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# *Paramecium caudatum* Avoids from Naloxone

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**Abstract** Protozoa aggregate to or avoid from chemical substances. We aimed to show the significance of nitric oxide (NO) in the performance of the eukaryotes to misuse drug exposure. The micro-organism *Paramecium caudatum* was collected from natural sources and properly isolated by repeatedly sub-cultivation in hay infusion. Number of cells per 1ml of pure medium culture was counted using Sedgwick-Rafter cell chamber. Doses of naloxone (0.05-0.4 µg/µl) solely or jointly with the NO agents were infused into the chamber. Cell response was recorded after drug infusion with intervals (0-180 sec). Negative control received distilled water (1 µl) instead of naloxone (legend 0). Along with, the Ca channels or cGMP pathway was banded to discuss the mechanism. According to the results, the *Paramecia* showed negative chemo-taxis to the naloxone, the response which is comparable with signs of withdrawal from abused drugs. This response was potentiated by activation of NO system, but, reversed after usage of the system blocker. The inhibition of Ca channels or cGMP signaling pathway markedly enhanced the avoidance. In conclusion, this study may clearly contribute the signal molecule NO in the dependence of the eukaryotes on sedative misuse drugs.

***Keywords:*** *paramecium, naloxone, evasion, nitric oxide*

## 1. Introduction

Movement of *Paramecium caudatum* a simple eukaryotic organism toward or away from a chemical is commonly seen. It provides the means for the organism to react to environmental gradients of nutrients or harmful materials by moving towards or away from them.

The ability of this micro-organism to respond to the concentrations of opioid drug morphine has been studied recently. It has been shown that morphine, the main abuse drug, aggregates Paramecia [1,2] by involving nitric oxide (NO) system. The opioid receptors [5,6,7] involve in the NO signaling [8,9] in free-living invertebrates. Even morphine-like compounds have been determined in protozoan parasites [10].

The NO which was described at first as endothelium-derived relaxing factor [3] is now known as organic molecule with great importance [4]. Researchers have shown that the NO plays important role in thermoregulation of *Paramecium* [11]. This simple unicellular organism senses temperature and move preferentially to temperature zones [12].

Studies with eukaryotic microorganisms that possess the chemical sensing and/or NO signaling systems give possibility of in vivo survey on the opioid ligand relation in contact of cells to the sedative misuse drugs. It also provides the ability to discuss the mechanism governing on the eager behavior toward the abuse drugs.

We presented a pure narcotic drug naloxone to the *P. caudatum* to argue on the opioid receptor-ligand relation in the unicellular organism. We more examined the action of opioid antagonist in presence of NO. We additionally studied the signal pathways in reaction to naloxone.

## 2. Materials and Methods

### 2.1. Subject (Collection and Cultivation)

Organisms were collected from the momentary fresh water bodies in Tehran region including small bogs and ponds. The cells were promptly cultivated in natural polyculture medium (hay infusions: 10g/l of tap water at 19 to 21 ℃). Hay infusions were boiled for about 5min; the boiled hay was then allowed to settle and the supernatant was used as a culture medium [13].

The specific attribution of protozoa was identified by data that have already been provided by this laboratory [14].

The little organisms were properly isolated from other subjects passed by the natural sources. They were repeatedly cultivated in hay infusion to colonize adequately. The media were adjusted to a pH of 6.8±0.2 and were maintained at 29 to 31 ℃. After about one week the media were refreshed.

### 2.2. Drugs

Naloxone hydrochloride (Tolid Daru, Co., Tehran, Iran), L-arginine (Sigma Chemical Co., USA) and NG-Nitro-L-arginine Methyl Ester (L-NAME; Research Biochemical Inc., USA) were prepared fresh in sterile distilled water (vehicle). The chemical gradients were purchased from

Merck Chemicals (Germany). $MgSO_4$ or Methylene blue was provided also by Merck Chemicals.

## 2.3. Calculation of Protozoan Population

The population of *P. caudatum* was daily counted by Sedgwick-Rafter cell counting chamber (Graticules, Ltd., UK). This chamber, holding a little more than 1 ml, can be covered with a thin coverslip, allowing microscopic examination with objectives up to 16X. The cells were counted by light microscopy using a 4X objective. A sample of the cell culture was placed in the chamber, which was then covered with a coverslip. The microorganisms were subsequently allowed to settle in the chamber. This step was performed as quickly as possible to ensure that the protozoa were randomly distributed and settled uniformly in the chamber [13,15]. The samples were accurately diluted to provide a definite number of cells per field (view) throughout the experiments. Each field was counted at least 5 times, and the mean of the counts was calculated and reported.

Cell populations were counted in 100 $\mu m^2$ units using an Olympus light photomicroscope at 4X magnification. Furthermore, the Image Tool program (UTHSCSA, version 2.03), the free image processing and analysis program for Microsoft Windows, was used for image analysis after spatial calibrations to provide quantification for an area of 100 $\mu m^2$.

## 2.4. Naloxone Effect Assessment

To measure the influence of naloxone in *P. caudatum* the experiments were designed and performed as following:

### 2.4.1. Pre-requirement Study

1ml of cultivation medium containing the cells was positioned in the chamber; permitting cell viability for about 30 min [13].The cells were transferred in the midline of the apparatus and allowed free access to the entire apparatus for 5 sec. The number of cells in the chamber was counted at low power (4X objective) using the protocol described above. In addition, all events were recorded using video microscopy (Olympus). The records later were reviewed by a blind observer and analyzed using the Image Tool program.

### 2.4.2. Effect of Naloxone on *Paramecia*

This effect was evaluated in time 0 or promptly after infusion of naloxone throughout the time intervals ranging 5-180 sec (Figure 1). The drug infusions at the desired concentrations (in a total volume of 1 µl) were performed using a glass 1-µl Hamilton syringe as consistently as possible over a definite time period. Control groups received distilled water solely (1 µl).

### 2.4.3. Joining of L-arginine with Naloxone

The cell population was measured (counts/view) after joining the L-arginine (1-8 µg/µl) with naloxone to participate the NO in response of *Paramecia* to naloxone.

### 2.4.4. Joining of L-NAME with L-arginine plus Naloxone

The infusion of L-NAME (1-8 µg/µl) was priorly performed to the infusion of L-arginine to discuss on the involvement of the NO system.

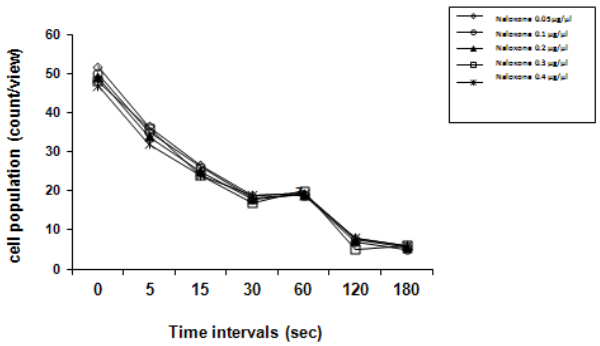


**Figure 1.** This figure shows the effect of naloxone (0 to 0.4 µg/µl upon infusion into the cell counter chamber). The narcotic drug induced avoidance within the cell populations (counts/view). As figure defines a point during which subsequent experiments were followed (the time point 60 sec) was chosen based on the data

## 2.5. Signaling Pathways Survey

Ca channel blocker, $MgSO_4$ (1-50 µg/µl), or Methylene blue (1-50 µg/µl), an inhibitor of cGMP signaling pathway was infused prior to L-arginine in the joining protocol with naloxone to clear the signal process.

## 2.6. Statistical Analysis

Data are presented as mean ± SEM. Groups were compared using one-way analysis of variance (ANOVA). Differences between groups were measured using the Tukey-Kramer post-hoc test. A p-value of $< 0.05$ was the threshold for statistical significance.

# 3. Results

## 3.1. Induction of Avoidance by Naloxone

Figure 2 shows a response curve to naloxone in *P. caudatum*. The narcotic drug induced a significant avoidance [$p< 0.0001$] in microorganisms. The maximum response in *Paramecia* was occurred at 0.4 µg/µl of the drug; thus, this dose was used for the subsequent behavioral tests.

## 3.2. Effect of L-arginine on Avoidance-Induced by Naloxone

Figure 3 shows the effect of the NO precursor, L-arginine, on naloxone effect in *P. caudatum*, which was statistically significant [$p < 0.05$]. The NO-generating agent potentiated the response in a dose-dependent manner. Based on the results, a dose of 2 µg/µl of L-arginine was used for subsequent behavioral testing.

## 3.3. Effect of Pre-infusion of L-NAME on L-arginine plus Naloxone Effect in *P. caudatum*

Figure 4 reveals the effect of the NOS inhibitor, L-NAME, on effect of L-arginine in joining with naloxone in *P. caudatum*. The effect of L-NAME was statistically significant [$p < 0.01$]. The NOS inhibitor blocked the observed effect significantly.

### 3.4. Nitric oxide signaling

Figure 5 and Figure 6 show that pre- infusion of Methylene blue or $MgSO_4$ prior to L-arginine adjoined with naloxone in little organisms. The responses were obtained at significant level [$p < 0.0001$]. The NO agent promoted the receptor-ligand signaling mechanisms as data show.

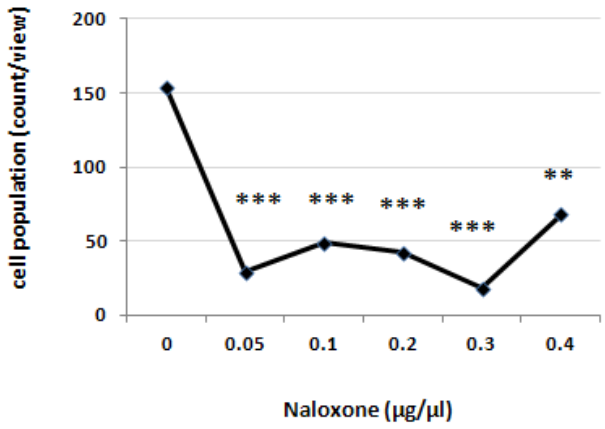


**Figure 2.** Dose response curve for naloxone in *P.caudatum*. Different doses of drug (0-0.4 µg/µl) were given in a schedule as detailed in the Materials and Methods. The negative control group legend 0 only received distilled water (1 µl). Score is defined as the cell counts/view at the 60-sec time point and is expressed as mean score±S.E.M. **p<0.01, ***p<0.001 significantly different from distilled water control group

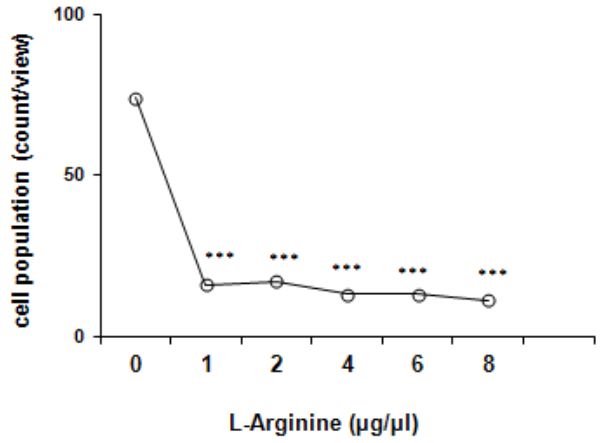


**Figure 3.** Effects of L-arginine on cell avoidance by naloxone. Cells received distilled water (1 µl) grouped as the control (legend 0). Others obtained L-arginine (1 to 8 µg/µl) prior to naloxone during the experiments. The control legend 0 group was treated naloxone (0.4 µg/µl) as alike as the other groups. Score is defined as noted in Figure 3.*p<0.05, **p<0.01 significant difference to other dose groups of naloxone.

## 4. Discussion

As the main results of this study show: (1) naloxone avoided the *P. caudatum*, (2) after infusion of L-arginine the microorganisms took out more potently, (3) L-NAME reversed the effect of L-arginine, (4) blocking of Ca channels or cGMP pathway significantly reversed the L-arginine plus naloxone response.

The naloxone-receptor relation blocked the opioid receptor action since the present evidence provides adverse response to the drug compared with that observed by morphine [1,2]. It should be notified that the number of *Paramecia* showed an increase when 0.4 µg/µl naloxone was used in the dose response assay. This evidence only demonstrates a dose dependent effect of the narcotic naloxone, but do not support a selective interaction with opioid receptors sub-types.

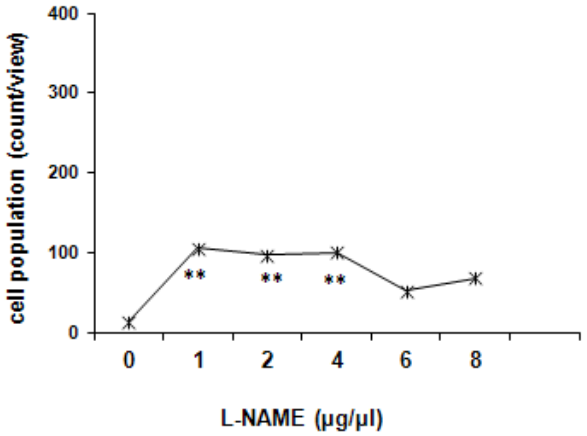


**Figure. 4.** Effect of L-NAME on *Paramecia* response to L-arginine plus naloxone. Cells were treated with distilled water (1 µl) or L-NAME (1 to 8 µg/µl) prior to L-arginine plus naloxone (0.4 µg/µl). The control legend 0 group was treated L-arginine plus naloxone as alike as the other groups. Score is calculated as defined in Figure 3. **p < 0.01 significant difference to other dose groups of naloxone plus L-arginine

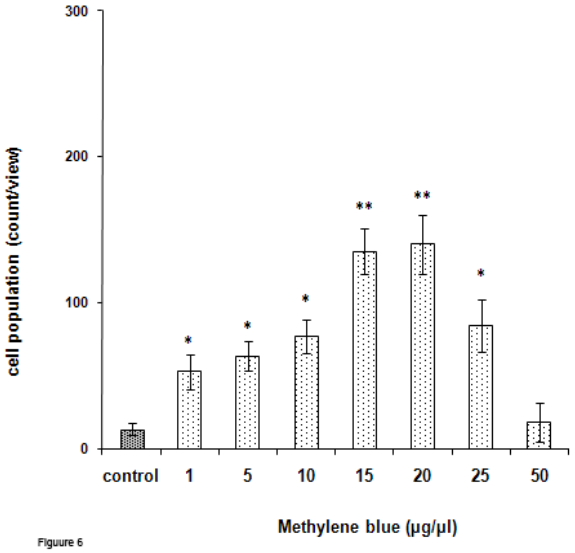


**Figure. 5.** Blockade of cGMP pathway due to pre-infusion of Methylene blue (1-50 µg/µl) to L-arginine plus naloxone. Cells received distilled water (1 µl) instead of Methylene blue that grouped as the control group or Methylene blue (1-50 µg/µl) prior to L-arginine (2 µg/µl) prior to naloxone (0.04 µg/µl) during the experiments. Score is defined as noted in Figure 3. *$p < 0.05$, **$p < 0.01$ significant difference to control group

The effect of naloxone on mobility of *Tetrahymena* has been previously studied [15]. Wu et al. (1997) [16] demonstrated that the drug was ineffective to inhibit the immobilizing effect- induced by an opioid in the organism. Josefsson and Johansson (1979) [17] demonstrated a naloxone-reversible opioid effect on pinocytosis in *Amoeba*, and similar observation was made by Chiesa et al. (1993) [18], as well as by Salaman et al. (1990) [19], who studied phagocytosis in *Tetrahymena*. In contrast to these data, we studied the opioid receptor-ligand relation in

accumulation or avoidance of *Paramecium*. The present results may demonstrate for the first time that the molecule NO is participated in avoiding of *P. caudatum* to the narcotic drug. Thus, this organism may be usable as a new model for measuring opioid withdrawal in living organisms. In agree, there is evidence showing that ultra-low-dose opioid antagonists block excitatory signaling of opioid receptors [20]. Indeed, because this unicellular model is low-cost and easy to manipulate, it is accepting to be used to interrogate the cellular mechanisms governing on opioid dependence. These cells, *Paramecia*, as been previously shown by this laboratory express a cell aggregation to morphine [1,2]. Our previous results have also implicated NO in the morphine effect by NADPH-diaphorase cytochemistry [1]. It has been postulated that several NO-sensitive targets, including guanylyl cyclase, potassium channels and voltage gated calcium channels [21,22,23,24,25] are stimulated in invertebrates. Other studies using the ciliate protozoan *Stentor* have demonstrated that these types of microorganisms express a G protein-mediated response to morphine when stimulated mechanically [26]. The L-type calcium channels are members of transmembrane ion channel proteins that are associated with calcium influx signal in many cells, including immune cells [27]. Regarding that Magnesium is a natural calcium antagonist and a potent L-type calcium channel inhibitor [28] rationally the usage of $MgSO_4$ antagonized the Ca influx signal in the organisms. In addition, considering that the depolarizing $Ca^{2+}$ inward current is coupled to the generation of cGMP [24,25,26], the works agree with ours that blockade of Ca channels or cGMP pathway significantly reverse the L-arginine plus naloxone response. Although the role of cGMP has not been elucidated in most systems, it mediates many NO physiological effects as a signaling molecule in the nervous and cardiovascular systems [11,29]. The effect of Ca channel blocker $MgSO_4$ was not dose responsive most likely because that decrease in $Ca^{2+}$ entry via voltage-dependent calcium channels varies in the presence of different concentrations of $MgSO_4$. This point remains elusive.

In addition, findings provided by this research denote that the inhibitor of NO system L-NAME reverses the L-arginine effect in the *Paramecia*. Previous data have shown that the *P. caudatum* produces the NO, and that the L-NAME inhibits the NO production [11]. Malvin *et al.* (2003) [11] have also suggested that the *P. caudatum* produces the NO by L-arginine through activation of a calcium-sensitive NO synthase (NOS). A role for L-arginine in NO production has been confirmed in a later study by examining the ability of *P.caudatum* to produce [3H] L-citrulline from [3H] L-arginine via a mechanism inhibited by L-NAME.

In brief, the contribution of NO in naloxone- induced avoidance of *P. caudatum* was shown by using a novel behavioral assay. Inhibition of Ca channels or cGMP signaling pathway markedly enhanced the avoidance. The findings may properly show the significance of the NO in the dependence of the eukaryotes on sedative misuse drugs, a precious data from the time when the signaling role of NO was known. So, changing of NO may bring us health problem, a point that offers a unique concentration to this molecule.

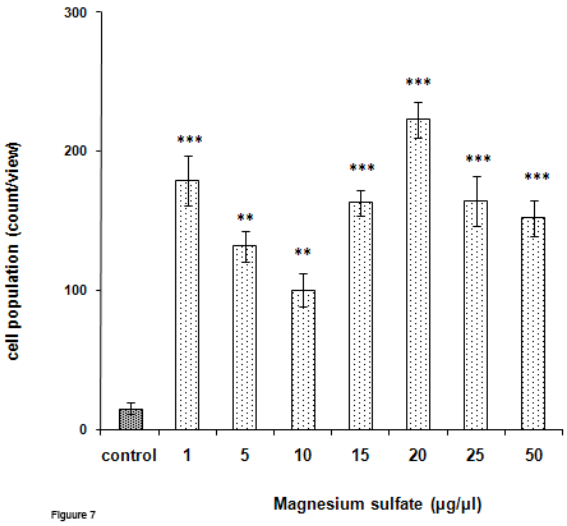


**Figure. 6.** Blockade of Ca channels pathway due to pre-infusion of MgSO4 (1-50 µg/ µl) to L-arginine plus naloxone. Cells received distilled water (1 µl) instead of Methylene blue that grouped as the control group or MgSO4 (1-50 µg/ µl) (1-50 µg/ µl) prior to L-arginine (2 µg/ µl) prior to naloxone (0.04 µg/ µl) during the experiments. Score is defined as noted in Fig 3. $^{**}p < 0.01$, $^{***}p < 0.01$ significant difference to control group

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