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Interaction of nicotine with morphine potency in *Paramecium caudatum*

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ABSTRACT

Aims: Many studies have been carried out about the interaction between nicotine and morphine in higher animals. Although previous behavioral and pharmacological evidence indicated the presence of opioid system in protozoa, there is no available data about nicotine effect on the potency of morphine in eukaryotic unicellular organisms such as protozoa. Hence, this work aims to investigate the interaction of nicotine with morphine in the protozoan *Paramecium caudatum*.

Main methods: According to our innovative model, the movement behavior of *P. caudatum* was investigated with a numerical scale using the Sedgewick-Rafter counting chamber at the field of view of 4X light microscope objective lens, such that the difference in number of Paramecia cells at definite moments after injection of drugs/substances was considered as a criterion for the behavioral response of *P. caudatum*.

Key findings: Results indicated the variations of morphine potency at the dose of 2 μg morphine accompanied by different doses of nicotine in *P. caudatum* so that the highest aggregation of Paramecia cells occurred at the dose of 2 μg morphine + 4 μg nicotine.

Significance: This confirmed that in eukaryotic unicellular organisms such as *P. caudatum*, nicotine can reinforce the morphine potency in a dose-dependent manner.

1. Introduction

Regarding the influence of opioids such as beta-endorphin, morphine and met-enkephalin in some ciliates like tetrahymena, stentor, and paramecium, previous pharmacological evidence indicated the presence of opioid receptors in these protozoa (Chiesa et al. 1993; Karami et al. 2013a; Karami et al. 2013b; Kostyra et al. 2002; O'Neill et al. 1988). Our behavioral studies have also revealed the ability of morphine to attract and aggregate Paramecia cells (Karami et al 2013a, 2013b).

It has been confirmed that morphine, as the head of opioid agonists, is an indicator for analgesic activities of other drugs. Several studies on the vertebrates have demonstrated that when morphine, as a metabotropic agent, deposits on opioid receptors (especially μ receptor), it attenuates cyclic adenosine monophosphate (cAMP) production via inhibitory G proteins (Gi), blocks the voltage-gated calcium channels, and triggers potassium moving out of the cell. This process leads to the hyperpolarization of cell membrane and causes analgesia as well as euphoric properties and physiological dependence (Al-Hasani and Bruchas, 2011).

On the other hand, nicotine, like morphine, is a narcotic alkaloid with ionotropic mechanism. It has numerous incontestable effects such as biosynthesis and release of neurotransmitters into the peripheral nervous system (PNS) and central nervous system (CNS) (Zarrindast et al. 2006). Clinical evidence and laboratory studies on the higher animals have demonstrated the influence of nicotine on learning, memory and cognition. In this regard, many studies have also been carried out about the interaction between nicotine and morphine in higher animals. The available data indicate that nicotine reduces physical and psychological signs of withdrawal syndrome induced by morphine (Rafsanjani et al. 2012); nicotine can potentiate the morphine-induced movement activity and conditioned place in mice (Vihavainen et al. 2008a) and improve the morphine-induced impairment of memory (Ahmadi et al. 2007). The reinforcing effect of acute and sub-chronic nicotine pretreatment on morphine state-dependent learning has also been confirmed (Zarrindast et al. 2006). Other studies mostly indicate positive and reinforcing effects of nicotine on morphine potency (Li et al. 2010; Talka et al. 2013; Vihavainen et al. 2008b).

However, there is no scientific report about the nicotine effect on morphine potency in protists such as protozoa. Hence, the present study is conducted based on the pharmacologic principle: agonist/antagonist-receptor interactions to explore the opioid and nicotinic systems, and

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the interaction of nicotine with morphine from physiological and behavioral perspectives in the protozoan *Paramecium caudatum*. For this purpose, we investigate the behavioral response of this microorganism to the exposure of morphine, nicotine, and trimethaphan, as a competitive antagonist of nicotine (Rose et al. 1999).

2. Materials and methods

2.1. Sampling, identification and cultivation

In our previous research, sampling and morphological identification of *P. caudatum* were performed; moreover, cultivation of this microorganism has been conducted in the laboratory until now (Karami et al. 2013b; Shahrokhi et al. 2013).

2.2. Optimum cultivation conditions

P. caudatum was cultivated in fresh natural medium (Bacterized hay infusion) and a specific one (Enriched yeast medium). The proper temperature for growth was adjusted within the range of 30 ± 2 °C. The pH of the culture medium was adjusted within the range of 6.8 ± 0.2 using 0.1 N acetic acid and sodium hydroxide solutions (Malvin et al. 2003; Shahrokhi et al. 2013).

2.3. Drugs

Morphine sulphate (TEMAD, Co., Tehran, Iran), nicotine hydrogen tartrate (Sigma, Pool, Dorset, UK) and trimethaphan camsylate (Pourateb, Co., Tehran, Iran).

2.4. Behavior analysis of P. caudatum

In this research, our previous innovative model (Karami et al 2013a, 2013b) was used to investigate drug potency in *P. caudatum*. In this method, the **movement behavior (Aggregation/Evasion)** of *P. caudatum* after exposure to the examined drugs/substances was analyzed with a numerical scale using the Sedgewick-Rafter counting chamber (Graticules, Ltd., UK), at the field of view of 4X light microscope objective lens (Fig. 1), such that the difference in number of Paramecia cells at definite moments after injection of drugs/substances was considered as a criterion for the behavioral response of *P. caudatum*. According to the following procedure, the behavioral and pharmacological experiments were performed:

First, 1 ml of the culture medium containing *P. caudatum* with the concentration of 2600 ± 250 Paramecia/ml was transferred into the Sedgewick-Rafter counting chamber. It should take 1 minute for Paramecia cells to adapt to the new place. Then, the determined dose of desired drug/substance was microinjected at the **injection spot** (the field of view of 4X light microscope objective lens in the counting chamber), by Hamilton syringe at once, and Paramecia cells were immediately counted at the 0th, 5th, 15th, 30th, 60th, 120th, and 180th second after the injection of drug/substance in the same place (Injection spot) (Fig. 1).

Finally, the difference in number of Paramecia cells in the 60th second proportional to the 5th second after injection of drug/substance (the first five seconds were considered as a time interval for the Paramecia cells to adapt with the injected drugs/substances) was considered as a criterion for the behavioral response of *P. caudatum* to the injected drugs/substances, in such a way that the positive values represent **aggregation** and the negative ones represent **evasion** of Paramecia cells. The injection of drugs/substances in each dose was repeated at least 5 times (n = 5), and data were recorded (Karami et al 2013a, 2013b).

2.5. Statistical analysis

First, data were examined with Kolmogorov-Smirnov (K.S.) test,

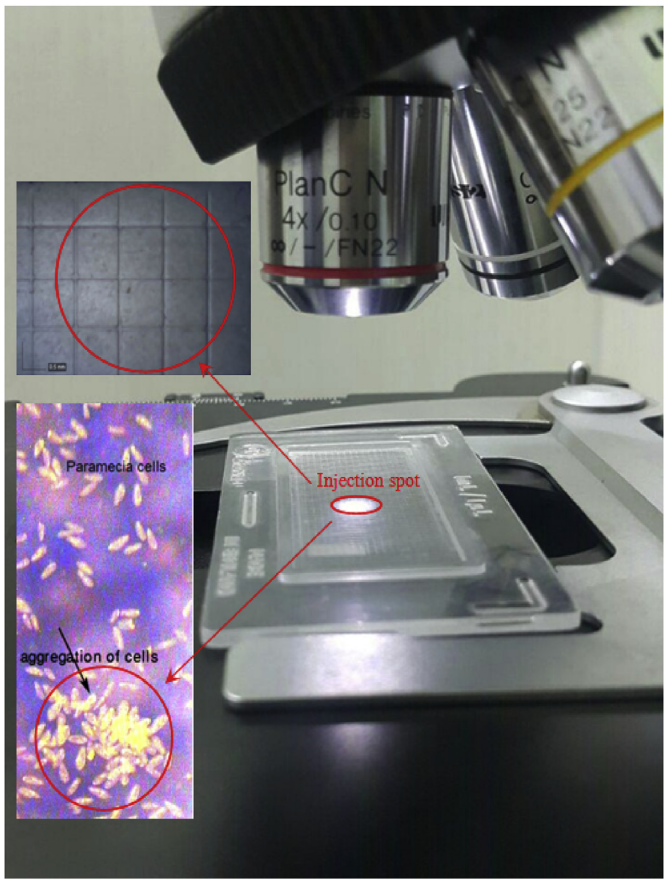

Fig. 1. Schematic illustration of the designed experimental method for analysis of movement behavior (Aggregation/Evasion) of *P. caudatum*. Injection spot: The area of Sedgewick-Rafter counting chamber observed via 4X light microscope objective lens, in which the examined drug/substance is microinjected and immediately, Paramecia cells are counted in the same place (*Injection spot*).

representing a normal distribution. Then, data were analyzed with one-way ANOVA. Post-Hoc tests such as Tukey and LSD were utilized to realize the differences among the related groups considering the alpha coefficient of 0.05 ($p < 0.05$).

3. Results

3.1. Morphine response

Fig. 2 shows the response of *P. caudatum* to morphine exposure. Data have been analyzed using one-way ANOVA, according to the test result: ($F(8, 36) = 246.475$, $p < 0.001$), significant differences have been observed between the groups compared with the control (without injection). Further analysis with Post-Hoc tests indicates that the higher increase in the number of *P. caudatum* takes place at the dose of 2μg morphine compared to other doses.

3.2. Nicotine response

The effect of different doses of nicotine (0.25, 0.5, 1, 2, 4, 8 and 16 μg/μl) on *P. caudatum* compared with the control has been demonstrated in Fig. 3. The difference in the number of cells at the 60th second proportional to the 5th second after the injection of nicotine was calculated at the view of 4X light microscope. Data have been displayed as Mean ± SD. Based on the test result: ($F(7, 32) = 592.524$, $p < 0.001$, significant differences have been observed between the groups compared with the control (without injection). Further analyses indicate the higher increase

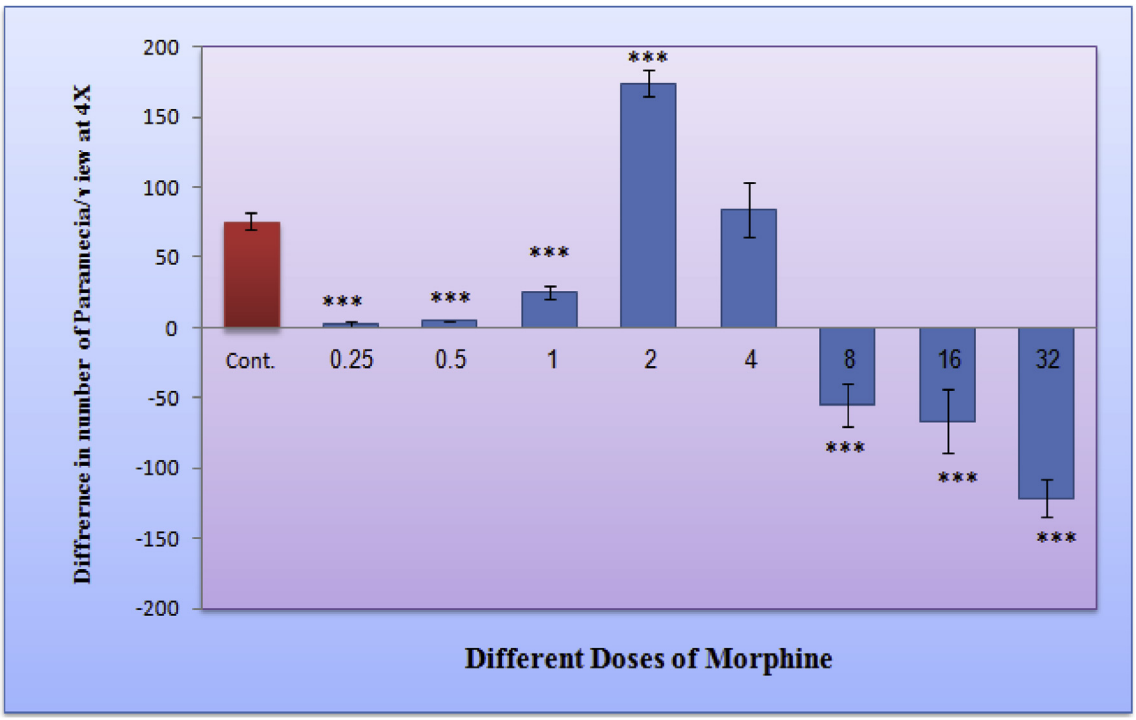

Fig. 2. The dose-response diagram of morphine: The horizontal axis represents different doses of morphine (0.25, 0.5, 1, 2, 4, 8, 16 and 32 μg/μl) (Dose); the vertical axis represents the difference in the number of *P. caudatum* at the 60th second proportional to the 5th second after the injection of morphine, at the view of 4X light microscope (as discussed in the Materials and methods) (Response). The control means no injection. Data have been depicted as Mean ± SD (n = 5). *** indicates $p < 0.001$ compared to the control.

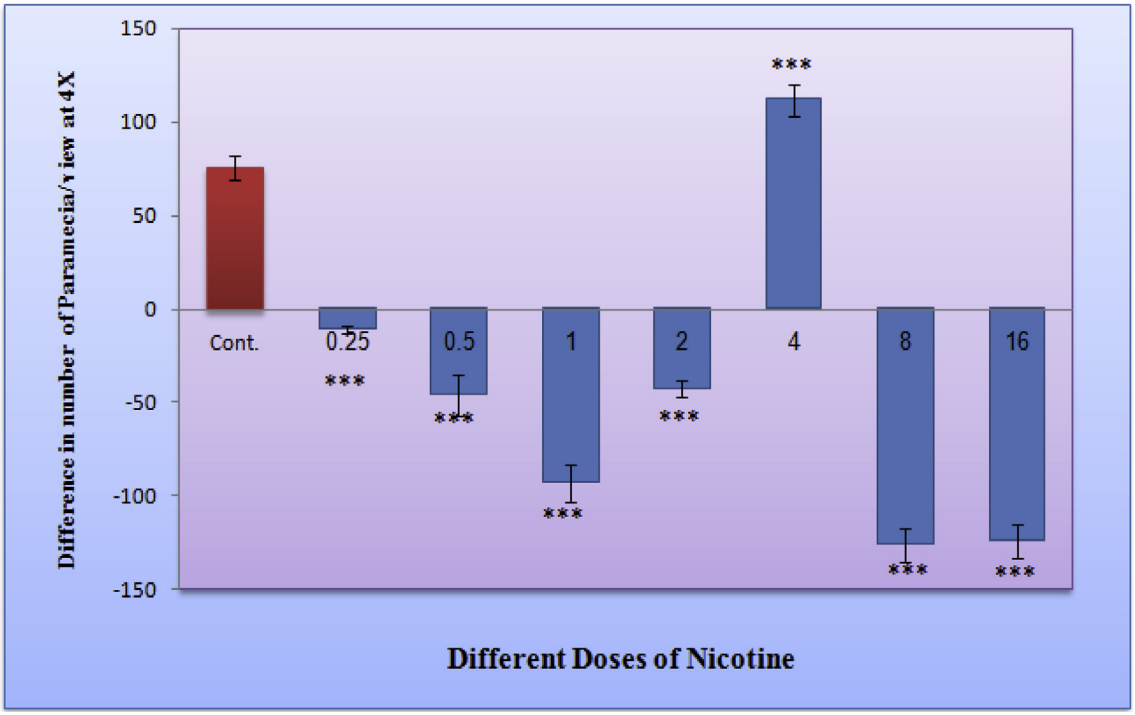

Fig. 3. The response to nicotine: Different doses of nicotine (0.25, 0.5, 1, 2, 4, 8, and 16 μg/μl) have been injected into the counting chamber. Data have been recorded as Mean ± SD proportional to the control (without injection). The difference in the number of *P. caudatum* at the 60th second proportional to the 5th second after the injection of nicotine, at the view of 4X light microscope (as mentioned in Materials and methods) has been presented (n = 5). Post-Hoc analysis indicates that *** is $p < 0.001$compared to the control.

of *P. caudatum* aggregation at dose of 4μg nicotine compared to other doses.

3.3. Trimethaphan effect on the potency of nicotine

Fig. 4 shows this response. The horizontal axis indicates that the dose

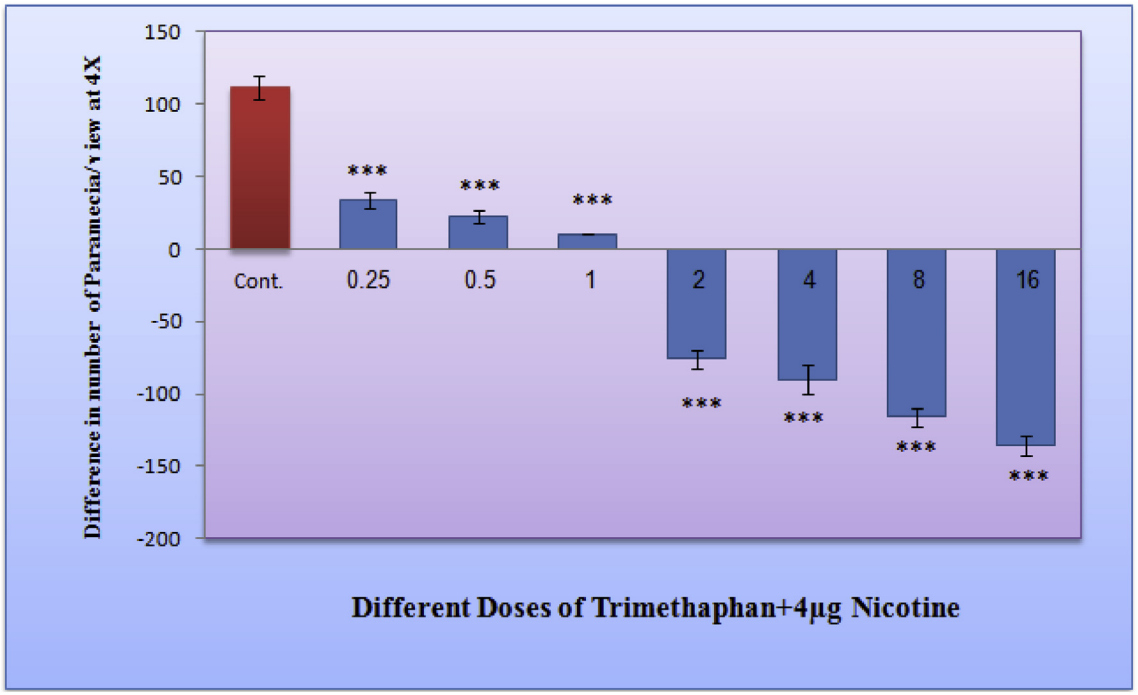

Fig. 4. The simultaneous response to trimethaphan and nicotine: The dose of 4µg nicotine prior to different doses of trimethaphan (0.25, 0.5, 1, 2, 4, 8 and 16 µg/µl) was injected. Then, the behavior of *P. caudatum* was recorded based on cell counting compared to the control (4 µg nicotine). Data have been presented as Mean ± SD of the difference in the number of *P. caudatum* at definite moments after injection of drugs (as mentioned in Materials and methods) (n = 5). Post-Hoc analysis indicates that *** is $p < 0.001$ compared to the control.

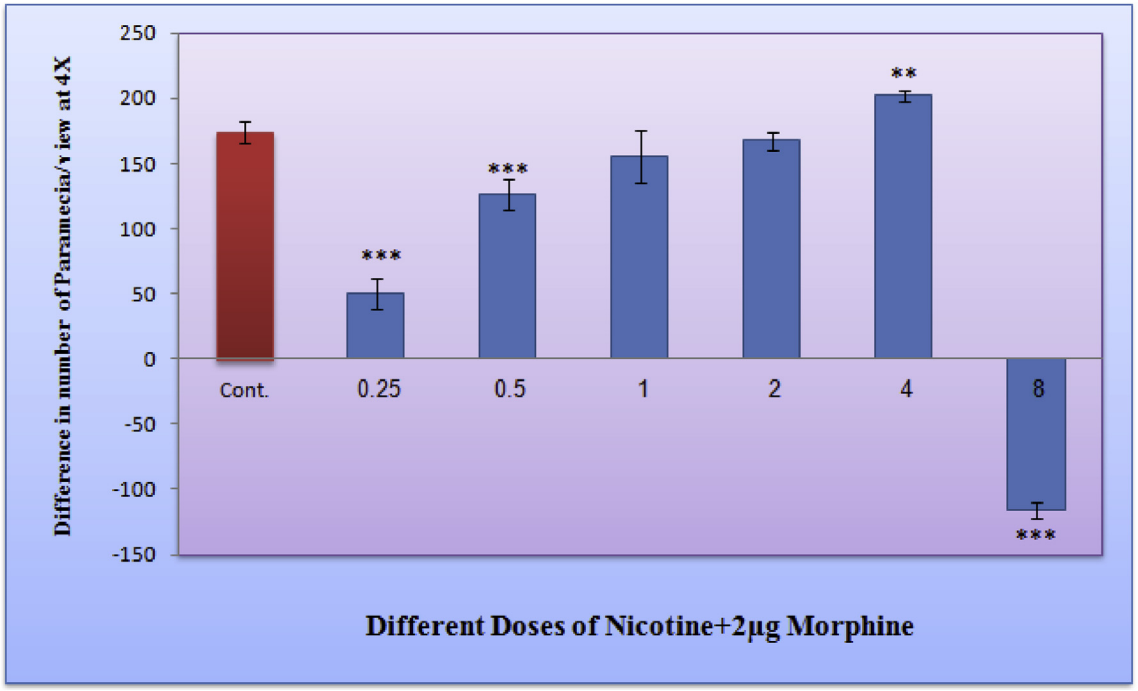

Fig. 5. The simultaneous response to morphine and nicotine: The effective dose of morphine (2 µg/µl) accompanied by different doses of nicotine (0.25, 0.5, 1, 2, 4 and 8 µg/µl) (Dose), in relation to difference in the number of Paramecia cells at definite moments after injection of drugs via 4X lens of light microscope (as discussed in the Materials and methods) (Response). The effective dose of morphine (2 µg/µl) has been considered as the control. Data have been presented as Mean ± SD (n = 5). $^{**}p < 0.01$ and $^{***}p < 0.001$.

of 4µg nicotine was injected prior to different doses of the trimethaphan (0.25, 0.5, 1, 2, 4, 8 and 16 µg/µl). According to the test result: ($F(7, 32) = 863.116$, $p < 0.001$), a fully significant difference has been recognized between the groups and the control (4µg nicotine). Further analyses have revealed the greater inhibitory effect of trimethaphan on the nicotine potency due to the higher doses. In other words, the dose of 16µg trimethaphan (compared to other doses) accompanied by 4µg nicotine caused the greatest decrease in the Paramecia cells (decreasing the nicotine potency).

3.4. Interaction of nicotine with morphine

The variations of morphine potency at the dose of 2 μg accompanied by different doses of nicotine (0.25, 0.5, 1, 2, 4 and 8 μg/μl) are presented in Fig. 5. Data were analyzed by one-way ANOVA. According to the test result: ($F(6, 28) = 489.162, p < 0.001$), the difference among the groups is quite significant. Further analysis confirms that the highest increase of Paramecia cells occurs at dose of 2 μg morphine accompanied by 4 μg nicotine; whereas considerable decreases of cells take place at doses of 0.25, 0.5 and 8 μg nicotine accompanied by 2 μg morphine.

4. Discussion and conclusion

Our previous studies (Karami et al 2013a, 2013b) and current research indicate that morphine aggregates Paramecia cells; while, in control mode (without injection), less aggregation is observed which can be due to the thermoregulation of these organisms (Malvin et al. 2003); however, from the behavioral and pharmacological aspects, the *P. caudatum* responds to the morphine differently, such that at the dose of 2μg, the highest morphine potency occurs to aggregate the Paramecia cells; but, higher doses of morphine (4, 8, 16 and 32 μg/μl) lead to adverse responses. This confirms the dose-dependent response of this eukaryotic unicellular organism to the morphine (Fig. 2).

Behavioral features of results confirm that the *P. caudatum* responds to the nicotine differently, such that the nicotine, like morphine, acts in a dose-dependent manner. That is, at 4μg nicotine, the aggregation of Paramecia cells has increased, though this aggregation is less than when it was exposed to the effective dose of morphine (2μg); albeit, in other doses of nicotine (0.25, 0.5, 1, 2, 8 and 16 μg/μl), aggregation is decreased (Fig. 3). However, at the molecular level, this finding likely indicates the existence of nicotinic system (receptor(s) and signaling molecules) in *P. caudatum*, as the negative response of *P. caudatum* to the trimethaphan strengthens this probability; because, trimethaphan, as a nicotinic competitive antagonist (Rose et al. 1999) reduces considerably the nicotine potency to aggregate the Paramecia cells, which is particularly obvious at 4μg nicotine + 16μg trimethaphan (Fig. 4).

Finally, it can be concluded that the injection of morphine accompanied by nicotine increases the morphine potency at the dose of 4μg nicotine, so that the number of *P. caudatum* at the dose of 2μg morphine + 4μg nicotine increases more significantly than 2μg morphine (Fig. 5). This confirms that in eukaryotic unicellular organisms such as *P. caudatum*, nicotine can also reinforce the morphine potency in a dose-dependent manner. These findings could lead to the behavioral and pharmacological identification of the opioid and nicotinic systems in the protozoa. Furthermore, it is possible to confirm that the similarity and originality of behavioral physiology in the genealogy of living organisms can evolve from the molecular similarity and originality of their ancestors.

Declarations

Author contribution statement

Seyed Sajad Shahrokhi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mahnaz Kesmati: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Bahram Kazemi: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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