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*EXPERIMENTAL Article*

# Inhalation versus intraperitoneal oxytocin administration in Swiss-Albino Mice

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## ABSTRACT

BACKGROUND: Oxytocin modulates several social behaviors, with research results focusing on its role as potential adjunctive dedication in psychiatric disorders. This raises the necessity for a less stressful, translational way of oxytocin delivery in animal models. The aim of this paper is to provide a comparison between mice's open field test (OFT) behavior after a single inhalator (Inh), respectively intraperitoneal (IP) oxytocin administration.

METHODS: Forty-four male Swiss-Albino mice were divided into three groups- oxytocin, no-procedure (control) and placebo, according to the administered substance. The substances were delivered either through IP injection, or through a 22 minutes nebulization. The mice in the oxytocin group received 1 UI of oxytocin, whereas those in the placebo group got the equivalent amount of saline. OFT was performed 30 to 90 minutes after the administration.

RESULTS: The time spent in the center varied among the groups ($F(4,44)=3.224$, $p=0.022$), due to the fact that the mice that received inhalatory oxytocin spent more time in the center than those in the control group and than those that were administered the substances intraperitoneally. The mice that received IP oxytocin had fewer boli emissions than all the other mice, whereas the mice that received Inh oxytocin had more boli emissions that those that received placebo, either inhalatory or intraperitoneally.

CONCLUSION: We may conclude that group inhalator delivery of oxytocin seems preferable to the intraperitoneal injection way, with higher anxiolytic effects at the same dose.

**Keywords**: oxytocin, mice behavior, inhalator administration, open field test

**Abbreviations:**
confidence interval (CI), inhalatory (Inh), intraperitoneal (IP/ip), inter-quartile range (iqr), international unit (IU), least significant differences (LSC), male (m), open field test (OFT)

## INTRODUCTION

Oxytocin, one of the most renowned and researched hormones of the past decade, is involved in modulating several social behaviors, such as pair-bonding and social cognition[1], as well as in

**CURRENT KNOWLEDGE:**

- ◊ oxytocin - effects on mood regulation, emotion perception, social interactions, anxiety
- ◊ several psychiatric disorders involve disregulations in oxytocinergic circuits
- ◊ wide animal models to study, but hardly translational results
- ◊ most common way of oxytocin delivery in humans – intranasal

**QUESTION: Is inhalatory delivery a good option for oxytocin delivery in mice?**

attitudes like empathy[2], optimism[3] or positive affect[4]. Moreover, it seems to regulate self-esteem[3], the need for social support[5] and the social adequacy of empathy triggered behaviours[6]. Its roles in pair bonding, paternal care and maternal behavior[7] have been widely studied, oxytocin being better known as "the hormone of attachment".

The popularity of the research results regarding oxytocin's functions in modulating anxiety-related responses[8], trust[9], generosity, altruism[10], risk taking[11], facial emotions recognition[12] and morality[13] have rendered it the renown of "confidence booster". Therefore, intranasal and sublingual formulations of oxytocin are currently largely available, without prescription, being marketed as self-confidence enhancers.

The inappropriate secretion of oxytocin or alterations in the expression of oxytocin receptor genes seem to be involved in various psychiatric disorders, such as anxiety disorders[14], autism spectrum disorder[15], schizophrenia[16,17], depression[18] and bipolar disorder[19], with several clinical studies investigating the potential role of oxytocin supplementation as adjuvant psychiatric treatment[20]. Moreover, it seems that oxytocin receptor regulation is one of the key facts in developing borderline personality disorder in the presence of a specific family behavior in the early life[21].

Given the potentially wide applicability of oxytocin administration as adjuvant treatment for several psychiatric conditions, it is very important to have an animal model of administration that will closely mimic the widely-used human intranasal delivery of oxytocin.

So far, in animal studies, the most used ways of administration have been intra-peritoneal injection, intra-cerebro-ventricular administration or targeted delivery in specific brain areas sub-cuteneous administration and per-nostril administration[14,22]. Optogenetics methods have also been employed in order to modulate oxytocinergic circuits activity14. However, the mere handling of the experimental subjects for any of these procedures is stressful for the animal[23,24], thus, there is a great necessity of developing a novel, less strenuous, way of administration.

The goal of this study was to investigate the effects on open field test (OFT) behavior of a single dose inhalator oxytocin administration in a group of Swiss-Albino mice in comparison to the classical intraperitoneal administration route.

## MATERIAL AND METHODS

*Experimental animals.* Forty-four Swiss albino mice were used in the following experiments. Table 1 summarizes the distribution of the animals and the procedures used. The animals were housed 5-7 per box, mixed from different litters after culling and weaning (post-natal day 20). The mice had free access to food and water and were maintained in a circadian light-day cycle, under standard light conditions, in an animal holding facility. An experimented researcher handled the animals, with minimal distress to the mice. The experimental procedures were developed with local ethical approval, in accordance with the European Communities Council Directive 2010/63/EC on the protection of animals used for scientific purposes.

*Acute intraperitoneal versus inhalator oxytocin administration.* The mice were divided into 3 groups – oxytocin, no-procedure (control) and placebo, according to the administered substance. Ten mice were included into the no-procedure (control) group - no procedure was performed prior to OFT testing. Ten mice from the oxyitocin and placebo groups received the substances through a 22 minutes inhalation.

**Table 1. The group distribution of the experimental animals and the substances that were administered**

| Number of animals/age/sex | Substance/amount | Way of administration |
|---|---|---|
| 10/ 60-90days/ m | none | - |
| 7/ 60-90days/ m | saline/0.2ml | IP |
| 7/ 60-90days/ m | Oxytocin Ferrig-Leciva sol 5UI/ml /1IU in 0.2ml | IP |
| 10/ 60-90days/ m | saline/0.2ml | Inhalatory-22min inhalation |
| 10/ 60-90days/ m | Oxytocin Ferrig-Leciva sol 5UI/ml /1IU in 0.2ml | Inhalatory-22min inhalation |

Five mice at a time (all mice from one cage) were placed in a custom-made closed plastic container. Oxytocin was nebulized through one whole, using a Nuvita Nebulizer. The air was ventilated through another whole in the opposite wall. The boxes were lined with fresh bedding for each nebulization and they were cleaned with water and 30% ethanol between trials. The actual nebulization took 4.5 minutes. The mice were kept in the box for the remaining 17.5 minutes so that each mouse would have enough time to breath in once the whole volume of the box (2L), considering the mouse average respiratory rate of 163 breaths per minute and the tidal volume of 0.15 ml[25]. The seven remaining mice from each group received an IP injection of the respective substance. Oxytocin group received 1 UI of oxytocin/mouse, while the placebo group received the equivalent volume of saline (**Table 1**).

OFT was performed 30-90 min after the Inh administration and at 60 min after the IP administration (**Figure1**).

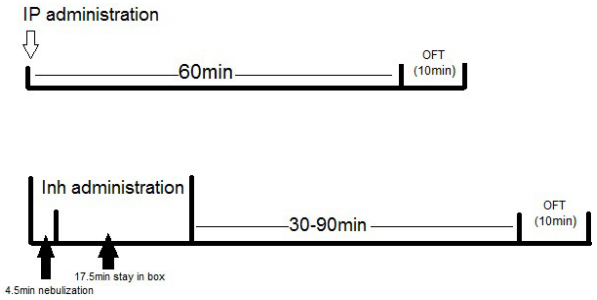


**Figure 1. The time siquence of the substance administration and OFT testing**

*Open field test (OFT).* The Open Field Test is a widely used behavioral analysis for various species of animals (rodents, fish, horses). It evaluates mobility and movement patterns, exploratory behavior, as well as anxiety-related attitudes. The total distance moved evaluates mobility, whereas the time spent in the center, the number of center entries and the fecal boli emissions are measures of anxiety behaviors. Anxious mice are going to spend less time in the center of the arena; they are going to have fewer center entries and more boli excretion than less-anxious subjects[26].

One mouse at a time was placed in the center of a custom made 40x40 $cm^2$ arena, surrounded by 40cm high walls. All the walls of the arena are black. The mouse is allowed to move freely inside the arena for a period of 10 minutes. During this time, it was tracked using the EthoVision 4 tracking software. Our custom-made OFT arena allows for two animals to be recorded simultaneously. The mice were brought into the testing room at least two hours before the experimental procedures, in order to accommodate. The total distance, the frequency of entries in the center of the arena and time spent in the center were automatically determined for each mouse by the tracking software. The experimenter counted the number of fecal boli emitted during the task. All the tests were performed between 2 PM and 5 PM, in a lit room, with controlled light exposure on the OFT arena.

*Statistical analysis.* The data were analyzed using SPSS 22.0 and Excel. The distribution of the continuous data (total distance, time spent in the center of the arena) was checked using the Shapiro-Wilk test. Parametric testing (univariate ANOVA with LSD post-hoc analysis) was employed for $p>0.05$. The ordinal variables were compared using non-parametric testing (Kruskall-Wallis with post-hoc Mann-Whitney U analysis). The reported p-values are those for the two-tailed test, unless otherwise specified.

## RESULTS

Both the total distance, as well as the time spent in the center of the arena had a normal distribution in all the five groups. There were no overall

differences between the groups (F(4,44)=1.643, p=0.183) with regards to the distance moved (**Figure 2**). However, LSD post-hoc analysis revealed the fact that the mice in the IP placebo group moved less than those in the control group. Less movement is associated with a decreased exploratory behavior, that can be due to anxiety or distress. In models of repeated or longer (30-120min) OFT recordings, gradual decreases in the total distance moved are markers of habituation.

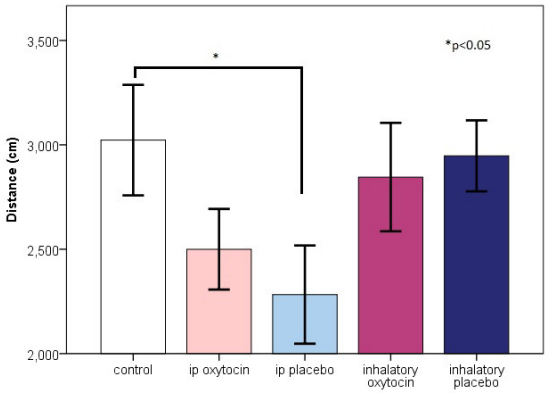


**Figure 2**. Bar graph representation of the average values of the distance moved by the mice in the different groups. The error bars represent the standard error. The mice that received the intraperitoneal placebo treatment moved less than those in the control group (average difference =739.9±342.87cm, %CI=[46.369cm,1433.432cm], p=0.037)

The time spent in the center of the OFT arena varied among the groups (F(4,44)=3.224, p=0.022), with an observed power of the difference of 78% (**Figure 3**). LSD post-hoc analysis revealed that the differences were because the mice that received inhalatory oxytocin spent more time in the center than those in the control group and than those that were administered the substances by means of IP injection. Anxious animals usually spend less time in the center of the arena, which is an opened, exposed area. The more time spent in the center, the less anxious the animal is.

There were no overall differences between the mice in the different groups in terms of number of center entries ($\chi^2(4)=6.675$, p=0.154). However, the mice in that received Inh oxytocin had more center entries that those with IP substance administration (**Figure 4**). Like the time spent in the center, the number of center entries is negatively correlated with the level of anxiety – the more anxious the animal is, the less number of center entries it will have. It also, indirectly, mirrors the mobility of the individual – a more mobile animal will have more center entries.

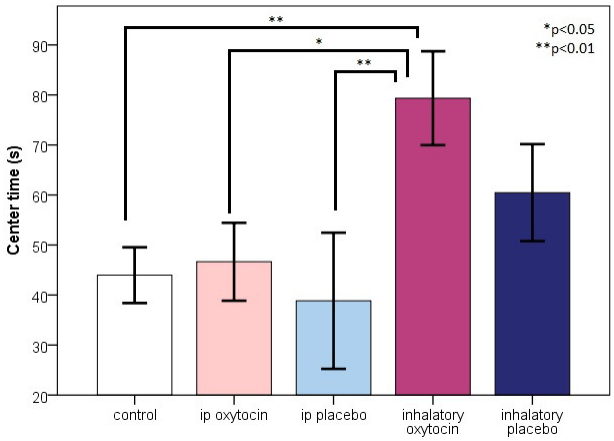


**Figure 3.** Bar graph representation of the average time spent in the center of the OFT arena. The error bars represent the standard error. The mice in the Inh oxytocin group spent more time in the center than those in the control group (average difference = 35.384 ± 12.3s, 95%CI= [10.504s, 60.263s], p=0.006), those in the IP oxytocin group (average difference = 32.7±13.554s, 95%CI= [5.284s, 60.116s], p=0.021) and those in the IP placebo group (average difference =40.506±13.554s, 95%CI= [13.09s, 67.922s], p=0.005).

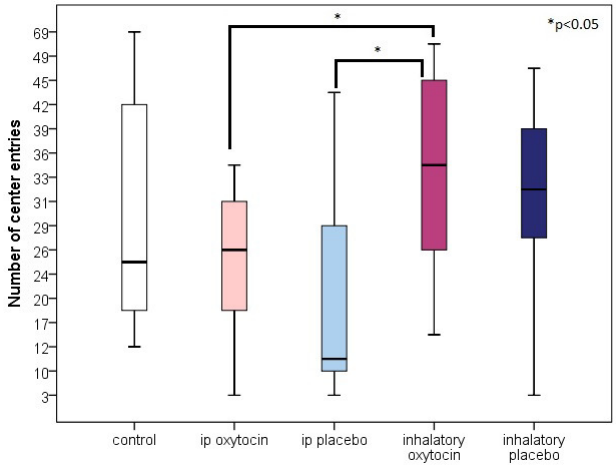


**Figure 4**. Box-plot of the number of center entries. The mice that received Inh oxytocin had more center entries that those that received IP oxytocin (Z=1.861, n1=10, n2=7, p(one-tailed)=0.035, median number of center entries in the Inh oxytocin group=34, IQR=20, median number of center entries in the IP oxytocin group=26, IQR=18) and than those that received IP placebo (Z=1.953, n1=10, n2=7, p(one-tailed)=0.027, median number of center entries in the IP placebo group=11, IQR=30)

The number of boli emitted during testing waried accros the groups ($\chi^2(4)=13.45$, p=0.009). The mice that received IP oxytocin administration had fewer boli emissions than all the other mice, whereas the mice that received Inh oxytocin had more boli emissions that those that received placebo, either inhalatory or intraperitoneally (**Figure 5**). The number of boli emissions is, generally, directly correlated with the degree of distress; however, the number of emissions strongly depends on several other factors, such as the intensity and duration of the stressor.

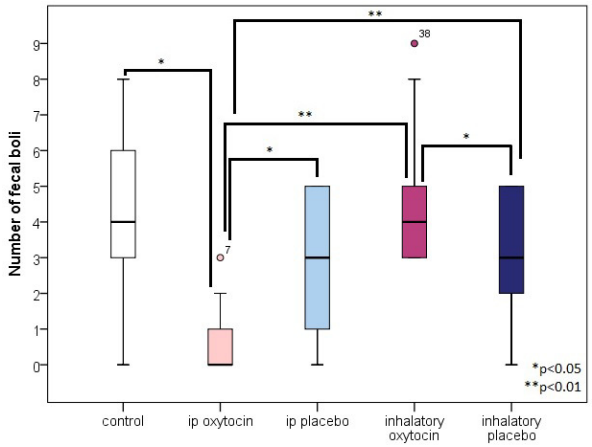


**Figure 5**. Box-plot of the number of fecal boli emissions, with significant outliers. The mice in the Inh oxytocin group emitted more boli than those in the IP oxytocin group (Z=3.352, n1=10, n2=7, p=0.001, median number of boli in the Inh oxytocin group=4, IQR=3, median number of boli in the IP oxytocin group=0, IQR=2) and than those in the Inh placebo group (Z=1.77, n1=n2=10, p(one-tailed)=0.044, median number of boli in the Inh placebo group=3, IQR=4). The mice in the IP oxytocin group emitted less boli than those in the control group (Z=-2.711, n1=7, n2=10, p=0.028, median number of boli in the control group=3.5, IQR=4), IP placebo group (Z=-1.778, n1=n2=7, p(one-tailed)=0.048, median number of boli in the IP placebo group=3, IQR=5) and Inh placebo group (Z=-2.2, n1=7, n2=10, p=0.001).

## DISCUSSION

The equivalent amount of oxytocin delivered intraperitoneally was 0.05-0.06 mg/kg, a dose smaller than previously reported anxiolytic doses[27]. Still, we observe a decrease in the boli emissions in the stressful OFT condition when compared to IP placebo delivery, as previously reported by Crine et al, in 1983[28], but for IP oxytocin doses ten times smaller than the ones that were used here. Moreover, the IP administration maneuver decreases mobility in our experimental animals, a result that is coherent with it being used as an experimental stressor model[29]. Even at this low concentration, the IP oxytocin administration counteracts this effect, as there are no differences in mobility between IP oxytocin administration group mice and control group ones.

As previous studies on anxiety with intra-nasal administration did not find any differences in OFT behavior[30,31], a higher dose than that previously reported was used for intranasal administration (50-60ug/kg). However, given the cortical availability of oxytocin after intranasal administration[32], the obtained concentration in the amygdala should be comparable to the one already reported as having anxiolytic effects on the OFT in central amigdala administration[33]. Our results are in concordance to those reported by Bale et al in 2010, for central amygdala delivery[33].

The increase in boli emissions post the higher inhalatory dose of oxytocin administration can be viewed as a confirmation of efficacy and bioavailability, with previous studies reporting that ICV oxytocin administration soothes stress-induced uncoordinated gastric motility, aiding in the delayed stress-induced gastric emptying[34].

The authors consider that this novel proposed method of delivery of oxytocin for experimental animals is more in concordance to the principle of the 3Rs used in animal research, in that it represents a refinement of previous administration methods, with subsequent reduction of the distress to the animal. The results obtained in this experiment sustain the inhalator way of delivery rather than the intraperitoneal one. These results are in concordance with previous reports[23,24] that show that the handling method and frequency influence the outcomes of behavioral assessments. These finding are in favor of the inhalator way of administering oxytocin, in terms of stress for the experimental animals and efficacy as an anxiolytic agent. However, there is still a great need for testing varied dosages on this protocol, as well as checking for single mouse administration, rather that group delivery of the active compound. At this point, we are in favor of the group delivery means of administration, seeing that it diminishes the stress related to a new environment, as is the administration chamber.

Our exhaustive literature search found no other recordings of the inhalator described in the text.

**Table 2. Comparative advantages and disadvantages of the two ways of substance delivery**

| INHALATOR DELIVERY | INTRAPERITONEAL DELIVERY |
|---|---|
| **ADVANTAGES** | |
| -group delivery – minimizes stress induced by isolation<br>-not painful<br>-minimal handling<br>-big amounts of substances may be delivered in a timely manner<br>-no immediate risks for the animal's bodily integrity<br>-mimics human delivery pattern<br>-doesn't require high experience from the experimenter for the procedure<br>-direct availability in the spinal fluid due to penetration through the cribriform plate | -rapid delivery<br>-precisely controlled amount of substance administered<br>-known bioavailability<br>-lots of literature studies about dose-effect relationship<br>-doesn't induce noise stress |
| **DISADVANTAGES** | |
| -rather noisy<br>-novel method<br>-no data about bioavailability<br>-no data about dose-effect relationship<br>-long duration of administration<br>-change of environment for the procedure | -somewhat painful<br>-strict, rather stressful handling<br>-requires experience for effective and rapid substance delivery<br>-possible iatrogenic pathologies – urinary bladder or intestinal loop perforation, peritonitis, etc.<br>-limited amount of substance that can be delivered<br>-hardly translational to human administration<br>-smaller bioavailability in the spinal fluid due to the selective permeability of the blood brain barrier |

The main advantages and disadvantages of each of the proposed ways of substance delivery have been summarized in **Table 2**.

## CONCLUSIONS

Based on the results presented in the text, it can be inferred that oxytocin induces an anxiolytic effect in the opened field test condition in a dose and way of administration dependent manner. Group inhalator delivery of oxytocin seems preferable to the intraperitoneal injection way, with higher anxiolytic effects at the same dose, most probably due to bioavailability. The authors consider that this novel way of drug delivery could represent a good applicability for translational models in oxytocin research, due to its minimization of animal-handling induced-stress and comparable results to those previously reported on anxiety levels. Moreover, the nebulization method also allows for higher amounts of substance to be delivered in amely manner, without acute volumetric or osmotic changes issues inherent to the current ICV, IP or intranasal way of delivery in mice. The authors consider that the impact of the study resides mainly in the novelty of the proposed way of oxytocin delivery. We look forward to testing more administration schemes, with various durations and amounts of substance delivered in order to further the proof of concept and to expand the knowledge in the constantly increasing field of oxytocin research.

**Acknowledgments**

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◊ **1IU inhalatory oxytocin – anxiolytic in OFT**

◊ **group inhalatory delivery – less stressful than IP delivery of oxytocin**

◊ **nebulization – future perspectives in oxytocin research due to translational potential**

**Conflict of Interest:**

The authors have no conflicts of interest to disclose in relation to this work.

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