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Small doses of morphine can enhance NK cell cytotoxicity in pigs ☆

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ABSTRACT

The effect of small and moderate doses of morphine (MF) on NK cell lytic activity (cytotoxicity, NKCC) (^{51}Cr release test) and the number of circulating large granular lymphocytes (LGL) was evaluated in *i.v.* catheterized Pietrain crossbred pigs. Simultaneously, plasma cortisol (COR) (RIA method) was measured. Blood samples were collected 15, 60, 120, 180, and 240 min after *i.v.* injections of 0.5, 1.0 and 5.0 mg/kg of MF alone or MF pretreated with naloxone (NX, 1.0 mg/kg, *i.v.*, 15 min before MF). It was found that MF induced dose- and time-dependent changes of NKCC. MF in a dose of 0.5 mg/kg evoked 4-fold increase in NKCC (in comparison to saline) without changes in the number of LGL/NK cells. Higher MF doses (1.0, 5.0 mg/kg) induced an early increase (up to 300Δ% and 29Δ%, respectively) followed by a decrease in cytotoxicity (to −76Δ% after 5.0 mg/kg), and in LGL number (−36Δ% after 5.0 mg/kg). These effects were concomitant with a marked rise in plasma COR (up to 234Δ% after 0.5 mg/kg and 567Δ% after 5.0 mg/kg of MF). NX pretreatment blocked all the changes in cytotoxicity but not in the LGL cell number and COR concentrations. The results indicate that MF, besides having well known immunosuppressive effects, can also enhance NKCC through the opioid receptors-dependent manner. The enhancement of cytotoxicity appears as a purely functional change independent of the recirculation of NK cells which occurs despite the high plasma concentrations of COR.

1. Introduction

Morphine (MF) and other opioids have been commonly thought to down-regulate the immune responsiveness. This effect of opioids has potentially serious consequences in the clinical environment. In a wide variety of the *in vivo* and *in vitro* experiments, primarily on rodents, both the acute and chronic depressive effects of exogenous opioids on various immunological endpoints have been shown.

MF has been reported to suppress mitogen-induced lymphocyte proliferative responses [1,2], macrophage activation [3], macrophage-mediated inhibition of tumor cell proliferation [4], phagocytosis [2,4], antibody formation [5], production of interferon-γ [2,6,7], and the number of circulating lymphocytes [8]. MF also suppresses natural killer (NK) activity [4,6,9,10]. The importance of these findings is that exogenous opioids may exacerbate the effects of infection thus impairing host homeostasis and survival [11].

As almost all the findings reported the immunosuppressive action of MF and other opiate alkaloids, it is surprising to find that endogenous opioids, particularly β-endorphin, besides suppression can also enhance some immune reactions, such as the lymphocyte proliferative response to mitogens both *in vivo* and *in vitro* [12,13], or the cytotoxic activity of NK cells (NKCC) [14–16]. This data might even suggest antagonism between the immunomodulatory action of exogenous opiate alkaloids and endogenous opioids. Our work provides evidence that low doses of MF can enhance NKCC, known to be crucial for the host's natural defense against viral and cancer transformed cells.

The effects of MF on NKCC and the number of large granular lymphocytes (LGL; that was treated as reflecting general tendency of changes of NK cells number) were evaluated in the peripheral blood of pigs. Plasma cortisol (COR) was measured as an index of the HPA axis activation [17]. An opiate antagonist, naloxone (NX), was used to evaluate the involvement of opioid receptors in the MF-induced effects. The experiments were done on pigs that possess many useful model properties. It has been reported that their immunological reactivity is close to that of humans [18,19]. The immunological similarity to humans makes pigs an attractive species to study immunomodulatory effects of opioids. Also, these large animals are very useful for long-lasting observations on dynamics of the drug-induced aftereffects.

Abbreviations: COR, cortisol; MF, morphine; NX, naloxone; NKCC, NK cell cytotoxicity; HPA, the hypothalamo-pituitary–adrenal axis; LGL, large granular lymphocytes.

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2. Materials and methods

2.1. Animals

The experiments were performed on 36 Pietrain crossbreed (synthetic line 890) male pigs (10–12 weeks old, 20–25 kg) obtained from The Pig Hybridization Center in Pawłowice (National Institute of Animal Production, Kraków, Poland). An automatically controlled 12-h photoperiod (lights on 0700 a.m. off 0700 p.m.) and thermostatically regulated ambient temperature (20–21 °C) were maintained throughout the experiment. The pigs were housed individually in stainless steel crates, fed the standard, completely balanced pelleted dry food twice a day (0700–0730 a.m. and 0200–0230 p.m.), with water available *ad libitum*. The animals were acclimated to their new environment for about 2 weeks before the experiments.

All efforts were made to minimize animal suffering and to reduce the number of animals used, with all the protocols being reviewed and approved by the Local Ethical Committee for the care and use of laboratory animals in Gdansk, Poland.

2.2. Catheterization and blood sampling procedure

All the pigs were fitted under general pentobarbital (Vetbutal, Biovet, Puławy, Poland) anesthesia with a chronic catheter according to the technique described previously [20]. In brief, the right external jugular vein was exposed and a polyvinylidine catheter was inserted into the vein. The free end of the catheter was then subcutaneously directed to the dorsal neck region and fixed between the scapulae. The catheters were filled through infusion plugs with heparin (1000 U/ml). During the next 5–7 days until the experiment began the pigs were accustomed to handling and to the blood collection procedure. In addition, the catheters were flushed daily with a fresh sterile heparinized (100 U/ml) saline.

2.3. Drugs and treatment protocols

Morphine hydrochloride (Sigma, St Louis, USA) and naloxone hydrochloride (Sigma, St Louis, USA) were dissolved in a sterile saline for *i.v.* administration. In the first experiment 24 pigs were divided into four groups (n=6 each) and injected with either MF (0.5, 1.0 or 5.0 mg/kg) or an equivalent volume (2 ml) of saline. Four days later, in the second experiment, the same animals were pretreated with 1.0 mg/kg of NX or saline (both in a volume of 2 ml) and then after 15 min injected with either MF or saline in the same doses and volumes as in the first experiment. All the injections were conducted in the home crate of each animal.

Venous blood samples (5 ml) were collected in the heparinized syringes through the catheters, with the blood being sampled 15 min before injections (time −15) and 15, 60, 120, 180, and 240 min after the injection of MF or saline.

The above experiment was preceded by a pilot study to establish an optimal (blocking effects of MF) dose of NX. Twelve pigs were divided into 3 groups (n=4), pretreated with saline and after 15 min injected with MF (0.5, 1.0 or 5.0 mg/kg). After 4 days, pigs in each group, before MF administration, were pretreated with different dose of NX (0.1, 0.5 or 1.0 mg/kg) every four days. All the procedures (injections, blood sample collection and immunological assays) were carried out the same way as in the main experiments.

As shown in Fig. 1 increasing doses of NX antagonized MF-evoked changes in NKCC with a dose of 1.0 mg/kg completely blocking the effects of all used doses of MF.

2.4. Natural killer cytotoxicity assay

The cytotoxicity of the peripheral blood NK cells was quantified using a ^{51}Cr-release assay according to the procedure described previously in [21].

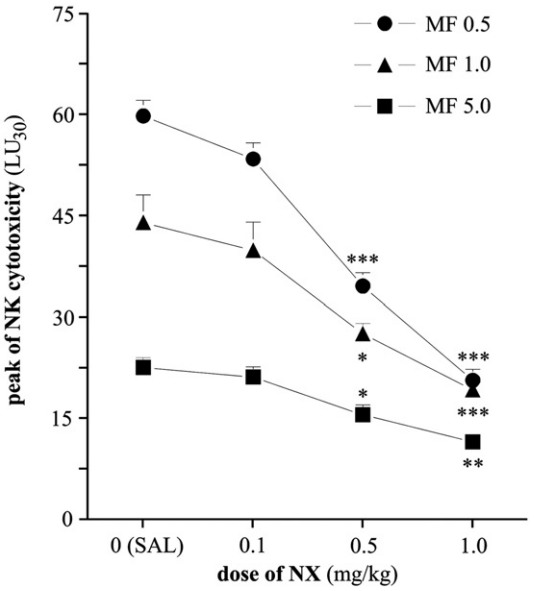

Fig. 1. Dose-dependent NX effect on natural killer cytotoxicity (NKCC) induced by different doses of morphine (MF). Pigs received *i.v.* MF in doses 0.5, 1.0 and 5.0 mg/kg (n=4 in each group) preceded by NX (in doses 0.1, 0.5 and 1.0 mg/kg) or SAL injection. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$ — significance of difference in comparison to SAL (NX 0 mg/kg) (*post-hoc* Tukey test).

2.5. Target cells

The YAC-1 tissue culture cell line of the YAC provided target cells for determining NKCC. This line was maintained in a stationary suspension culture in complete medium: RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma, St Louis, USA), penicillin (Sigma, St Louis, USA) (100 U/ml) and streptomycin (Sigma, St Louis, USA) (100 mg/ml) in a 5% CO_2 humidified incubator (standard culture conditions). Washed in complete medium, the YAC-1 target cells (5×10^6) were labeled with 100 µCi of $Na_2^{51}CrO_4$ (Radio Chemical Center, Otwock-Świerk, Poland) at 37 °C for 1 h. The labeled target cells were then washed five times using RPMI containing 2% FBS and adjusted to 1×10^5/ml in complete medium. In the NK cytotoxicity assay the peripheral blood mononuclear cells were used as the effector cells.

2.6. Effector cells

Peripheral blood mononuclear cells (PBMC) were used as effector cells. PBMC were separated from the heparinized blood by Ficoll 400 (Pharmacia, Uppsala, Sweden) and Uropolinum (Polfa, Starogard, Poland) density centrifugation method [22]. After centrifugation (1113 ×g, 30 min at 4 °C) the isolated cells were collected with a Pasteur pipette, washed with phosphate-buffered saline three times (322 ×g, 10 min at 4 °C), counted and adjusted to 1×10^7 cells/ml in complete medium.

2.7. NKCC assay

The targets cells (2×10^4) were cultured in round-bottomed microwells plates (Nun, Roskilde, Denmark) with various concentrations of effector cells (effector: target cells ratios: 50:1, 25:1 or 12:1, in a total volume of 200 µl) in triplicate, under standard culture conditions for 4 h. Spontaneous ^{51}Cr release wells had target cells plus 100 µl of complete medium and the maximum release wells contained target cells plus 100 µl of complete medium with 5% Triton X-100 (Sigma, St Louis, USA). The assay was terminated at the 4th h by centrifuging the plates (500 ×g, 5 min). Then 100 µl of supernatant was

removed from each well. An isotope count and the percentage of specific lysis (specific ^{51}Cr release) were performed with a gamma counter (Baker System 9120, Allentown, USA) as follows:

$$\text{specific } ^{51}\text{Cr release} = \frac{\text{experimental } ^{51}\text{Cr release} - \textit{spontaneous } ^{51}\textit{Cr release}}{\text{maximum } ^{51}\text{Cr release} - \textit{spontaneous } ^{51}\textit{Cr release}} \times 100.$$

All results are presented in lytic units (LU), which were calculated using the formula:

$$LU_{30} = \frac{E_{STD}}{E : T_{30} \times T_{STD}},$$

where E_{STD} (predeterminated standard number of effector cells) = 10^7, T_{STD} (specified number of target cells) = 2×10^4 and $E{:}T_{30}$ is the effector: target cell ratio needed to lyse 30% of the target cells. One LU_{30} indicates the number of effector cells necessary to kill 30% of the target cells. The total LU per 10^7 effector cells were calculated and used in the figures representing NKCC.

2.8. Large granular lymphocytes number

The percentage and total number of LGL were determined in the peripheral blood mononuclear cells of each individual. This procedure was performed according to the method of Timonen et al. [23] with minor modifications described previously [24]. Cell smears for morphological analysis were prepared by centrifuging for 10 min of 0.2×10^6 PBMC in 200 µl of complete medium on a microscope slide at 350 ×g with a Wescor 7120 cytocentrifuge (Wescor, Logan, USA). After air drying, the cells were fixed in methanol for 10 min, air dried and stained for 10 min with May-Grünwald (Merck, Darmstadt, Germany) diluted in a pH 7.2 phosphate buffer (Merck, Darmstadt, Germany) in distilled water. Subsequently, the cells were stained for 10 min with Giemsa 20% (Merck, Darmstadt, Germany) in a pH 7.2 phosphate buffer. After washing twice with the buffer (pH 7.2) and tap water, the slides were inspected under oil immersion microscopy. At least 200 cells were examined in each slide. The percentage of LGL was obtained per number of all the lymphocytes counted on the blood slide. The total number of LGL in the blood was calculated on respective counts of the total lymphocyte number.

2.9. Lymphocyte number

The total lymphocyte number in the peripheral blood was estimated using a hematology analyzer (Baker System 9120, Allentown, USA).

2.10. Cortisol measure

The plasma COR concentration was measured by radioimmunoassay using a commercially available kit, Spectria Cortisol [^{125}I] (Orion Diagnostica, Espoo, Finland) and Wizard 1470 gamma counter (Pharmacia-LKB, Turku, Finland). The sensitivity of the assay was 1.45-2.55 ng/ml at the 90% B/B_0. The intra- and interassay coefficients of variation were 4.5% and 5.5% respectively.

2.11. Statistical analysis

The results are presented as a mean ± SE. A two-way analysis of variance (ANOVA) was used to test for the effects of doses of MF and time followed by the *post hoc* Tukey test. To test for the effect of NX and time the two-way ANOVA and Tukey test were used separately for each dose of MF or saline.

3. Results

3.1. Changes in NK cytotoxic activity

The smallest dose of MF (0.5 mg/kg) evoked a long-term stimulation of NKCC (maintained throughout the observation period) (Fig. 2). After 1.0 mg/kg of MF the early increase in NKCC was replaced by its suppression. A dose of 5.0 mg/kg MF led to a decrease in NKCC without the stimulation phase characteristic of lower doses of MF.

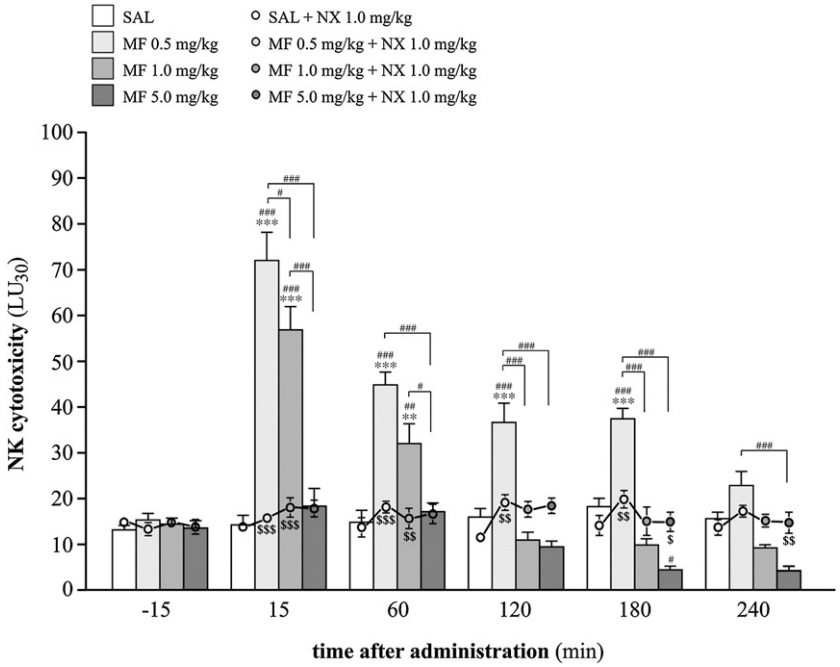

Fig. 2. Dose- and time-dependent response to morphine (MF) of natural killer cytotoxicity in the peripheral blood (bars) and the effect of naloxone (NX) on MF-induced changes (horizontal lines). Pigs received *i.v.* MF in doses 0.5, 1.0 and 5.0 mg/kg or saline (SAL) (n = 6 in each group). NX (1 mg/kg) preceded (by 15 min) MF or SAL injection. ***, $P<0.001$; **, $P<0.01$ — significance of differences in comparison to control value (−15 min); ###, $P<0.001$; ##, $P<0.01$; #, $P<0.05$ — in comparison to SAL or differences between MF doses; $$$, $P<0.001$; $$, $P<0.01$; $, $P<0.05$ — in comparison to SAL or MF (without NX pretreatment) (*post-hoc* Tukey test).

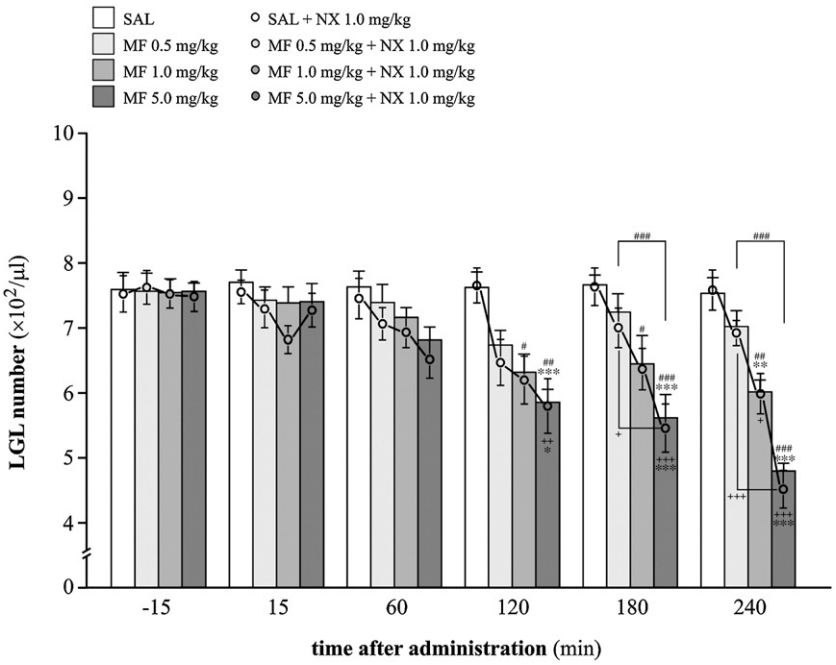

Fig. 3. Dose- and time-dependent response to MF of large granular lymphocyte (NK cells) number in the peripheral blood (bars) and the effect of NX on MF-induced changes (horizontal lines). Procedure and abbreviations as in Fig. 2. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$ — significance of differences in comparison to control value (−15 min); ###, $P<0.001$; ##, $P<0.01$; #, $P<0.05$ — in comparison to SAL or differences between MF doses; +++, $P<0.001$; ++, $P<0.01$; +, $P<0.05$ — in comparison to SAL+NX or differences between MF doses (with NX pretreatment) (*post-hoc* Tukey test).

The two-way ANOVA yielded reliable effects for a dose ($F_{3,143}=116.129$, $p<0.001$) and time after injection ($F_{5,143}=59.382$, $p<0.001$) as well as the dose×time interaction ($F_{15,143}=16.272$, $p<0.001$). A significant interaction between dose and time after injection indicates that the main effect (NKCC changes) recovered at different paces across the doses. A high, above 2- to 4-fold, increase in NKCC induced by the lowest dose of MF (0.5 mg/kg) was seen in each time-point after injection: 508% of the control (saline injection) value ($p<0.001$) after 15 min, 303% ($p<0.001$) after 60 min, 230% ($p<0.001$) after 120 min, 206% ($p<0.001$) after 180 min and 146% ($p>0.05$) after 240 min. This dose of MF did not decrease NKCC below the control level in any animal tested. A significant increase in NKCC in response to 1.0 mg/kg of MF was observed after 15 min (400% of the control value, $p<0.001$) and 60 min (217%, $p<0.01$). After 5.0 mg/kg of MF the early changes were insignificant. Later, between 120 and 240 min, after

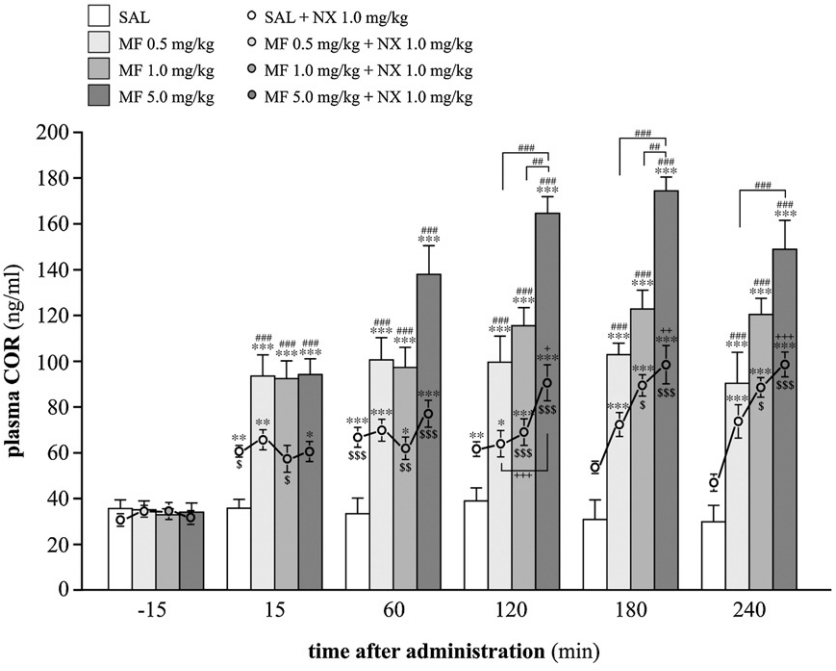

Fig. 4. Dose- and time-dependent response to MF of plasma cortisol (bars) and the effect of NX on MF-induced changes (horizontal lines). Procedure and abbreviations as in Fig. 2. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$ — significance of differences in comparison to control value (−15 min); ###, $P<0.001$; ##, $P<0.01$ — in comparison to SAL or differences between MF doses; +++, $P<0.001$; ++, $P<0.01$; +, $P<0.05$ — in comparison to SAL+NX or differences between MF doses (with NX pretreatment); $$$, $P<0.001$ — in comparison to SAL or MF (without NX pretreatment) (*post-hoc* Tukey test).

1.0 as well as 5.0 mg/kg of MF there was a fall in NKCC, maximally at the 180th min to 54% ($p<0.05$) and 24% ($p>0.05$) of the saline value, respectively.

NX pretreatment antagonized the MF-induced changes in NKCC. As shown in Fig. 2 (horizontal solid lines across the bars), MF in any dose administered to NX-pretreated (1.0 mg/kg) pigs did not affect NKCC. Analysis of the variance revealed the highly significant effect of the treatment (MF after pretreatment with NX and MF alone in all doses tested) ($F_{1,71}=170.585$, $p<0.001$ for 0.5 mg/kg MF; $F_{1,71}=17.029$, $p<0.001$ for 1 mg/kg MF; $F_{1,71}=19.213$, $p<0.001$ for 5 mg/kg MF).

3.2. Changes in the peripheral blood LGL (NK cell) number

The two-way ANOVA yielded reliable effects for a particular dose ($F_{3,143}=33.099$, $p<0.001$), time after injection ($F_{5,143}=17.223$, $p<0.001$) and interaction (dose×time) ($F_{15,143}=3.952$, $p<0.001$). As Fig. 3 shows, the significant effect of time after MF administration concerned mainly the effect of LGL/NK number reduction evoked by doses of 1.0 and 5.0 mg/kg, which became significant only in the later periods (120–240 min) after MF. After 240 min LGL number was reduced to 80% ($p<0.01$) and 64% ($p<0.001$) (1.0 mg/kg and 5.0 mg/kg, respectively) of the saline value. The lowest used dose of MF (0.5 mg/kg) had no significant effect on peripheral LGL number. Whereas no significant relationships between NKCC and LGL number were noted after smaller doses of MF (0.5 and 1.0 mg/kg), a positive correlation was observed only after the largest (5.0 mg/kg) dose of MF ($r=0.730$, $p<0.001$). NX pretreatment had no significant effect on MF-induced changes in LGL number. In the ANOVA there was no treatment effect (MF alone *vs* MF after NX).

All the MF doses caused an insignificant fall in lymphocyte number.

3.3. Changes in the plasma cortisol level

All the doses of MF elevated blood plasma COR (Fig. 4). The COR level rose maximally at the 180th min to: 334% ($p<0.001$), 499% ($p<0.001$), 667% ($p<0.001$) of the saline value after 0.5, 1.0, 5.0 mg/kg of MF, respectively. The ANOVA yielded reliable effects of a dose ($F_{3,143}=135.283$, $p<0.001$), time after injection ($F_{5,143}=45.506$, $p<0.001$) and interaction (dose×time) ($F_{15,143}=8.474$, $p<0.001$). No significant changes in plasma COR concentration were observed in control (saline) animals. There was a negative correlation between plasma COR and LGL number after 1.0 mg/kg and 5.0 mg/kg of MF ($r=-0.629$ and $r=-0.715$, respectively, $p<0.001$ in both cases) with no significant relationships after the smallest dose of MF. A significant correlation between COR and NKCC was found only after the largest (5.0 mg/kg) dose of MF ($r=-0.442$, $p<0.01$).

As Fig. 4 shows NX alone induced a marked rise in the level of plasma COR, maximally to 200% ($p<0.001$) of the saline injection value. NX pretreatment had no significant effect on MF-induced changes in plasma COR. In the ANOVA there was no treatment effect (MF alone *vs* MF after NX).

4. Discussion

The results obtained showed that the doses of MF used evoked dose- and time-dependent and NX-related changes in the lytic activity of LGL/NK cells. The lowest dose (0.5 mg/kg) evoked a marked increase in the cytotoxicity without a significant change in LGL cell number maintained for the whole observation period (240 min). In the case of 1.0 and 5.0 mg/kg, this increase was smaller and shorter (15-60 min) and was followed by a decrease in the activity and number of LGL cells. Modulation of NKCC (both stimulation and suppression) was concomitant with a marked elevation of COR level.

Majority of the literature treats MF action as exclusively immunosuppressive. However, there are several reports indicating the possibility of the immunostimulatory effect of MF, particularly on NKCC. For example, Kraut and Greenberg [25] showed stimulation of splenic natural killer cell activity after irregular *i.p.* treatment with 20 mg/kg of MF in rats, in contrast to the suppressive effects of regular daily injections of the same dose, whose influence was suppressive. Carr and France [26] obtained an enhancement of splenic NKCC after *s.c.* 3.2 mg/kg of MF in monkeys that were drug-abstinent for 2 days, in contrast to daily MF-injected subjects which displayed suppressed NKCC. Data obtained by the same authors [10] suggested a tendency to enhance cytotoxic activity after *i.v.* 10 mg/kg of MF in mice (the effect neglected by the authors as insignificant) in contrast to a decrease in NKCC evoked by 32 mg/kg of MF.

The problem of the opposite effects of different doses and schedules of administration concerns also other drugs, including psychotropic ones [27]. In our previous studies with amphetamine [21,28], a low amphetamine (1 mg/kg) dose induced a marked increase in NKCC in rats, which did not support the common view on the unidirectional, immunosuppressive action of this drug. Our data correspond with the bimodal, dose-related response (hormesis) described by Calabrese monography [27] as a universal phenomenon, the mechanism of which is not yet clear. It seems that conclusions about the immunosuppressive effects of MF derived from the studies that omitted the low, subanalgetic doses of MF that are of little interest to the clinicians. Beside, the differences between our data and those of other authors may depend on compartments from which the effector cells are derived as well as species differences.

Most of research on the immunomodulatory effects of opioids has been done in rodents that are more sensitive to immunosuppression than humans, monkeys and pigs. These differences in susceptibility to immunosuppression (among other to the suppressive effects of steroids) have been for long used to differentiate between steroid resistant and steroid susceptible species [29]. For example, pigs injected with CRH displayed markedly lower tendency to reduction of NK cytotoxicity then much greater effect observed in rats [30–33]. In humans, which are among the steroid resistant species, catecholamines (mediators of the immunomodulatory effects of opioids) and different stressors (mental or physical) known as the immunosuppressors can also enhance immune functions including NK cytotoxic activity [34–37]. The possibility of the immune function enhancing properties of catecholamines has been shown also in other animals [38–41].

In the present experiment changes in the activity and number of NK cells have been assessed in peripheral blood and may differ from results obtained with splenocytes. It was reported that sympathetic blockade was more effective in affecting the immunosuppressive effects of stress in the case of splenocytes than blood lymphocytes [42]. Similar to other reports [43,44] the assessment of the functionality of NK cells was based on the measurements of the cytotoxic activity and the number of LGLs. Yet, NK cytotoxicity is not an exclusive property of LGL cells. For instance, in pigs peripheral blood, lymphocytes contain about 20% of γδ T cells exhibiting natural killer cytotoxic activity [45,46]. Exact functional properties of these and other lymphocyte subsets, e.g. subsets of NK cells in spleen and peripheral blood under different stressors and drugs, have not been studied. It should be considered that the cytotoxic activity of splenocytes, the largest sources of lymphocytes, does not always reflect changes that can be observed in the case of repeated sampling of peripheral blood. It may be that a cell in peripheral blood is activated by a number of factors that are quite limited in an organ. Such a possibility has been suggested by studies of Schedlowski et al. [47], showing that catecholamines mobilize human NK cells circulation and function via spleen independent B2-adrenergic mechanism.

Pretreatment with a nonselective opioid antagonist, NX, blocked cytotoxicity changes induced by all the doses of MF used suggesting that the observed effects may depend on different types of opioid receptors. Despite preferences for μ receptors, MF also binds to other subtypes [48]. According to Lockwood et al. [49] μ and κ receptors may

be responsible for immunosuppressive while δ and ε for immunoenhancing effects. Furthermore, there is a possibility that the same receptor could mediate different effects of opioids on the immune system in a dose-dependent way [14,50,51]. The opposite effects of MF may also result from different ways of its indirect influence on peripheral immune cells. Substantial literature indicates that MF does not act directly on the peripheral cells but that its *in vivo* effects are mediated through the central opioid receptors [52]. Two main pathways from the central opioid receptors to the peripheral endpoint (NK cell) are suggested: the sympathetic nervous system and the COR releasing HPA axis [53–55].

Available data indicate that stimulation of opioid receptors especially at peri-aqueductal grey matter increases sympathetic outflow that may results in completely different effects [52,56]. It has been suggested that immonosuppressive effects can be realized through indirect activation of α-adrenoreceptors on peripheral cells [56,57]. Carr et al. [57,58] showed that preatreatment of mice with α-adrenoreceptor antagonists blocked the MF-induced suppression in NK cell activity. On the other hand, Fecho et al. [59] demonstrated that β-adrenergic antagonist did not block the suppressive effects of morphine on NK cell activity in the rat. The finding suggests that β-receptors may mediate enhancement of NKCC by the MF (opioids)–COR–catecholamine–immune competent cells chain. The possibility of the immunostimulatory effects of catecholamines has been confirmed by the studies concerned with direct effects of adrenaline and noradrenaline on the adrenergic receptors and, also, their indirect role in the immunoenhancing effects of stress. Some studies have suggested the pivotal role of β-receptors (β-adrenergic mechanism) in mobilization of NK cells (increase in the peripheral blood NK activity). Schedlowski et al. [47] showed that β-adrenoreceptors antagonist, propranolol, but not selective β1-adrenergic antagonist bisoprolol, inhibited adrenaline- and noradrenaline-induced increases in NK cell number and functions. These data were in accordance with other reports that propranolol blocked mobilization of NK cells elevated by physical exercise [60], mental stress [61,62] and single dose of amphetamine [28].

Our results indicate the activation of the HPA axis by MF. MF evoked a marked increase in COR whose dynamics depended on MF doses and postinjection time. However, despite the widely accepted view of the immunosuppressive role of COR [17] we observed an enhancement of NKCC simultaneously with an intense rise of COR. Thus, we received further support for our earlier findings [24] and those of other authors [49,63] suggesting that the negative correlation between COR and the immune reagents is not always the rule. It also occurred that the function of NK cells, their number and changes in the level of COR may be modulated independently. A relatively small COR rise (early action of the lower MF doses) was concomitant with an intense enhancement of the cytotoxicity not accompanied by significant changes in NK cell number (a lack of changes or a tendency to a decrease). Only at the paramaximal increase of COR (the later period of action of the higher MF doses) was there a fall of NKCC simultaneous with a decrease in NK cell number. It seems that under the low MF doses the lack of the immunosuppressive action of COR may be related to the well known and recently recalled permissive influence of glucocorticoids on immunostimulatory action of catecholamines [64–66].

Similar dissociation between the functional changes (cytotoxicity) and the number of cells (cellularity) has been described in the clinics. Many patients with cancer show decreased levels of NK cytotoxicity despite a consistent increase in the number of NK and natural killer T cells [67–69] (the mechanism of this phenomenon remains unclear). There are speculations that the cytotoxic activity (of a single cell), which depends on perforin system, may be modulated by affecting the excretion of cytoplasmic granules. This mechanism would make the whole cytotoxic activity independent to a large extent of NK cells changes [70–72].

Lack of relationship between changes in cytotoxicity and cellularity and/or COR level was evident in different responses to NX. In contrast to MF-induced modulation of NKCC, the level of COR and LGL and NK cell number appear to be NX insensitive. Similar data suggesting dependence of MF-induced changes in cytotoxic activity on the opioid receptors (sensitivity to NX) without a corresponding blockade of NK cell number, were reported by Nelson et al. [73] and Tseng et al. [74]. The results may suggest that MF affects atypical receptors. This could mask the effects of NX. However, recent data point that NX insensivity to COR rise does not exclude involvement of opioid receptors because NX alone can increase COR through activation of the HPA axis [75–77].

There are certainly many other ways through which opioids can both suppress NKCC as well as counteract its potentiation. For example, Madden et al. [78] and Carr et al. [10] suggest that NX-sensitive lymphocyte receptors are not membrane bound on the resting cells but that they are cytoplasm associated with the microsomes and the mitochondria. It is tempting to speculate that only large doses of MF are likely to interact with cytoplasmic or genomic receptors [10]. It has been also suggested that opioids may function as "primitive cytokines" which are able to modulate directly the activity of immunocompetent cells [55,79].

To summarize, although the immunomodulatory effects of opioids, including MF, have been described for several years, the mechanisms are still unclear. Our data suggest that it is possible to find such a small dose of MF that potentiates NKCC, influencing only the function of NK cells. Also, it seems that NK cytotoxicity and LGL/NK cell number can be controlled independently. Surprisingly, the immunostimulatory effect occurs simultaneously with a relatively high peripheral blood COR level which indicates activation of the HPA axis. It appears that small doses of MF can stimulate NK-related mechanisms for the defense against neoplastic transformations and viral infections; this is not to be ignored while assessing the complexity of the mechanism of immune control and should be taken into consideration in clinical practice.

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