Original Article

Assessment of the effects of *Zincum metallicum* in maternal deprivation-induced behavioral disturbances in female rats

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Abstract

**Background:** Clinical studies have shown that adverse events in early life are quite stressful and can lead to the development of psychiatric disorders, such as anxiety, in adolescence and adulthood in humans. Experimental models of maternal deprivation (MD) in rats can be a useful tool for the understanding how these events in the early period of development can lead to behavioral changes in adulthood. Aims: The objective of this study was to evaluate the long-term effects of *Zincum metallicum* (Zinc met) homeopathic treatment in rats subjected to maternal deprivation (MD) in their early days of life. **Methods:** Newborn female rats were subjected to MD during 10 days, one hour per day, from PND10 to PND21, and treated from the 10th day of lactation (PND10) up to weaning (PND21). On the PND21 the rats were divided in 4 groups (N=8): a) treated with Zinc met 30c; b) treated with Zinc met 6c; c) treated with 10% hydroalcoholic solution (HS); and “blank control”, rats without any treatment nor experienced deprivation (ND). The medicines were administered in blind trials, identified by codes. The animals were weighed weekly, from weaning until the end of the experiment, and evaluated in the Open Field (OF) and in the Elevated Plus Maze (EPM) devices, to evaluate mobility, emotionality and anxiety, in 3 moments of life: in PND21 (childhood), during puberty (PND 40) and adulthood (PND75). Data were analyzed statistically by ANOVA, followed by the Bonferroni’s Multiple Comparison Test, being p<0.05. **Results:** 1) relative to ND group the Zinc met 6c showed reduced body weight while no differences were observed between the other groups; 2) In the OF, the MD group showed increased rearing behavior at PND 40. The Zinc met 6c group reversed this effect showing similar profile as the ND group. Moreover, Zinc met 6c group showed increase in the immobility time at this age; 3) In the EPM, the MD group showed increased time in closed arms and decreased head dips relative to ND group in PND21 period of observation. Treatment with Zinc met 6c but not with Zinc met 30c was effective to reduce this anxiety-like behavior in PND21. Conclusion: According to the proposed model, *Zincum metallicum* 6c seems to be able to prevent in anxiety-like behavior induced by maternal deprivation in the childhood, mainly in behavioral models related to anxiety. However, other studies still need to be developed to understand the physio-pathological basis of these effects.

**Keywords:** Homeopathy, High Dilutions, *Zincum metallicum*, Stress, Anxiety, maternal deprivation.
1. Introduction

Clinical studies have shown that adverse events in early life can lead to the development of psychiatric disorders, such as anxiety\(^1\), in adolescence and adulthood in humans. Laboratory animal models, such as maternal deprivation (MD), can be a useful tool for the understanding how these events in the early period of development can lead to behavioral changes in adulthood\(^2,3,4\).

Zinc plays an important role at immune system\(^5\), therefore, it is widely recommended to treat many diseases, including respiratory and digestive infections\(^5,6\). Zinc in prenatal treatment has proven to reduce the stress response in rats with sickness behavior\(^7\).

The homeopathic treatments employ high diluted medication, which create controversy in so many aspects to modern science\(^8\), but biological effects in experimental models are described in research\(^9,10,11,12,13,14,15,16\). Studies about the use of homeopathic ultrahigh dilutions as tools to minimize stress and anxiety are found in the literature\(^17,18,19,20\). There are so many employed medication as for example *Ignatia amara*\(^21\), *Atropa Belladona*\(^17\), *Anax imperator*\(^19\), *Passiflora*\(^20\), *Gelsemium sempervirens*\(^17,18\), this last one, commonly used in behavior models, also regulates emotional and conduct response\(^22\).

The homeopathic medicine *Zincum metallicum* is used for the treatment of neurological and behavioral symptoms, including: weakening of intellectual functions with brain and nervous exhaustion, loss of vitality, slow comprehension, memory disorder, general tremor and constant movements\(^23,24\). These alterations can be characterized as stress-related phenomena in different species. This same medication has even been used in studies to treat respiratory illness\(^25\) and cirrhosis of the liver\(^26\).

**Aims:** The objective of this study was to evaluate the long-term effects of homeopathic treatment in animals subjected to stress in their early days of life (maternal deprivation). The homeopathic medicine *Zincum metallicum* is a possible medicine to improve the quality of life of animals that suffered maternal deprivation.

2. Material and methods

This study was approved by the Ethics Committee on the use of Animals (CEUA-UNISA), according to process number 11/2014 on 18.06.2014 (Appendix 1 a,b).
According to the Arrive guidelines (www.nc3rs.org.uk/ARRIVE) and checklist, all procedures performed in this study was in accordance to the 3Rs rules. The complete filled checklist can be seen in Appendix 2.

The 3Rs were respected in this study:

1) “Reduction”, only eight animals were used in each experimental group, this is the minimal number considered statistically representative in most published studies which used maternal deprivation model or behavioral models.

2) “Refinement”, the experimental design was built up based on recent studies of maternal deprivation that contribute to the deep understanding of the behavioral and physio-pathological effects related to maternal deprivation in humans and animals, including the evaluation of therapeutic possibilities. Moreover, the behavioral observation of rats is minimally invasive.

3) “Replacement”, it is known that there is no in vitro or alternative method to study certain variables, such as behavioral effects. Even clinical trials have difficulty in revealing specifically this kind of effects, since in these cases, samples are submitted to a great sort of variables and the distinction of a real therapeutic effect from a placebo effect is generally hard, what does not happen in experimental situations.

All procedures were in accordance to the CONCEA Brazilian guidelines for animal experimentation (version 2013, when the study was developed), that is based on the EU Directive 2010/63/EU.

**Animals, house conditions and treatments**

A total of 32 female Wistar rats were housed in polypropylene cages (38 X 32 X 16 cm; 4 rats per cage) at a controlled temperature (22°C ± 2°C) and humidity (65–70%) with artificial lighting (12hr light/12hr dark cycle, lights on at 6:00 AM).

All females were maintained in cages with ad libitum access to food and water during the experimental period. In this study, newborn female rats were subjected to maternal deprivation (MD)- during 1 h daily, from PND10 until PND21. During the MD each litter were maintained in the same box with in of 22°C ± 2°C; the dam was isolated in other cage and maintained in another room. This procedure was based, in the study of KIKUSUI; MORI (2009)²⁷ and PINHEIRO, et al. (2011)²⁸. Treatments were performed during the MP, once a day (0,1 ml) and by oral route. Thus, the rats
were divided in 4 groups: 8 treated with *Zincum metallicum* 30 CH (Zn30cH group); 8 treated with *Zincum metallicum* 6 CH (Zn6cH group); 8 treated with 10% hydroalcoholic (HS group) (the vehicle of *Zincum metallicum*), medicines in blind trials, identified by codes; and 8 animals not submitted to maternal deprivation and without any treatment (ND group—“blank control”). Medications were made based on the Brazilian Homeopathy Pharmacopeia (3rd Edition, 2011).

At weaning (PND21) the female rats of each group housed in polypropylene cages under the same conditions as their parents and the male rats used in another study. The animals were weighed weekly, from weaning until the end of the experiment, and evaluated in the Open Field (OF) and in the Elevated Plus Maze (EPM) devices to measure mobility, emotionality and anxiety, in 3 moments: in PND 21 (childhood), during puberty (PND 40) and adulthood (PND 75) (Figure 1).

**Figure 1** Experimental protocol. PND- post-natal day. PND21: childhood, PND 40: Puberty and PND75: adulthood. OF: Open Field; EPM: Elevated Plus Maze.

**Open Field (OF)**
Rats general action was observed in the Open Field Test (OF). In this test, animals were put in the open field always during the same period of day. Each animal was placed individually in the center of open field equipment, being watched for five minutes. The bottom of this equipment is composed of twenty-five parts almost equals. Between one animal to next, the equipment was cleared with a 5% alcohol solution to avoid smell interference from the previous animal.

The following parameters were registered: frequency of locomotion, rearing, freezing time and the number of bolus fecal. The unit of locomotion is defined as the act in which the rat penetrates with
its four legs in one of the floor divisions; the unit of raise refers to position in which animals get supported by inferior legs with perpendicular position of the trunk to the floor, leading its head upwards, touching or not open field sidewalls with the superior legs; the number of fecal bolus produced by the animals during the period of test is also registered as a unit of defecation. Immobility time is also measured and it is recognized when animal show no locomotion or sniff movements.

**Elevated Plus Maze (EPM)**

Elevated-plus maze (EPM) is an apparatus first conceived by the British psychologist Sheila Handley's group as a model to evaluate anxiety and it is one of the most used for that purpose. Rats are placed at the junction of the four arms of the maze, and entries/duration in each arm are observed for 5 min. Other ethological parameters (i.e., head dips) can also be observed. An increase in open arm activity (duration and/or entries) reflects anti-anxiety behavior. These values were interpreted as indexes of anxiety in mice.

**Statistical analysis**

Homogeneity was verified using Bartlett’s test. Normality was verified using the Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) followed by by the Bonferroni’s Multiple Comparison Test. The results are expressed as the mean ± SEM. In all cases, the results were considered significant at p≤0.05.

**3. Results**

Concerning body weight, rats treated with Zn6cH group showed lower bodyweight compared to ND rats. No differences were observed between Zn30cH group relative to ND and HS group (p≤0.05) (Figure 2).

In the OF, at PND21 no difference was observed between the parameters (Locomotion, Periferal Locomotion, Rearing, Immobility and Fecal boli), between the groups. The PND40, Zn30c group showed increase Rearing frequency relative to ND group, and Zn6c group showed increase in Immobility frequency (seconds) relative to HS group and Zn30c group, no difference was observed between the others parameters. The PND75, no difference was observed between the parameters. (Table 1)
Figure 2. Weight gain among the groups, from the beginning to the end of the experiment. One way ANOVA followed for Bonferroni’s Multiple Comparison Test *p< 0,05 relative to ND group.

Table 1. Effects of *Zincum mettalicum* treatments (6c and 30c) in pups maternal deprived from PND10 to PND21 and observed in the open field at weaning (PND 21), in the juvenile (PND 40) and adult age (PND 75). Data are presented as means ± SEM. N= 8/group. One way ANOVA followed for Bonferroni’s Multiple Comparison Test *p< 0,05 relative to ND group; a p< 0.05- relative to HS group; b p< 0.05- relative to *Zinc met* 6c; c p< 0.05- relative to *Zinc met* 30c.

| Days of age | ND group | HS group (MD) | Zinc met 6c | Zinc met 30c |
|-------------|----------|---------------|-------------|-------------|
| PND 21      |          |               |             |             |
| Locomotion (Frequency) | 74,2 ± 15,5 | 60,8 ± 6,8 | 77,7 ± 8,2 | 74,2 ± 10,4 |
| Peripheral locomotion (Frequency) | 55,8 ± 10,7 | 50,0 ± 6,3 | 62,2 ± 6,3 | 52,25 ± 7,7 |
| Rearing (Frequency) | 20,0 ± 4,7 | 11,3 ± 2,8 | 20,8 ± 2,4 | 19,8 ± 5,1 |
|                | PND 40 | PND 75 |
|----------------|--------|--------|
| **Locomotion** (Frequency) | 47.6 ± 9.5 | 102.3 ± 15.3 |
| **Peripheral locomotion** (Frequency) | 46.0 ± 9.3 | 88.7 ± 13.4 |
| **Rearing** (Frequency) | 5.7 ± 1.3 | 20.2 ± 4.9 |
| **Immobility** (seconds) | 36.8 ± 9.8 | 22.8 ± 14.7 |
| **Fecal boli** (Unit) | 2.6 ± 0.7 | 2.6 ± 0.7 |

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In the EPM, at PND21, the Zn30c group showed increased time in closed arms, decreased head dips and in the time in the open arm and decrease closed arm entry relative to ND group. The Zn6c showed increased Head dips (frequency) relative the HS group and Zn30c. HS group (Maternal deprivation) showed increased time in the closed arm, also in head dips and decreased time in the open arm and open arm entry relative to ND group. PND40 and PND75 no difference was observed between the parameters. Observations suggesting increased anxiety-like behavior and decreased risk assessment were present (p≤0.05); treatment with Zn6c but not with Zn30c was effective to reduce the anxiety-like behavior in the childhood (Table 2).

Table 2. Effects of Zincum mettalicum treatments (6c and 30c) in pups deprived from maternal contact from PND10 to PND21 and observed in the Elevated Plus Maze (EPM) at weaning (PND 21), in the juvenile (PND 40) and adult age (PND 75). Data are presented as means ± SEM. N= 8/group. One way ANOVA followed for Bonferroni's Multiple Comparison Test *p<0.05 relative to ND group; a p< 0.05- relative to HS group; b p< 0.05- relative to Zinc met 6c; c p< 0.05- relative to Zinc met 30c.

| Days of age | ND group | HS group (MD) | Zinc met 6c | Zinc met 30c |
|-------------|----------|---------------|-------------|--------------|
| PND 21      |          |               |             |              |
| Open Arm Entry (Frequency) | 6,7 ± 0,3 | 3,2 ± 0,7^ab | 6,6 ± 0,6   | 2,66 ± 0,7^ab |
| Time in the Open Arm (seconds) | 124,9 ± 23,11 | 50,8 ± 23,5^a | 96,1 ± 9,1  | 37,6 ± 11,57^a |
| Closed Arm Entry (Frequency) | 6,5 ± 0,6  | 6,0 ± 0,9     | 8,2 ± 1,3   | 4,0 ± 0,8^b  |
| Time in the Closed Arm (seconds) | 136,5 ± 18,9 | 217,3 ± 26,7^a | 178,3 ± 8,8 | 248,5 ± 11,9^a |
| Head dips (Frequency) | 11,6 ± 1,2  | 4,3 ± 1,6^c   | 11,3 ± 0,8^bc | 4,5 ± 1,8^a  |
| PND 40      |          |               |             |              |
| Open Arm Entry (Frequency) | 4,1 ± 0,6  | 4,3 ± 1,1     | 5,0 ± 0,9   | 4,7 ± 0,8    |

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### Table 1: Behavioral Data

|                         | Time in the Open Arm (seconds) | Time in the Closed Arm (seconds) | Head dips (Frequency) |
|-------------------------|--------------------------------|---------------------------------|-----------------------|
|                         | 31,4 ± 8,5                     | 76,1 ± 21,7                     | 3,7 ± 1,2             |
| Closed Arm Entry (Frequency) | 7,6 ± 0,8                      | 5,6 ± 1,0                       | 5,5 ± 2,2             |
|                         | 67,8 ± 11,1                    | 6,6 ± 0,9                       | 7,5 ± 2,5             |
|                         | 76,2 ± 13,76                   | 7,1 ± 1,1                       | 9,3 ± 2,3             |
|                         | 235,6 ± 18,14                  | 226,4 ± 21,8                    | 10,7 ± 1,9            |
|                         | 217,1 ± 14,0                   | 205,1 ± 17,3                    |                      |
|                         | 205,1 ± 17,3                   |                                 |                      |
|                         | 217,1 ± 14,0                   |                                 |                      |
|                         | 205,1 ± 17,3                   |                                 |                      |
|                         | 76,2 ± 13,76                   |                                 |                      |
|                         | 7,1 ± 1,1                       |                                 |                      |
|                         | 9,3 ± 2,3                       |                                 |                      |
|                         | 10,7 ± 1,9                     |                                 |                      |
|                         | 14 ± 2,6                       |                                 |                      |
|                         | 12,0 ± 2,6                     |                                 |                      |
|                         | 10,0 ± 1,5                     |                                 |                      |

### 4. Discussion

Maternal deprivation, also referred to as maternal separation (PINHEIRO, 2001), is a model for stress\(^{32-34}\), this model has been used for decades\(^{29}\) indicating that, stress in childhood will produce alterations in adult emotional behavior, also producing physical damage\(^{35-37}\). These data, demonstrating that private mother females in childhood have anxiety behavior are illustrated in studies using animal models of maternal deprivation\(^{38-41}\). Mother-pup relationships were reported as pivotal for final neural development, emotional behavior later in life and homeostasis\(^ {38}\). Maternal deprivation during the first day of life was sufficient to modify the hypothalamus-pituitary-adrenal (HPA) axis activity, leading to behavioral changes indicative of increased emotionality and fear\(^ {42,43}\).
In rodents, the critical period for maternal separation effects occurs during the first 2 weeks of life, a time when HPA maturation takes place. Concerning the OF data MD increased rearing behavior without interferences with the immobility time. No effects of MD on locomotor activity in all ages of female rats were found in the open field. These findings are in line with a number of previous studies. Li et al. (2003) showed no effect of MD on locomotor activity of adult female rats in a novel environment. In contrast, Slotten et al. (2006) showed that female rats displayed higher levels of activity in a novel environment compared to their male counterparts, an indication of increased arousal and exploratory behaviour. Similarly, a 6 h MD protocol employed to evoke a hyperactive/impulsive phenotype resulted in female rats displaying increased rearing behaviour in the open field compared to male rats and female rats spending more time in the centre zone of a familiar open field environment. Thus, we suggest that MD in our study increased rearing behavior by an increased arousal an exploration of the OF. The Zn6c administration reversed this behavior but not the Zn30c treatment at the adolescent age. The lack of effects at PND21 and PND75 needs further investigations.

Also, in the present study maternal deprivation increased anxiety-like behavior at PND21 because female rats remained more time in closed arms. These effects were not observed at PND40 and PND 75. In this respect, early exposure to maternal deprivation decreases the activity of the HPA axis. And the serum corticosterone later in life. In this vein, the early increase in HPA activity induced by maternal separation would result in later hyporesponsiveness to environmental stimuli, explaining the lack of anxiety-like behavior at PND40 and PND75.

In addition, only the Zn6ch treatment reduced the anxiety-like behavior in PND21 in our study.

Thus, we proposed that treatment with the Zn6ch was effective in reverse the effects of early stress related to maternal deprivation in female rats. In addition, it was observed a biphasic effect of both doses of Zincum metallic were the Zn6ch reduced the effects of stress and the Zn30ch treatment did not.

Unlike other experiments where maternal deprivation may be a factor in the adult behavioral disorders. The results presented here in contradict Oitzi et al., in which maternal deprivation effects can reach even the senescence time. On the other hand, therapeutic possibilities were studied to revert the maternal deprivation-induced disturbances in adulthood, such as physical activity, as shown by Mello et al..
A slight decrease of the weight gain was seen in maternal-deprived groups, as related in other experiments\textsuperscript{40,51}. Zinc met\textsuperscript{6c} improved the weight gain (p<0,05) and Zinc met\textsuperscript{30c}, even better, kept the same weight gain, compared to the control group, which might indicate greater physiological adaptability comparing to rats submitted to maternal deprivation stress.

As in present study, Mutlu et al\textsuperscript{19} intended behavioral models, like open field test and also EPM, to evaluate anxiety, dealing with Anax imperator\textsuperscript{30c} and 200c via intraperitoneal. Here in, we obtained the same results with Zinc met\textsuperscript{6c} supplied orally, showing that possible variabilities between medications, via of administration and potencies can reach the same results, according to the context.

Similiarly to Zincum metallicum, other studies have also demonstrated anxiolytic effects of Gelsemium sempervirens using the behavioral methods, such as open field and light / dark tests\textsuperscript{18}; Marzotto et al\textsuperscript{21} also used the same behavioral tests to evaluate the drug Ignatia amara and some emotion-related observed symptoms in laboratory mice. All of these medications, including Zincum metallicum did not alter the mobility between groups. Interestingly, the best results seem to be reached with low potencies, as Ignatia amara\textsuperscript{9c} and Zinc met\textsuperscript{6c}.

5. Conclusion

According to the proposed model, Zincum metallicum\textsuperscript{6c} seems to be able to prevent in anxiety-like behavior induced by maternal deprivation in the childhood, mainly in behavioral models related to anxiety. However, other studies still need to be developed to understand the physio-pathological basis of these effects.

Conflict of interest

The authors declare no conflict of interest regarding financial or personal support for the work carried out in this article.

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