NS	0.970	0.159	0.432 (NS)	0.018 (*)
UREA (mg/dl)	24 h: *** 48 h: *** 72 h: ***	24 h: NS 48 h: * 72 h: NS	24 h: NS 48 h: NS 72 h: NS	24 h: NS 48 h: *** 72 h: NS	0.812	0.247	0.345 (NS)	0.012 (*)
CREAT (mg/dl)	24 h: *** 48 h: *** 72 h: ***	24 h: NS 48 h: NS 72 h: ***	24 h: NS 48 h: * 72 h: **	24 h: * 48 h: *** 72 h: NS	0.935	0.638	0.450 (NS)	0.286 (NS)
AST (IU/l)	24 h: NS 48 h: NS 72 h: ***	24 h: *** 48 h: *** 72 h: ***	24 h: * 48 h: *** 72 h: ***	24 h: *** 48 h: *** 72 h: *	0.771	0.790	0.676 (NS)	0.110 (NS)
ALT (IU/l)	24 h: NS 48 h: NS 72 h: NS	24 h: *** 48 h: NS 72 h: NS	24 h: *** 48 h: ** 72 h: *	24 h: NS 48 h: ** 72 h: ***	0.505	0.552	0.147 (NS)	0.117 (NS)

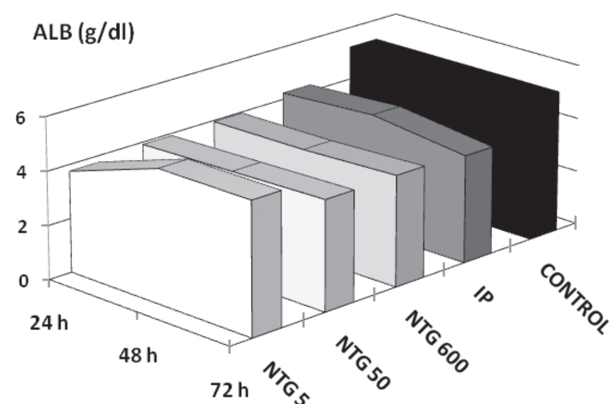

Fig. 3a. The influence of three doses of NTG on the albumin (ALB) concentration during experimentally induced pleuritis in rats

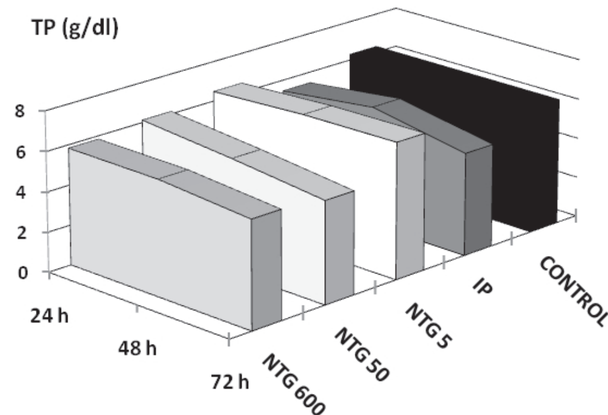

Fig. 3b. The influence of three doses of NTG on the total protein (TP) concentration during experimentally induced pleuritis in rats

and NTG600 groups at the 48 h, when the TP values are lower than the proper values of the IP group. The third NTG application contributes to further emphasizing the TP value changes. The TP still increases in the NTG5 group and decreases in the NTG50 and NTG600 groups at the 72 h of pleuritis (Fig. 3b). The dose of 5 µg NTG/kg b.w., applied each 24 h within 3 days after carrageenin injection, causes the highest rise in the level of TP relative to the other NTG groups. The statistically significant rise in the TP level in the NTG5 group compared to the IP group is observed during the total experimental time (Tab. 2). Levene's tests for equality of variances (the mean *vs.* time and NTG dose) show that the equal variances assumption is executed because p-values are higher than 0.05 (Tab. 2). A two-way ANOVA analysis (the mean *vs.* time and NTG dose) demonstrates a statistically significiant difference in variation of the NTG doses. Tukey's tests show statistically essential differences in the NTG5 group compared to the IP, NTG50 and NTG600 groups (Tab. 2). A lack of differences between the control group and the IP, NTG50 and NTG600 groups was not proved.

C3 and C4 complement proteins

C3 and C4 proteins are complement and acute phase proteins. The concentration of these proteins changes during inflammation and tissue damage. The C3 level rises during the inflammatory reaction. The C4 concentration changes in sinusoid way, which is a result of C4 synthesis and consumption processes. The C4 protein is responsible for erythrocyte coating, leading to their hemolysis, which causes C4 consumption during inflammatory reactions [8].

A significant rise in C3 in the IP group relative to the control group is observed during the total experimental time (Tab. 1). Each NTG dose causes a rise in the levels of C3 relative to the IP group at the 24 h of pleuritis initiation. This increase occurs in the following order: NTG5 > NTG50 > NTG600. The second NTG application at the 48 h causes a subsequent C3 rise only in the NTG5 group (Fig. 3c). A contrary effect is observed in the NTG50 and NTG600 groups at the 48 h, when C3 values are lower than the proper values of the IP group. The third NTG application is responsible for a further rise in the levels of C3 in the NTG5 group, and C3 drops in the NTG50 and NTG600 groups at the 72 h of the inflammatory reaction. Even a single injection of 5 µg NTG/kg b.w causes a change in the inflammation character. Two-time and three-time doses of NTG5 enhance this inflammatory reaction. A drastic C3 decrease at the 72 h of inflammation is observed in the NTG600 group, in which the C3 value is lower than the proper value of the control group. The rapid drop in the C3 concentration in the NTG600 group probably leads to a limitation in the formation of antigen-antibody complexes in the walls of blood vessels and the gathering of neutrophils and necrotic changes (Fig. 3c). Levene's tests for equal variances (the mean *vs.* time and NTG dose) show that the equality of variances assumption is executed because p-values are higher than 0.05 (Tab. 2). Two-way ANOVA analysis (the mean *vs.* time and NTG dose) demonstrates an essential dif-

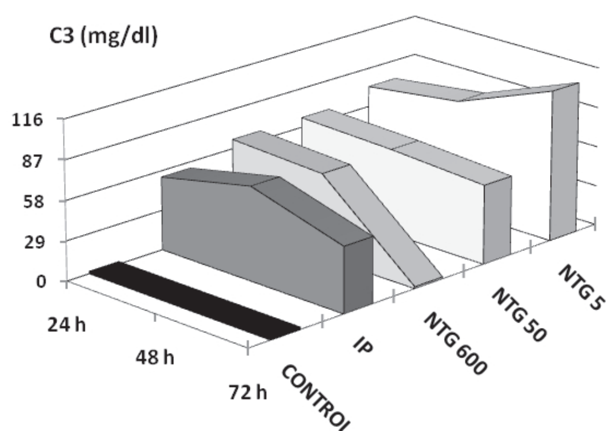

Fig. 3c. The influence of three doses of NTG on the C3 complement protein concentration during experimentally induced pleuritis in rats

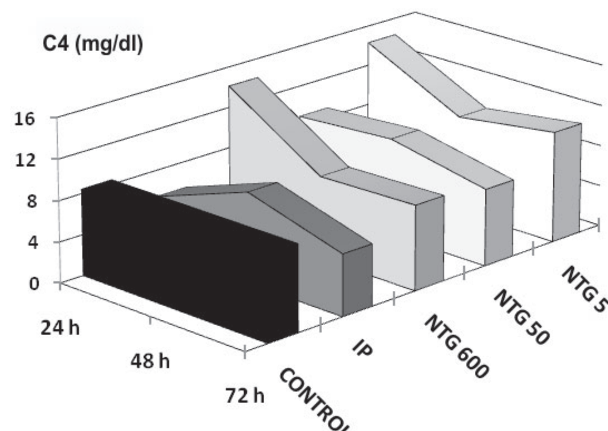

Fig. 3d. The influence of three doses of NTG on the C4 complement protein concentration during experimentally induced pleuritis in rats

ference in the variation of NTG doses. Tukey's tests show statistically essential differences between the control group and the IP, NTG5 and NTG50 groups. Tukey's tests are not able to interchangeably show differences between the NTG5 and NTG50 groups or between the NTG600 and control group.

The C4 value drops in the IP group relative to the control group at the 24 h and 72 h and increases at the 48 h of inflammation (Tab. 1). The C4 level rises at the 24 h of the experiment in the following order: NTG5 > NTG600 > NTG50 (Fig. 3d). The second NTG application contributes to C4 decreases in the NTG5 and NTG600 groups and a C4 rise in the NTG50 group at the 48 h after pleuritis initiation. However, these values are similar to the C4 level in the control group (intact group). The third NTG application causes a further rise in C4 levels in all NTG groups relative to the IP group at the 72 h of inflammation in following order: NTG5 > NTG600 > NTG50 (Fig. 3d).

The C4 concentration is statistically significantly higher only in the NTG5 group at the 24 and 72 h compared to the IP group (Tab. 2), which indicates an immunomodulating ability of NTG at the dose of 5 μg/kg b.w. Levene's tests for equality of variances (the mean *vs.* time and NTG dose) show some changeability; however, an equal variances assumption is executed because p-values are higher than 0.05 (Tab. 2). Two-way ANOVA analysis (the mean *vs.* time and NTG dose) does not demonstrate an essential difference in the variation of NTG doses. There is no reason to discard the hypothesis of equal means.

Transferrin (TRF) and haptoglobin (HAPT)

TRF is a protein that negatively reacts during inflammation and binds iron from erythrocyte hemolysis, which prevents bacterial growth. HAPT is a positive protein that also protects against iron loss. HAPT binds hemoglobin, protects against lipid peroxides or free radical formation and inhibits prostaglandin synthesis [8].

A statistically significant TRF rise in the IP group relative to the control group is observed during the total experimental time (Tabs. 1, 2). The first NTG application causes a rise in the levels of TRF in all NTG groups relative to the IP group at the 24 h of pleuritis initiation. This increase occurs in the following order: NTG50 > NTG600 > NTG5 (Fig. 3e). The second NTG administration causes a contrary effect: the TRF level is lower in all NTG groups than in the IP group. These decreases occur in following order: NTG5 > NTG600 > NTG50. These TRF drops, noticed at the 48 h after pleuritis induction, are statistically essential in all NTG groups compared to the IP group (Tab. 2). The third NTG application causes an elevation in the levels of TRF in all NTG groups. However, these TRF levels are lower than the proper values of the IP group in the NTG5 and NTG50 groups. A consistently higher TRF concentration is observed in the IP, NTG5, NTG50, and NTG600 groups (without the NTG5 group at the 48 h of pleuritis) compared to the control group (intact group) throughout the 3 days of the experiment (Fig. 3e). Levene's tests for equal variances (the mean *vs.* time and NTG dose) show that an equal

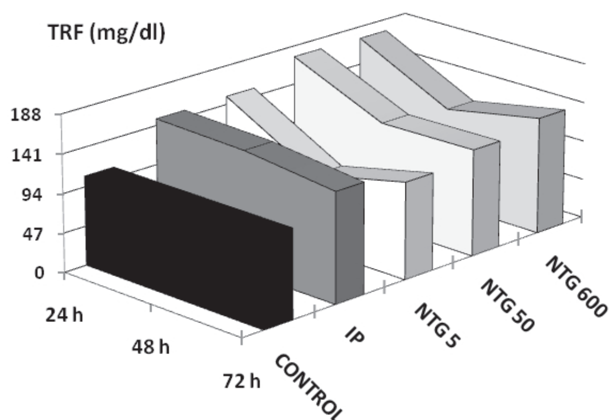

Fig. 3e. The influence of three doses of NTG on the transferrin (TRF) concentration during experimentally induced pleuritis in rats

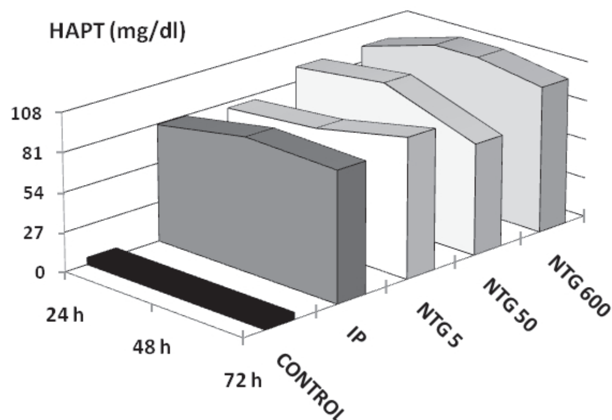

Fig. 3f. The influence of three doses of NTG on the haptoglobin (HAPT) concentration during experimentally induced pleuritis in rats

variances assumption is executed because p-values are higher than 0.05 (Tab. 2). Two-way ANOVA analysis (the mean *vs.* time and NTG dose) demonstrates an essential difference in the variable of time. Tukey's tests do not show statistically essential differences between the 24 h and 72 h or between the 48 h and 72 h of the experiment. The statistically essential difference between the 24 h and 48 h of the experiment is not obvoius. Although we can suppose that this difference exists (according to ANOVA analysis), Tukey's and Fisher's tests do not interchangeably prove it.

The HAPT level increases in the IP group compared to the control group during the total experimental time, but the highest rise occurs at the 48 h of the experiment (Tab. 1). The concentration of HAPT increases about 20 times in every group with inflammation at the 24 h of the pleuritis initiation relative to the control group (intact group). The HAPT level is higher only in the NTG50 and NTG600 groups compared to the IP group after the first NTG application. This HAPT rise at the 24 h of the experiment occurs in the following order: NTG600 > NTG50 > NTG5 (Fig. 3f). Although a second NTG injection further enhances the HAPT increase in the NTG50 and NTG600 groups relative to the IP group, the value of this biochemical parameter for the NTG5 group at the 48 h is lower than the corresponding value in the IP group. A third NTG application causes contrary effects in all NTG groups at the 72 h of inflammation: the HAPT level rises in the NTG5 and NTG600 groups (Fig. 3f). However, the HAPT concentration is lower only in the NTG50 group than in the IP group. This decrease is the most statistically significant HAPT drop, which is observed within 3 days of the experiment. On the other hand, the most statistically significant HAPT changes are observed between the control and IP groups and between the NTG600 and IP groups at the 24 and 48 h of induced pleuritis. In the NTG600 group, both the TRF and HAPT concentrations change statistically significantly compared to the IP group between the 24 and 48 h of the inflammatory reaction (Fig. 3e, f). Levene's tests for equality of variances (the mean *vs.* time and NTG dose) show that an equal variances assumption is executed because p-values are higher than 0.05 (Tab. 2). Two-way ANOVA analysis (the mean *vs.* time and NTG dose) demonstrates an essential difference in the variation of NTG doses. Tukey's tests show statistically essential differences in the control group compared to the IP, NTG5, NTG50 and NTG600 groups. Tukey's tests are not able to interchangeably show a difference between the NTG5 and NTG600 groups.

Fibrinogen (FBR)

Fibrinogen reacts very strongly in inflammatory reactions as an acute phase protein [8, 39].

The FBR level increases in the IP group compared to the control group during the total experimental time; however, an almost 2-fold increase occurs at the 48 h of the experiment (Tab. 1). The first NTG application causes a drop in the FBR levels in all NTG groups relative to the IP group at the 24 h of inflammation. These decreases occur in the following order:

NTG50 > NTG5 > NTG600 (Tab. 1, Fig. 3g). However, none of the FBR values determined for the NTG groups at the 24 h of pleuritis are lower than the corresponding FBR value in the control group. The second NTG application causes a rise in FBR levels in all NTG groups at the 48 h of inflammation in the following order: NTG5 > NTG600 > NTG50. However, the FBR level in the experimental groups is still lower than the corresponding FBR value in the IP group. These changes are practically not statistically significant (Tab. 2). A third NTG application is responsible for contrary effects occurring at the 72 h of pleuritis: the FBR level is higher for all NTG groups relative to the IP group. These differences are not statistically significant, except in the NTG50 group in which the FBR value is about 68% higher than the corresponding FBR value in the IP group. The lack of essential changes in the FBR level within the 3 days of pleuritis shows that NTG probably does not influence fibrinogen synthesis (Fig. 3g). Levene's tests for equality of variances (the mean *vs.* time and NTG dose) show that an equal variances assumption is executed because p-values are higher than 0.05 (Tab. 2). Two-way ANOVA analysis (the mean *vs.* time and NTG dose) demonstrates essential differences in the variation in the NTG doses. Tukey's tests show statistically essential differences in the control group compared to the IP, NTG50, and NTG600 groups. Tukey's tests are not able to interchangeably show a difference between the NTG5 and control group.

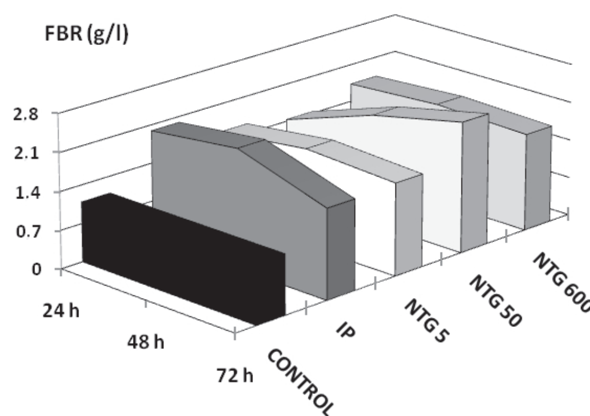

Fig. 3g. The influence of three doses of NTG on the fibrinogen (FBR) concentration during experimentally induced pleuritis in rats

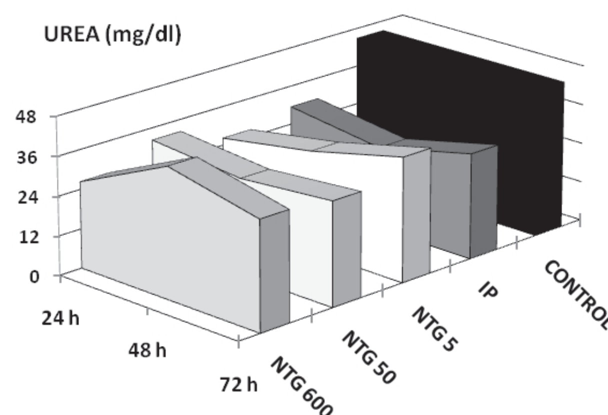

Fig. 3h. The influence of three doses of NTG on the urea (UREA) concentration during experimentally induced pleuritis in rats

Urea and creatinine (CREAT)

Monitoring of the urea levels allows the observation of protein catabolism changes and shows the efficiency of the uropoietic system. CREAT also mediates the control of skeletal muscle metabolism. The concentration of CREAT rises during catabolic reactions and kidneys disorders [8].

Although the urea level changes differently in the IP group during the total experimental time, its values compared to the control group are mainly higher within 3 days of pleuritis (Tab. 1). The most significant drop occurs in the IP group at the 24 h of inflammation (Fig. 3h). The first NTG application causes urea rises in the NTG5, NTG50, and NTG600 groups at the 48 h of inflammation compared to the IP group (Tab. 1). A second NTG application is responsible for the urea rise in all NTG groups at the 48 h of inflammation compared to the IP group in the following or-

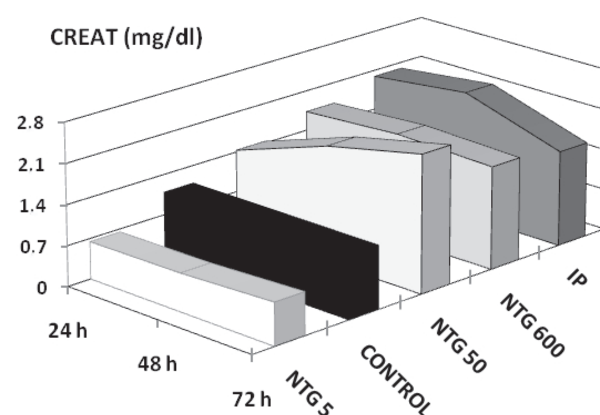

Fig. 3i. The influence of three doses of NTG on the creatinine (CREAT) concentration during experimentally induced pleuritis in rats

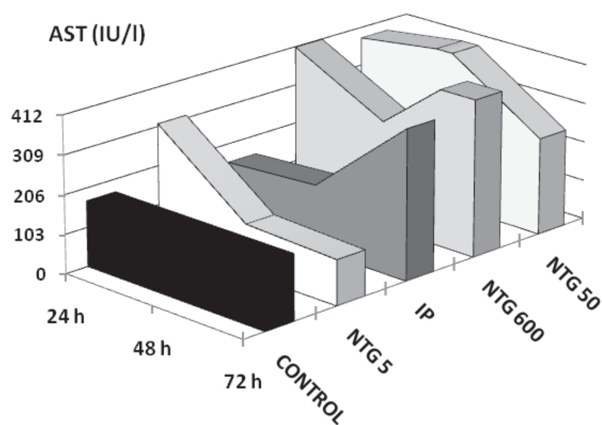

Fig. 3j. The influence of three doses of NTG on the aspartate aminotransferase (AST) activity during experimentally induced pleuritis in rats

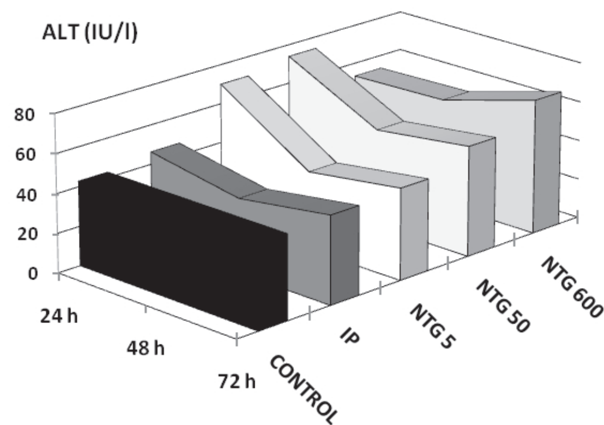

Fig. 3k. The influence of three doses of NTG on the alanine aminotransferase (ALT) activity during experimentally induced pleuritis in rats

der: NTG600 > NTG5 > NTG50. The urea values are higher at the 72 h of inflammation than the corresponding urea values in the IP group for all experimental groups. Statistically essential changes are characteristic of the 48 h of pleuritis in the NTG5 and NTG600 groups (Tab. 2). Levene's tests for equal variances (the mean *vs.* time and NTG dose) show that the equal variances assumption is executed because p-values are higher than 0.05 (Tab. 2). Two-way ANOVA analysis (the mean *vs.* time and NTG dose, respectively) demonstrates an essential difference in the variation of NTG doses. Tukey's tests show statistically essential differences in the control group compared to the IP, NTG5, and NTG50 groups. Tukey's tests are not able to interchangeably show a difference between the NTG600 and control group.

The CREAT value drops in the IP group relative to the control group during the total time of the experiment (Tab. 1). The first NTG application causes a decrease in CREAT in the NTG50 and NTG600 groups at the 24 h of inflammation and an increase in CREAT in the NTG5 group relative to the IP group (Fig. 3i). After the second NTG injection at the 48 h of inflammation, CREAT levels rise in all NTG groups relative to the control and IP groups (Fig. 3i). The CREAT increases are followed at the 48 h in the following order: NTG600 > NTG5 > NTG50. These changes (except in the NTG5 group) are statistically significant (Tab. 2). A third NTG application enhances the CREAT rise in the NTG5 and NTG50 groups at the 72 h of inflammation and is responsible for a drastic CREAT drop in the NTG600 group, in which the CREAT value is lower than the corresponding value in the IP group. Levene's tests for equality of variances (the mean *vs.* time and NTG dose) show that the equal variances assumption is executed because p-values are higher than 0.05 (Tab. 2). Two-way ANOVA analysis (the mean *vs.* time and NTG dose) demonstrates an essential difference in the variation of the NTG doses. Tukey's tests show statistically essential differences in the control group compared to the IP, NTG5, and NTG50 groups. Tukey's tests are not able to interchangeably show a difference between the NTG600 and control group.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

The presence of AST and ALT in blood indicates damage to liver cells. These non-specific biochemical parameters are not characteristic for the inflammatory reaction course, but they show the metabolic reaction state in different diseases [8].

The AST level increases in the IP group compared to the control group between the 48 h and 72 h of the pleuritis induction (Tab. 1). The AST concentration in the IP group significant drops during the first 24 h of the experiment relative to the control group. The first NTG application causes a greater than 2-fold AST rise at the 24 h of inflammation in all NTG groups relative to the IP group. These increases occur in the following order: NTG600 > NTG50 > NTG5 (Fig. 3j). A second NTG application is responsible for a further rise in AST in the NTG50 group at the 48 h of inflammation relative to the IP group. The drastic drop in the AST level in the other NTG groups, especially in the NTG5

group, leads to lower levels than the corresponding AST value in the IP group. A third NTG application causes AST decreases in the NTG5 and NTG50 groups at the 72 h of pleuritis. The AST levels in these groups are lower than the corresponding AST value in the IP group in this time interval. In contrast, the AST value increases in the NTG600 group compared to the IP group at the 72 h of pleuritis. Levene's tests for equality of variances (the mean *vs.* time and NTG dose) show that the equal variances assumption is executed because p-values are higher than 0.05 (Tab. 2). Two-way ANOVA analysis (the mean *vs.* time and NTG dose) does not demonstrate an essential difference in the variation of the NTG doses (Fig. 3j).

The ALT value is higher in the IP group compared to the control group during the first 24 h and between the 48 h and 72 h of the pleuritis initiation (Tab. 1). The ALT concentration drops between the 24 h and 48 h of the experiment in the IP group relative to the control group. However, these changes are not statistically significant. The first NTG application causes an elevation in ALT in all NTG groups relative to the IP group at the 24 h of inflammation. These increases occur in the following order: NTG50 > NTG5 > NTG600 (Fig. 3k). The second NTG application decreases the ALT level at the 48 h of inflammation in the NTG5 and NTG50 groups, but the ALT concentrations in these groups are still higher than the corresponding ALT value of the IP group. The ALT level after the second NTG application at 48 h causes a further ALT rise only in the NTG600 group. A third NTG dose causes an ALT rise in all NTG groups, and these values are higher than the corresponding ALT value in the IP group. Almost all ALT values in NTG50 and NTG600 are statistically significant (except the first NTG application in the NTG600 group). Levene's tests for equality of variances (the mean *vs.* time and NTG dose) show that the equal variances assumption is executed because p-values are higher than 0.05 (Tab. 2). Two-way ANOVA analysis (the mean *vs.* time and NTG dose) does not demonstrate an essential difference in the variation of NTG doses.

Discussion

NTG demonstrates hepatogenic properties and interacts with DNA in dividing cells [5]. These effects can significantly influence the dynamics of the inflammatory reaction, which is determined by changes in biochemical and hematological parameters and the level of interactions of acute phase protein synthesis [Całkosiński I.: Habilitation thesis: The course of experimentally induced acute pleuritis with use of Nitrogranulogen (NTG) and 2,3,7,8-tetrachlorodibenzo-p-dioxin, Department of Publishing of Wroclaw Medical University, 2005, ISBN 83-7055-038-X]. Other studies have demonstrated that the application of NTG at doses of 1–10 µg/kg b.w. has immunomodulating properties [26, 44]. The small NTG doses probably contribute to the changes in the course of the induced inflammatory reaction. We studied the characteristic biochemical parameters important for induced pleuritis in rats as well as these parameters after NTG administration at three different doses. The monitored biochemical parameters showed that the three different doses of NTG (5, 50 and 600 µg/kg b.w.) given every 24 h during 3 days after pleuritis initiation can change the course of the experimental-induced carrageenin pleural edema in various ways. The NTG dose of 5 µg/kg b.w. has immunomodulating properties [26, 44]. We supposed that every NTG dose demonstrates immunomodulating properties on the synthesis of albumins in the liver. The ALB and TP levels change in the same way during pleuritis. Although we observed an increase in the concentration of albumins in the blood serum of all NTG groups after 3 days of the experiment (which is characteristic for anti-inflammatory reactions), the TP level was the most statistically significant only in the NTG5 group at the 72 h of the experiment. Our studies show that NTG administration caused opposite effects on ALB and TP metabolism. These effects are probably a result of the consumption of other serum proteins (e.g., globulin fraction) during the inflammation process.

The concentrations of C3 and C4 undergo changes during inflammation. Although the C3 level rises, the C4 concentration alters itself in a sinusoid manner [8]. These alterations are a result of the synthesis and consumption of C4 during erythrocytes coating, for example. In our studies, both C3 and C4 levels drop in the NTG50 and NTG600 groups within 3 days of pleuritis relative to the IP group. However, the C4 concentration decreases in the NTG5 group during 3 days but is at a higher level than in the IP group. The level of C3 protein is much higher for this group than in the IP group. This high C3 level indicates the development of a defense reaction. This reaction is necessary to inhibit the inflammatory process.

Pleuritis initiation is responsible for intensifying the synthesis of TRF and HAPT. NTG application at doses of 50 and 600 µg/kg b.w. causes a rise in the level of TRF in the first 24 h after pleuritis initiation. During the following time intervals, TRF drops are noticed in all group of animals compared to the IP group. Higher doses of NTG stimulate HAPT and TRF synthesis during inflammation. However, the NTG5 dose is too low and certainly cannot induce any change in the levels of HAPT and TRF. The doses of NTG 50 and 600 µg/kg b.w. have the same influence on the dynamics of concentration and increase of HAPT parameter. The intensification of HAPT and TRF synthesis may be connected with strong erythrocytes hemolysis during inflammation in the NTG50 and NTG600 groups. The fibrinogen concentration changes itself very rapidly during inflammation because it is an acute phase protein. The level of fibrinogen is almost doubled at just the 24 h after carrageenin injection. None of the NTG doses (even given in three repetitions) significantly decreases the value of this biochemical parameter during the 3 days of pleuritis. NTG probably does not influence fibrinogen synthesis during first 24 h. However, only the NTG dose of 50 µg/kg b.w. causes a significant rise in the level of FBR synthesis at later times of inflammation, which may have anti-inflammatory properties and cause disseminated intravascular coagulation syndrome (DIC).

Non-specific biochemical parameters, such as changes in the concentration of urea and creatinine, in the NTG groups occur in a similar manner as in the IP group during the 3 days of inflammation (Tab. 1). The administration of NTG at a dose of 600 µg/kg b.w. causes an increase in the concentration of urea and creatinine at the 48 h of the inflammatory reaction compared to IP only, which indicates the cytotoxic properties of NTG600 (Fig. 3h, i). The significant drop in the level of urea and CREAT proves the inflammatory reaction in experimental animals in the IP group. Even a low NTG dose such as NTG5 probably has slight anti-inflammatory properties.

The rise in the concentration of ALT and AST is characteristic of the inflammatory reaction. The dynamics of these changes in enzyme activity have significance for many pathologies [8]. Although we observed a consistent rise in the AST level in the IP group relative to the intact group, the ALT level was practically constant throughout the 3 days of the experiment. Although the AST activity rises at the 72 h of pleuritis in the IP group, the ALT activity does not change at this time for this group. The application of NTG influences the synthesis of these liver proteins. The NTG5 and NTG50 doses cause a drop in the level of ALT and AST. These decreases may indicate that the NTG5 and NTG50 doses have anti-inflammatory properties. In contrast, injection of the NTG600 dose increases the AST and ALT concentration, which is a result of the strong cytotoxic action on the liver of such a large NTG dose.

Our studies show that the different influences of NTG on the course of inflammation depend on the dose of NTG. NTG at a dose of 5 µg/kg b.w. has immunomodulating properties. Changes in some biochemical parameters, such as ALB, TP urea, CREAT, TRF, AST, and ALT, indicate the anti-inflammatory features of this NTG dose. On the other hand, HAPT and C3 levels demonstrate the pro-inflammatory properties of NTG5. The 5 µg/kg dose of NTG is not enough for use as an anti-inflammatory agent during pleuritis. The application of NTG at a dose of 50 µg/kg b.w. during 3 days of inflammation showed its anti-inflammatory properties. Almost every biochemical parameter (except ALB and CREAT) reached a significant lower level between the 48 and 72 h of inflammation than in the IP and control groups. The decrease in the concentrations of TRF, HAPT, C3, ALT, and AST demonstrate the anti-inflammatory properties of the NTG50 dose. The increase in ALB and CREAT also confirm this NTG50 feature. A second NTG50 application at the 48 h of inflammation caused a pro-inflammatory effect. The biochemical parameters of HAPT, C4, and AST levels rise and urea drops. These changes are probably a result of the inflammatory response of the organism. A subsequent NTG50 application (third dose) causes a total anti-inflammatory reaction at the 72 h of inflammation. The TRF, HAPT, C3, C4, AST, and ALT levels rapidly drop and the ALB and CREAT concentrations significantly rise. The NTG dose of 50 µg/kg b.w. during the 3 days of inflammation does not significantly influence the urea or TP concentrations.

Our studies confirm that NTG at a dose of 600 µg/kg b.w. (used as the positive control group) demonstrates its cytotoxic action. For example, high AST and ALT activity was observed in the NTG600 group at the 72 h of inflammation (after 3-fold NTG application every 24 h), which points to acute liver damage. Similar cytotoxic properties of NTG doses of 400–600 µg/kg b.w. have been given in other studies [17, 33]. The NTG600

group also has a significantly higher C3 concentration than the control and IP groups between the 24 and 48 h of inflammation. A third dose of NTG600 at the 72 h of inflammation rapidly causes both the C3 and C4 concentrations to drop below the control value (in the intact group). This NTG action proves the cytotoxic properties of NTG at a dose of 600 μg/kg b.w. Furthermore, the ALB, HAPT, UREA, and CREAT levels drop significantly between the 48 and 72 h of the experiment. These changes, as well as the rapid drop in TRF, prove that NTG at a dose of 600 μg/kg b.w. is too large and cannot be used as an anti-inflammatory agent.

Conclusions

Three doses of NTG were used to study the behavior of the course of inflammation during 3 days. The detailed analysis of biochemical parameters changes allowed the observation of the influence of cytostatic agents, such as NTG, on the synthesis of acute phase proteins or the modification of liver enzyme activities. These NTG properties are a result of its ability to strongly alkylate DNA. An NTG applied at a dose of 5 μg/kg b.w. has immunomodulating properties. Some parameters change upon NTG administration, such as TRF, ALB, TP, UREA, and CREAT. These changes probably result from the anti-inflammatory action of the lowest dose of NTG. The other parameters (AST, ALT, C3, and HAPT) fluctuate, indicating that the NTG5 dose is too low to abolish the inflammation. In other words, the inflammation reaction is complex.

The administration of NTG at a dose of 50 μg/kg b.w. causes characteristically moderate effects on the cells (intermediate between the immunomodulatory and cytotoxic effects seen at a dose of 600 μg/kg b.w., which was also applied in the experiment as a positive control). The changes in almost all biochemical parameter values testify that the NTG50 dose has anti-inflammatory properties.

The application of NTG at a dose of 600 μg/kg b.w. shows a strong cytotoxic effect. The rapid rise in the ALB, urea and CREAT concentrations and the significant drop in the TRF, C3, and C4 levels prove that this dose of NTG has a strong anti-inflammatory effect. On the other hand, the rise of HAP, AST, and ALT point to a pro-inflammatory character of this NTG dose. These two features demonstrate that NTG600 has strong cytotoxic action.

The inflammatory reaction initiation doubles the fibrinogen concentration. None of the NTG doses (even given in three repetitions) drop the value of this biochemical parameter during 3 days of pleuritis. NTG probably does not influence fibrinogen synthesis.

Acknowledgments:
The project was financially supported by Grant No. PB-768 from Wroclaw Medical University. The authors would like to thank Ewa Biłyk and Maciej Karczewski from Statistic Laboratory “Manteion” in Wrocław (Poland) who kindly carried out the analysis of variance (ANOVA) and for their skilled statistic assistance.

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Received: January 6, 2010; **in the revised form:** October 20, 2010; **accepted:** November 18, 2010.