

## Effect of Morphine on Thioglycollate-Induced Peritonitis in Chickens

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### Abstract

**BACKGROUND:** Morphine exerts immunomodulatory effects dependent on several factors including species and parameter examined. The aim of this study was to assess the influence of morphine on experimental peritonitis and leukocyte activity in young chickens of both sexes.

**METHODS:** Peritonitis was elicited by intraperitoneal injection of thioglycollate (TG) alone or supplemented with morphine; additional chicken groups were injected with morphine alone. Morphine-treated birds (with or without elicited peritonitis) were pretreated with naltrexone, an antagonist of opioid receptors. Control groups were intact or PBS-injected. At specific postinjection intervals peritoneal leukocytes (PTL) were obtained by flushing the peritoneal cavity, counted and used for *in vitro* assays of activity (respiratory burst). Vascular permeability was measured using Evans blue solution.

**RESULTS:** Inflammatory reaction and morphine influence were gender-dependent: the extent of TG-induced peritonitis was higher in males and it was additionally stimulated by morphine. This pro-inflammatory effect of morphine was not seen in females. PTL collected from morphine-treated chickens of both sexes exhibited a stronger nitroblue tetrazolium reduction than in non-treated birds and this effect was antagonized by naltrexone.

**CONCLUSIONS:** The effect of morphine on peritonitis in chickens appears different to that in other vertebrate species, although the mechanism(s) of its influence on leukocyte activity are similar

**Abbreviations**

|      |                           |
|------|---------------------------|
| b.w. | body weight               |
| DMSO | dimethyl sulfoxide        |
| i.p. | intraperitoneal           |
| M    | morphine                  |
| MEM  | serum-free Eagle's medium |
| N    | naltrexone hydrochloride  |
| NBT  | nitroblue tetrazolium     |
| PBS  | phosphate-buffered saline |
| PFA  | paraformaldehyde          |
| PMA  | phorbol myristate acetate |
| PTL  | peritoneal leukocyte      |
| TG   | thioglycollate            |
| WBC  | white blood cell          |

**Introduction**

There is a large body of evidence that the endogenous opioid system produces a variety of effects within the immune system. These signal molecules act via well-defined receptor types, also mediating the effects of exogenous opiates, such as morphine [for review see 1]. On the other hand, endogenous opioids may participate in the influence of melatonin, a principal pineal hormone, on immune system function [for review see 2]. Administration of morphine results in a variety of physiological, pharmacological and behavioral changes [3], including the effects on immunity. This last morphine influence is exerted in different ways; it alters various immune parameters indirectly through the central nervous system and directly via opioid receptors on immune cells in both vertebrates and invertebrates [4]. The effects of morphine on inflammatory processes in mammals have been studied in several experimental systems. The majority of data were obtained from paw inflammation [e.g. 5], with a few studies done on experimental inflammation of the ear [6], digestive system [7], respiratory tract and the peritoneum [8].

Experimental peritonitis is a good model for comparative studies of inflammatory processes, including modulatory effects of various factors on the kinetics of this process [8, 9]. Peritoneal exudates containing leukocytes are also easily collected from inflamed peritoneal cavities and may be thus accessible for study [10]. Chickens provide a particularly convenient animal to study these cells involved in the inflammatory response because of the absence, in comparison with other vertebrates, of any significant number of harvestable resident leukocytes [11, 12].

The aim of this study was to examine the kinetics of inflammation elicited by intraperitoneal injection of sterile irritant and its modification by morphine in male and female chickens.

**Materials and methods*****Birds***

Experiments were performed on male and female Hi-Line chickens obtained from a local hatchery. Chickens were kept from hatch under controlled light and temperature conditions, with free access to standard

food and water. Birds were maintained under 12h light/dark cycles (LD 12:12, lights on at 0800) at an ambient temperature of  $32 \pm 2^\circ\text{C}$  during the first week and a temperature of  $24 \pm 2^\circ\text{C}$  thereafter. Chickens were submitted to the treatments according to the Polish regulations concerning experiments on animals.

***Reagents***

The following substances were obtained from Sigma Chemical Co. (St. Louis, MO, USA): thioglycollate (TG), naltrexone hydrochloride (N), phorbol myristate acetate (PMA), nitroblue tetrazolium (NBT), dimethyl sulfoxide (DMSO), Evans blue. Heparin was obtained from Biochemie (Vienna, Austria) and paraformaldehyde (PFA) was purchased from Serva Electrophoresis GmbH ANOVEX Co. (Heidelberg, Germany). Phosphate-buffered saline (PBS) and serum-free Eagle's medium (MEM) were purchased from Biomed (Lublin, Poland). *Morphinum hydrochloricum* (M) was obtained from Polfa (Kutno, Poland) and crystal violet from Buchemia (Chorzow Batory, Poland).

***Experimental peritonitis***

Two/three-week-old chickens with a body weight (b.w.) ranging from 100 to 150 g were subjected to treatments as described by Chadzińska et al. [13]. Groups of animals were injected i.p. with a single dose of 2 ml/100 g b.w. of 3% thioglycollate alone [TG-group] or supplemented with different doses of morphine [TGM-group]. An additional group of animals was injected i.p. with 2 ml/100 g b.w. of PBS supplemented with morphine at a dose of 50 mg/kg b.w. [M-group]. For both morphine-treated groups (i.e. M and TGM) there were introduced additional, naltrexone-pretreated groups of chicken, namely NM and NTGM. Naltrexone was injected i.p. 30 min. earlier in a single dose of 5 mg/kg b.w. Animals either untreated [INT-group] or injected i.p. with PBS [PBS-group] were used as control groups. Behavioral changes were observed immediately and up to 3 h after treatment.

***Cell number***

At specific postinjection intervals, animals from each group (5–7 individuals per group) were weighed and sacrificed. Blood obtained from the jugular vein was used to prepare smears and the total white blood cell (WBC) number was counted by the method of Natt and Herrick [14]. Differential count of lymphocytes and granulocytes was made using smears stained by the standard method of Pappenheim. Peritoneal leukocytes (PTLs) were obtained by flushing the peritoneal cavity with 7–10 ml of sterile medium with heparin. The isolated PTLs were counted in a haemocytometer, centrifuged for 15 min. (400 g), and suspended in MEM prior to further analysis.

***PTLs activity assay in vitro***

PTLs were allowed to adhere in two 96-well tissue culture plates,  $10^6$  cells in 200  $\mu\text{l}$  MEM per well [15]. One of the plates was used to estimate the level of adhesion, and the other to measure the respiratory burst according to the method of Secombes [16]. Briefly, after

1 h of incubation at 41°C and 5% CO<sub>2</sub>, nonadherent cells were removed from both plates by washing 3 times with PBS. Adherent cells were fixed on the first plate with 2% PFA, stained with crystal violet and the level of adhesion was measured colorimetrically (OD estimation at 570 nm) in a multiscan spectrophotometer, using 70% methanol as a blank. The respiratory potential of adherent leukocytes was measured as the level of intracellular O<sub>2</sub><sup>-</sup> in the second plate. After removal of nonadherent cells, 100 µl of NBT alone or with PMA (1 µg/ml) was added to each well and the plate incubated at 41°C and 5% CO<sub>2</sub> for another 1h. During incubation, NBT was reduced by O<sub>2</sub><sup>-</sup> into insoluble blue formazan. After this time, the medium was removed, the cells fixed with 70% methanol, washed several times and allowed to air dry. The intracellular formazan was dissolved by mixing with 120 µl of 2M KOH and 140 µl of DMSO, estimated colorimetrically at 630 nm in a multiscan spectrophotometer (OD630 nm/OD570 nm) and adjusted to the same number of adhering cells.

#### Vascular permeability measurement

Three hours after injection, 3-5 birds from PBS, M, TG and TGM groups were injected, *via* a wing vein, with Evans blue solution in saline (10 mg/ml), 0.1 ml/100 g b.w. Intact birds (INT) received the same injection of Evans blue solution. Thirty minutes later, the animals were killed and the peritoneal cavity lavaged with 7 ml of PBS and centrifuged for 10min. Absorbance at 630 nm, measured in a multiscan spectrophotometer using PBS as a blank, was used as an indicator of protein extravasation into the peritoneal cavity [17].

#### Statistical analysis

All data are expressed as mean ± SE. Data were analyzed by one-way analysis of variance (ANOVA), followed by the least-significant difference Student-Newman-Keuls test, if significance was indicated by the ANOVA.

## Results

### Dose-dependent effect of morphine on peritoneal inflammation

In preliminary experiment on male chickens, the highest morphine dose used (50 mg/kg b.w.) significantly ( $p < 0,001$ ) increased the number of PTLs in comparison to the effect of TG alone and to both lower morphine doses (10, 20 mg/kg b.w., Fig. 1). Therefore, this morphine dose was used in subsequent experiments performed on chickens of both sexes.

### Kinetics of inflammatory reaction

The beginning of inflammation (increased number of PTLs in comparison to PBS group) was observed in females 3 h and in males 6 h after i.p. injection of TG. The number of PTLs in males increased significantly up to 12–15h after the induction of inflammation and thereafter decreased gradually to the level in PBS injected birds at 18–24 h (Fig. 2A). Application of mor-

phine together with TG caused in males an earlier increase in PTL number, which was also significantly higher than in the PBS and TG-treated groups. This effect was observed as early as 3 h after i.p. injection and PTL number continued to be higher in TG group up to 36 h after reaction initiation (with the exception of 12 h postinjection). In females the number of PTLs increased up to 18 h after induction of inflammation (was significantly higher than in PBS group), and then decreased gradually (Fig. 2B). This pattern of kinetics of peritonitis was not modified by morphine application. The magnitude of peritoneal inflammation expressed as a surface integral exhibited a 2.5 fold increase in the TGM group *vs* the TG group in male chickens (Fig. 2A insert) and was not influenced in females (Fig. 2B insert).

### WBC number (data not shown)

TG-induced peritonitis in males was accompanied by the changes in the WBC number: increasing up to 12 h after TG injection and thereafter decreasing gradually, though remaining higher than in control animals. In TGM males the increase in the WBC number was smaller. Both TG and TGM treatments evoked a well-pronounced percentage changes in the main subpopulations of WBC: a significant increase in the percent of granulocytes simultaneously with a decrease in the percentage of lymphocytes. The control level was observed 48 h after induction of inflammation. In females, peritonitis induced by injection of both TG and TGM did not affect either WBC number or the percentage of main subpopulations of WBC.

### PTL activity during inflammatory reaction

PTL activity was measured *in vitro* at two time-points: at the beginning (3 h) and towards the end of the inflammatory reaction (24 h). Stimulation index (calculated as a ratio of mean results obtained with to those without PMA addition) measured 3 h after the induction of inflammation was highest in PTLs from the TGM group both in males (Fig 3A) and females (Fig 3B), and naltrexone pretreatment decreased these values. When the peritonitis was already finished (24h postinjection) the effect of morphine was not significant.

### Influence of morphine alone on PTL number and activity

In male and female chickens, the injection of morphine significantly increased ( $p < 0,001$ ) the number of PTLs (24 h after injection) in comparison to the effect of PBS alone (Fig. 4), and in males this effect was antagonized by naltrexone pretreatment. A stimulation index of leukocytes isolated from the peritoneum of chickens of both sexes treated with morphine alone was higher than that from control groups (INT, PBS) and pre-treatment with naltrexone antagonized the stimulatory effect of morphine (Fig. 4).

### Vascular permeability

The extravasation of plasma protein into the peritoneal cavity, used as a measure of vascular perme-

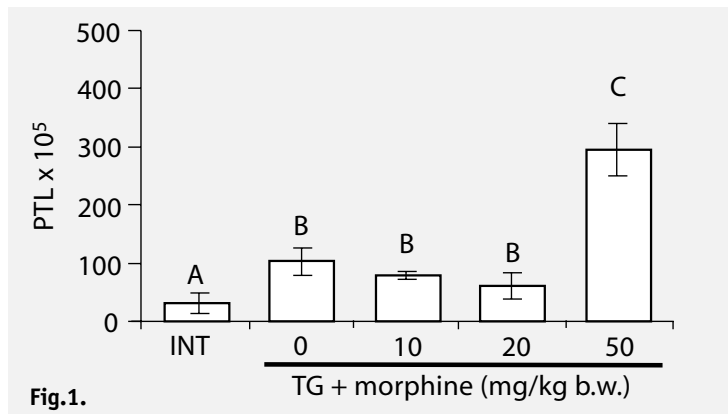


Fig.1.

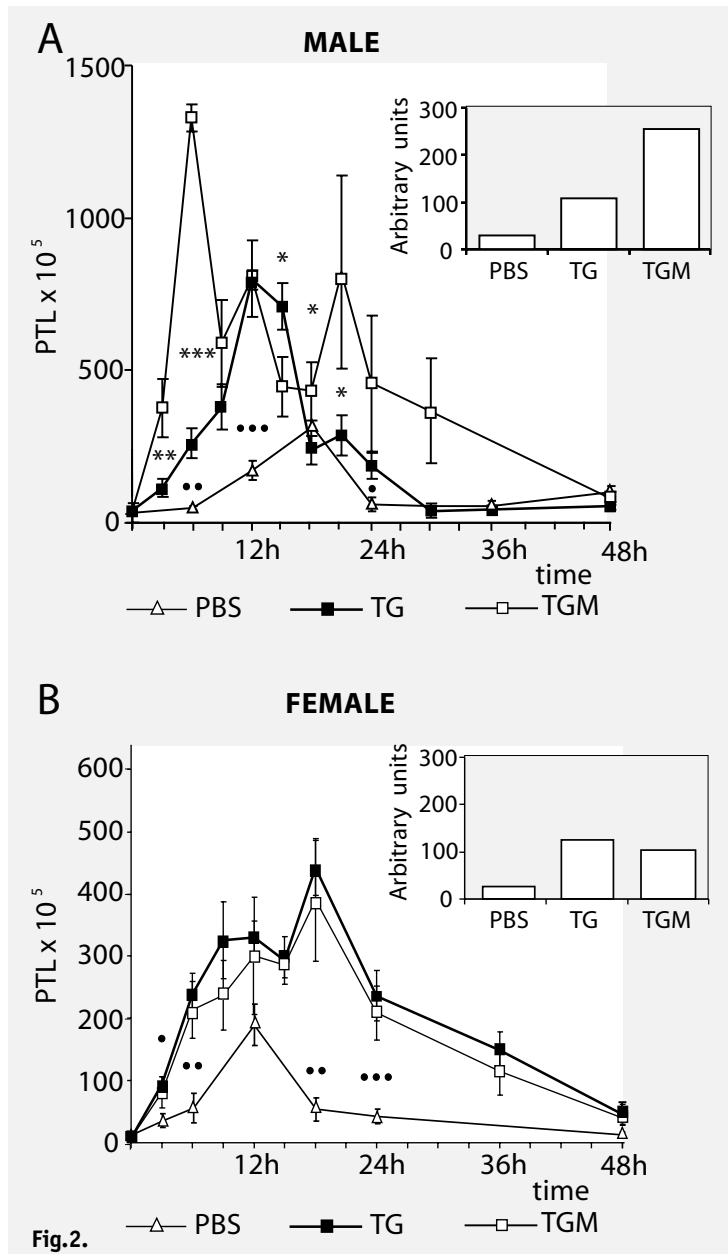


Fig.2.

**Fig.1.** Dose dependent effect of morphine on the male chicken peritoneal leukocyte number (PTL) measured 24h after i.p. injection of thioglycollate (TG) or thioglycollate supplemented with morphine in the dose of 10, 20 or 50mg/kg b.w. INT – control, untreated birds. Values are means ( $\pm$ SE) of 5–7 birds per group. Columns with different letters are significantly different.

**Fig. 2.** Effect of i.p. injection of morphine on the kinetics of TG-induced experimental peritonitis in male (A) and female (B) chickens. Animals were untreated (time 0) or injected i.p. with PBS (control group), TG alone or supplemented with morphine, 50mg/kg b.w. (TGM). Values are means ( $\pm$  SE) of 5–7 birds per group. \*, \*\*, \*\*\* indicate significant difference be-

ability, was greatest in TG-treated male chickens, and differed significantly from both controls and morphine (M and TGM) injected birds. Morphine alone did not influence the vascular permeability whereas injected together with TG counteracted the increase caused by TG (Fig. 5).

*Behavioral observations*

Within 10-15 min. following administration of morphine the birds of both sexes had become quiet, and successively fell into sleep. This behavior was maintained for at least 120 min. after injection and 60 min. later the chicken’s behavior became essentially normal. After PBS and TG treatment the chickens did not display any behavioral changes. The behavior of animals pre-treated with naltrexone did not differ from TGM-treated chickens.

**Discussion**

The cellular mechanism of TG-induced peritonitis appears to be relatively well characterized in mammals, amphibians and fish [8, 13, 18] but to our knowledge, it has not yet been examined in birds. Recently, we have shown [19, 20] that in young chickens TG-induced peritonitis was modified by the pineal hormone melatonin in two subsequent steps: firstly the inflammation was blocked because of the anti-oxidant properties of melatonin, and next a pro-inflammatory effect was observed. This last phenomenon was most probably mediated *via* some compound(s) [cytokines or/and endogenous opioid(s)] synthesized and secreted by immune cells under melatonin influence. It has been demonstrated in several experimental approaches that among the mechanism(s) involved in melatonin action within immune system its influence on the synthesis of endogenous opioids has to be taken into consideration [2, 21]. Our own unpublished data have demonstrated that also in chickens treated with exogenous melatonin, immune cells expressed both  $\beta$ -endorphin and dynorphin synthesis [22]. In the present study we have, therefore, asked the question what kind of effects on experimental peritonitis in chickens may be exerted by morphine, an exogenous agonist of opioid receptors.

In experiments presented here, intraperitoneal injection of TG evoked an acute inflammation in male chickens, and morphine at different doses applied together with the irritant caused

tween TG and TGM, •, ••, ••• indicate significant difference between PBS and TG values at particular time points,  $p < 0.05$ , 0.01 and 0.001, respectively. The insert shows the effect of morphine on intensity of inflammatory reaction expressed as surface integral (surface under curve =  $\int_0^1 f(x) + \int_1^3 f(x) + \dots + \int_{36}^{48} f(x)$ ).

a dose-dependent increase in the number of PTLs. The highest concentration of morphine (50 mg/kg b.w.) significantly increased the number of PTLs in comparison to the effect of TG alone. This preliminary result obtained in male chickens differed from those in mammals, in which a decline in peritoneal leukocytes after morphine treatment was observed [23]. In subsequent experiments we have applied this highest morphine dose to examine its effect on TG-induced peritonitis in chickens of both sexes, and it has appeared as gender-dependent. TG injection evoked an inflammatory reaction in birds of both sexes but in males this process was more acute than in females, i.e. in males the PTL number was almost double than that seen in females. Also the pro-inflammatory effect of morphine was only seen in male chickens. This effect consisted of an advanced onset, a higher increase as well as a prolonged duration of the inflammatory reaction. The calculated surface integrals including changes in the PTL number up to 48h after injection of TG and TG with morphine better demonstrate the magnitude of peritoneal inflammation. In TGM treated males, a 2.5-fold increase was seen in comparison with animals treated with TG alone, but in TG and TGM female groups there was no difference in this parameter. TG induced inflammation in male chickens was accompanied by a change in both the WBC number and the percentage of leukocyte subpopulations in blood, not influenced in females under the same treatment. All those results may suggest that the female immune system can more easily fight pathogens. Sexual dimorphism within the immune system is already a well-accepted phenomenon [24]. Immune organ size is sex dependent, possibly through the influence of sex hormones for which the immune cells have specific receptors [25]. Sex-related differences in phagocyte function have also been described in humans, with a lower respiratory burst activity observed in females, explained by an anti-inflammatory property of estrogens [26].

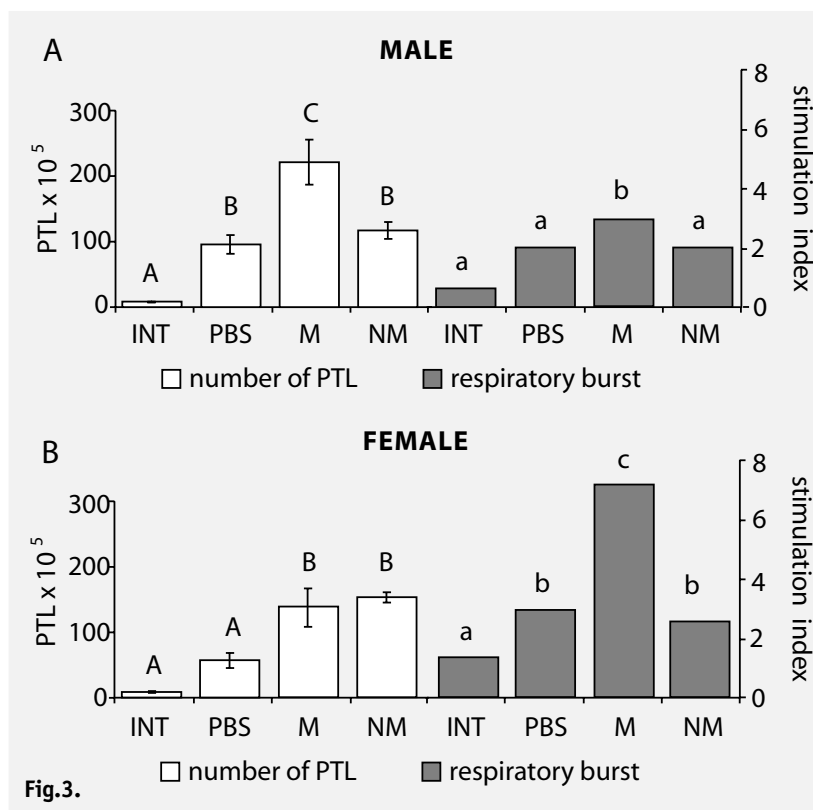


Fig.3.

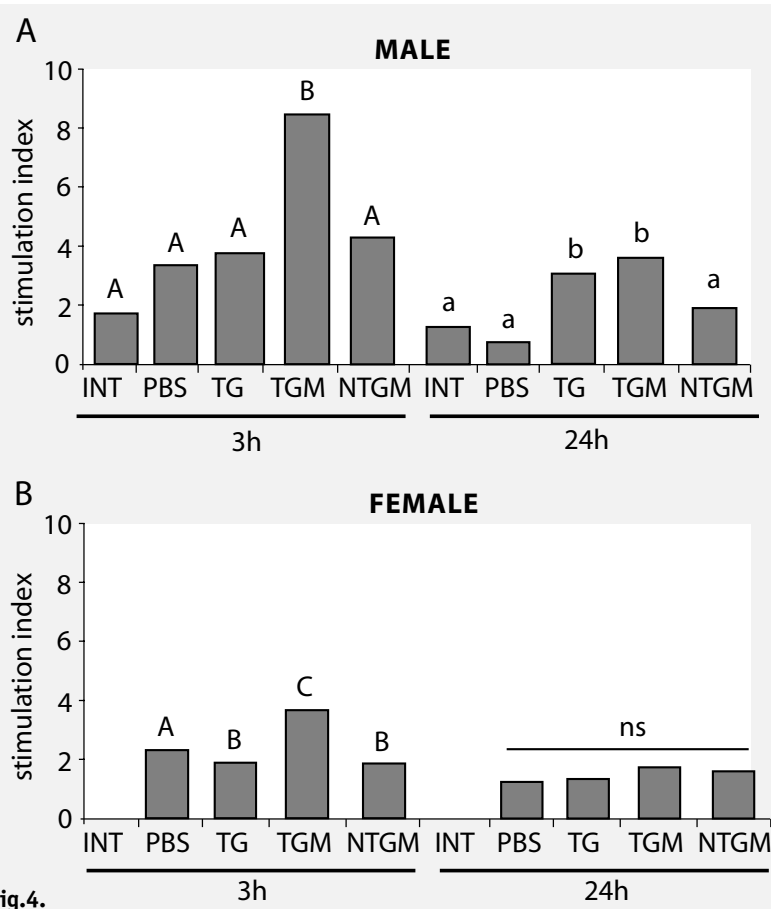


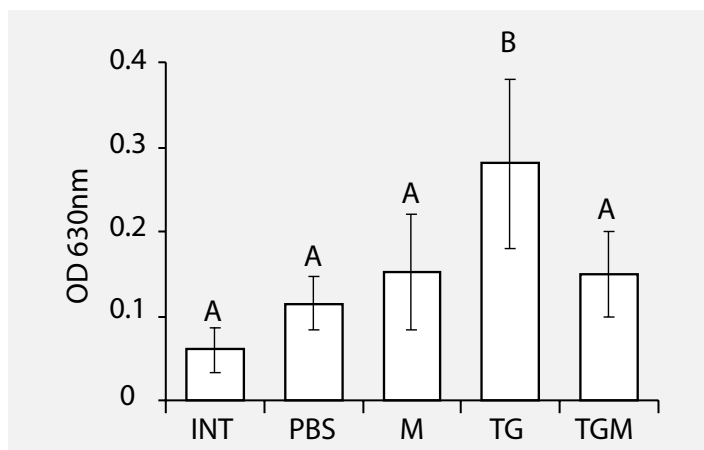
Fig.4.

**Fig. 3.** Effect of i.p. injection of morphine (M) or pretreated (30 min.) with naltrexone (NM) on the number (□) and the respiratory burst (■) of peritoneal leukocytes, in male (A) and female (B) chickens. Measurement was done 24h after injection. Untreated (INT) and in-

jected with PBS animals (PBS) were used as a control. Stimulation index was calculated as a ratio of the results obtained in cells stimulated with PMA to those without PMA. Columns with different letters are significantly different.

**Fig. 4.** Effect of i.p. injection of morphine

(TGM) and naltrexone pretreatment (NTGM) on the respiratory burst of peritoneal leukocytes 3h and 24h after induction of inflammation (TG) in male (A) and female (B) chickens. Other explanation as in Fig. 3.



**Fig. 5.** Effect of i.p. injection of morphine (M) and TG alone or supplemented with morphine (TGM) on the vascular permeability 3h after injection in male. Untreated (INT) and injected with PBS animals (PBS) were control groups. Results are expressed as mean  $\pm$  SE of 3-5 animals per group. Columns with different letters are significantly

In both sexes of chickens the inflammatory reaction had finished 24 h after induction, and its duration appeared to be species-dependent. In mammals [8] and birds (Fig. 2A and B) a peritonitis induced by the same dose of TG finished after 72 h and 24 h, respectively, however in amphibians [23] and fish [13] it lasted several days. Additionally, the influence of morphine alone on the number of PTLs was species-dependent. Morphine alone evoked (24 h after injection) a well evident increase in the number of PTLs in male and female chickens. In different vertebrate species, treatment with morphine either caused a decrease in the PTL number in mammals (Swiss and CB6 mice), amphibians (green frogs) and fish (goldfish and salmon) or was ineffective (yellow-bellied toads and common frog) [8,13,23]. Behavioral observations are also consistent with these species-related differences. Administration of morphine caused a sedation and sleep, which persisted for at least 3 h in chickens of both sexes. Whereas, morphine-treated male and female rats became quiet within 10–15 min, but 30–35 min. after i.p. administration of morphine they showed an improved reflex and displayed straub tail. Two hours post-injection rats appeared essentially normal [3]. All above results indicate a phylogenetical difference in the defense mechanisms within vertebrate species.

The increase of PTL number in males treated with morphine (alone or together with TG) was not connected with any increase in vascular permeability, measured as the level of plasma protein extravasations into the peritoneal cavity (Fig. 5). Moreover, in both human and fish leukocytes it was demonstrated that morphine alone acts as a strong chemoattractant [18,27]. Our preliminary results (not presented) also confirmed these morphine properties in the case of male chickens: although plasma isolated from morphine treated male chickens was not a better attractant than that from control animals, morphine addition *in vitro* caused an increase in the migratory cell activity. This kind of morphine activity as well as its influences on PTL adhesion

is at present being examined extensively in our laboratory.

There are a number of studies on the effect of morphine and other agonists of opioid receptors on the production of superoxide radicals by leukocytes, but results are contradictory [28-30], probably because of the species-specific and experimental system-dependent effects [9]. As  $O_2^-$  is the first product released during the respiratory burst, the measurement of  $O_2^-$  has been accepted as a direct and accurate way of quantifying leukocyte activity [16]. Stimulated with PMA leukocytes from TG-treated chicken groups produced a higher amount of superoxide anions (expressed as stimulation index on Fig. 3) in comparison to those from non-treated animals, and this activity was additionally increased in TGM-treated birds, especially 3 h after the beginning of inflammation (exceptionally, 24 h after treatment the effect of morphine on PTL activity in females was not observed, Fig. 3B). Moreover, i.p. injection of morphine alone caused a similar increase in PTL activity (Fig. 4). All these morphine effects were basically reversed by a pre-treatment with naltrexone, a specific antagonist of opioid receptors, comparably to those observed in mice and fish [8]. In the case where the effect of morphine was not antagonized by naltrexone (in females, 24 h after treatment with morphine alone, Fig. 4), it could be probably attributed to the experimental model used (i.e. naltrexone dose and time of pre-treatment, taken from experiments on other vertebrate species [8]).

Therefore, we suggest that morphine exerts a stimulatory effect on chicken PTL activity by a mechanism(s) involving opioid receptors, similarly as on peritoneal leukocytes retrieved from TGM-treated salmon and female CB6 mice (in comparable conditions) and stimulated *in vitro* with PMA [8, 9]. There are also some data indicating the opposite effects of morphine and other opioid agonists. Peterson et al. [1] described that morphine and  $\beta$ -endorphin in cultured human peripheral

blood mononuclear cells suppressed oxygen metabolism in response to respiratory burst stimuli, but both of them induced an increase in resting  $O_2^-$  and  $H_2O_2$  generation. Ortega et al. [29] has shown that  $\beta$ -endorphin *in vitro* did not modify the ability of macrophages to produce superoxide anions in male mice. In another *in vitro* study  $\beta$ -endorphin modified PMA-stimulated superoxide production by rabbit alveolar macrophages in a dose-dependent way (a decrease and an increase caused by high and low  $\beta$ -endorphin concentration, respectively [30]).

In conclusion, our results suggest that in chickens morphine modulates a TG-induced peritoneal inflammatory reaction in a gender-dependent way. In males, morphine increases the number of PTLs but the same morphine dose remains without effect in females. The immunomodulatory effect of morphine may be exerted in two ways. First, it stimulates an influx of leukocytes into the peritoneal cavity from the blood most probably acting as a chemoattracting factor, and secondly, morphine activates leukocytes to more efficient phagocytosis, as expressed by an increased free radical production. Opioid  $\mu$ -receptors are involved in both functions because an injection of naltrexone counteracts the effect of morphine. Therefore, the effect of morphine on the inflammatory reaction in chicken *in vivo* appears different to that reported in other vertebrate species (stimulatory in birds *vs* inhibitory in mammals and fish), but the mechanism(s) of its influence on leukocyte activity may be similar to those in other vertebrate species so far examined. Additionally, results presented herein suggest that morphine mimicked the action of melatonin on peritonitis in chickens [19], supporting our previous hypothesis that, comparably as in mammals, endogenous opioids are involved in the effect of melatonin on the avian immune system function.

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