Preliminary Data Regarding the Effects of Oxytocin Administration on the Oxidative Stress Status of Zebrafish (Danio Rerio)

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Zebrafish are important animal models widely used in molecular biology research. To our best knowledge, no previous study on OT effects in zebrasishes' oxidative stress status was performed. Moreover, while the zebrafish are naturally producing isotocin, the investigation of its homologue OT effects may provide consistent evidence of the possible influence of oxytocin in isotocin naturally producing teleosteans. In this way, we aimed to analyse the influence of water exposure (e.g. branchial and tegmental exposure) on two different doses of OT (33.2 ng/mL and 66.4 ng/mL) on the adult zebrafish (n = 15) oxidative stress markers. Significant differences and correlations were found in the zebrafish exposed to a higher OT concentration, although lipid peroxidation tended to decrease as the OT concentration increased. Thus, it seems that oxytocin is cross-reacting and exerts clear effect on oxidative stress status main parameters in zebrafish.

Keywords: oxytocin, superoxide dismutase, glutathione peroxidise, enzymatic activity, malondialdehyde, zebrafish, oxidative stress

Oxidative stress is the most known biological process which occurs in every living being leading to important physiological imbalance, including ageing, degeneration, and apoptosis. Oxidative stress can be easily defined as being the biochemical condition resulted from the imbalance between reactive oxygen species (ROS) production and the antioxidant systems activity [1]. Shortly, several important ROS for the living body are the superoxide anion (O²⁻), the hydroxyl radical (HO·), the hydrogen peroxide (H₂O₂), the nitric oxide (NO), the peroxy radical (ROO·), and the reactive aldehyde (ROCH) [2]. From a certain point of view, all of these ROS are usually produced in the body due to their participation in the normal metabolism execution and modulation [3]. Their accumulation prevention and catabolism following usage are assured by the antioxidant system which consists in antioxidant enzymes (e.g. superoxide dismutase-SOD catalyzing superoxide radicals to hydrogen peroxide conversion, catalase – CAT which converts the latter in water and glutathione peroxidise- GPx which also converts hydrogen peroxide to water and reduces lipid hydroperoxides in the correspondent alcohols) [4-8] and non-enzymatic antioxidant such as several vitamins and biologically active molecules [9]. Moreover, as our group previously demonstrated on different occasions, oxidative stress is an important component of many disorders and a key mechanism involved in neurological degeneration [10-16].

During the last decade, a great interest has been developed in regarding zebrafish investigation [17]. By contrast to rodents and bigger and more evolved animals usually used in neuroscience research, zebrafish investigation is rather rudimentary and easy possessing easily assessable behavior and simple molecular compenence. However, they are an important tool in both molecular biology and, became intensely discussed as a behavior model [18]. As most of the conventional research done using zebrafish is directed towards biomedical research and transgenesis, it was shown that zebrafish can be extremely useful in behavior research [19] therefore they can be used as neurobiology animal models.

Oxytocin (OT) is an important neuropeptides which is known to alter both behavior and physiological functions [20]. A growing interest on the OT beneficial effects behavior and other important biological processes was noted, considering its stress reliever and social bonding potentials in healthy, but also in individual with various neurological and psychiatric alterations [21-28]. Although oxytocin structure was demonstrated as highly conserved in placental mammals, several variants were described. For an instance, fish by contrast to mammals do not produce oxytocin, but a similar neuropeptide named isotocin (glutamine-4 changed to serine and arginine-8 changed to isoleucine) [29]. Isotocin was demonstrated to stimulate smooth muscle contractions and to influence several behavioral and physiological processes such as neurological processes, reproduction, and body fluids regulation [30, 31]. However, no clear evidence of isotocin effects on oxidative stress was brought by recent research.

In our best knowledge, no previous study on OT effects in zebrasishes’ oxidative stress status was performed. In this way, we aimed to analyze the influence of OT exposure on the adult zebrafish oxidative stress status.

Material and methods
Animals and treatments
A group of 15 wild type long-tail striped zebrafish specimens (Danio rerio) were selected in a random manner from an adult population of 200 specimens (both males and females) and used for this study. Prior the experimental conditions were applied all of the animals’ behavior was assessed for memory, mobility and social performance homogeneity. The animals’ accommodation was insured by a 100 L glass tank filled with constant 21°C water kept...
in 12 h light/12 h dark cycle. Following selection procedure, the animals were regrouped in three experimental groups (Saline; OT 33.2 ng/mL; OT 66.4 ng/mL) and scheduled for study treatments. Individual saline or OT exposure was performed to 50 mL single-use plastic flasks filled with saline or OT solutions of mentioned concentrations (in saline) for 90 s. At 30 minutes after OT exposure, the animals were extracted from the water tank and instantly frozen. The experimental procedures were carried out in accordance with the mandatory principles of the ethics [32, 33].

Sample preparation
Tissue extracts were prepared from samples of zebrafish total body ranging from 0.4 to 0.5 g. After thorough grinding, the samples were washed by previously cooled (4°C) animal tissue extraction buffer (Tris-HCl 0.025 M, KCl 0.175 M, pH = 7.4) in a 1:10 ratio and transferred to centrifugation tubes. Following a 15 minutes centrifugation (3000 rpm, 4°C), the supernatant liquid was separated and used for biochemical analysis.

Biochemical determinations
Biochemical analysis included superoxide dismutase, glutathione peroxidase, malondialdehyde, and total protein assessment. SOD enzymatic activity was determined using a spectrophotometric SOD Assay Kit (Sigma, Germany) according to the manufacturer’s instructions. Based on the WT (water soluble tetrazolium) salt reaction with superoxide anion producing a water-soluble formazan dye, the indirect measurement of SOD activity is obtained. The results for antioxidant enzymes’ activity were normalized by total sera proteins concentration and transformed in enzyme specific activity units. GPx enzyme activity was assessed using GPx Cellular Activity Assay Kit (Sigma, Germany), an indirect determination method kit based on the NAPDH concentration decrease measurement in the reaction media, correspondent to the GPx activity during which NAPDH is oxidized to NADP+. MDA levels were assessed using thiobarbituric acid-reactive substances (TBARS) determination method. Trichloroacetic acid (50%, 0.25 mL), thiobarbituric acid (0.73%, 0.255 mL) and tissue extracts (0.05 mL) were mixed and vortexed. Afterwards, a 20 minutes incubation at 100°C (boiling water bath), and a 10 minutes centrifugation (3000 rpm) were performed. The supernatants were exposed to a 532 nm spectrometry system and the absorbances were read against a MDA standard curve (the results were expressed as mmols MDA/mL tissue extract).

Statistical analysis
Statistical analyses were carried out using Minitab 17 (Minitab Inc., 2013) software and standard statistical analysis (One-way Anova) was firstly applied. Post hoc analyses were performed using Tukey’s honestly significant difference test. F values for which p < 0.05 were regarded as statistically significant. All results are expressed as mean ± S.E.M. Pearson’s correlation coefficient and regression analysis were used to evaluate the connection between antioxidant defense, and lipid peroxidation.

Results and Discussion
In the present study we measured several important oxidative stress markers in the whole body extract of zebrafish exposed to different concentrations of OT (33.2 ng/mL and 66.4 ng/mL), compared to similar individuals exposed to vehicle solution (0.9% NaCl solution). Biochemical analysis for the studied oxidative markers revealed significant differences regarding all the evaluated parameters, except for the total protein concentration. In this way, the present work provides evidence that OT administration in zebrafish may lead to oxidative stress status change. It is important to note that no previous work reports oxidative stress status evaluation in adult zebrafish. Also, although zebrafish are naturally producing isotocin, OT is active in terms of oxidative stress status changes.

In this way, we observed that SOD activity in the OT-treated fish was lower (0.80 ± 0.045 U SOD/mL body extract) for the smaller OT concentration, as compared to the control animals (0.90 ± 0.007 U SOD/mL body extract), and higher (1.34 ± 0.07 U SOD/mL body extract) (Figure 1). Statistical analysis of the results showed a significant difference between the groups (overall one-way ANOVA: [F (2,21) = 20.01; p < 0.001]. As stated before, SOD is an important enzyme which participates in superoxide anion conversion to lesser active ROS. The fact that increased SOD activity occurs while a higher concentration of OT is administered may lead to the conclusion that a superoxide producing pathway may be enhanced or may be the effect of a ROS highly producing organ or metabolic pathway.

For the second antioxidant enzyme, we obtained an interesting pattern of variation in terms of enzymatic specific activity by difference to OT concentration. In this way, for the 33.2 ng/mL OT concentration we observed a decrease in GPx activity (0.014 ± 0.0009 U GPx/mL body extract), as compared to control group (0.019 ± 0.002 U GPx/mL body extract). However, the increase of OT concentration changed the variation pattern, for the 66.4 ng/mL OT, the GPx activity being more intense (0.021 ± 0.001 U GPx/mL body extract), than that of the enzyme measured in the control animals. The analysis of variance showed statistical differences between the groups (overall one-way ANOVA: [F (2,18) = 4.76; p = 0.021]) (fig.2). Similarly to SOD, a complementary increase in GPx activity can be observed. In this case, it seems that superoxide over production or SOD over-reaction leads to GPx over reaction as a result of excessive hydrogen peroxide. It is interesting to note that even though the MDA concentrations decrease, GPx activity increases, therefore its activity is not the result of the lipid hydroperoxides reduction.

Also, we obtained different results for the lipid peroxidation marker depending on the used OT concentration. In this way, we observed a significantly higher MDA concentration for the lower OT concentration treatment (31.09 ± 2.65 mmols MDA/mL body extract), and non-significant increase in MDA concentrations for the other OT concentration used (20.72 ± 1.76 mmols MDA/mL body extract), as compared to the controls (18.95 ± 2.63 mmols MDA/mL body extract). Overall one-way
ANOVA analysis showed statistical significant differences between the groups \( [F (2.21) = 7.53; p = 0.003] \) (fig. 3).

However, by contrast to all of those variations in all the studied oxidative stress markers, we obtained no statistically significant difference in regarding the total protein content of the whole body extract (Overall One-way ANOVA: \( [F (2.9) = 1.19; p = 0.34] \) (fig. 4).

Post hoc analysis was also performed in order analyze the correlations between the oxidative stress markers. In this way, by running Pearson’s correlation test and Spearman’s correlation indices, we found statistical significant correlations between SOD and GPx activities. While comparing the Pearson’s coefficient \( r = 0.408; p = 0.048 \) with the Spearman’s \( \rho = 0.452; p = 0.026 \), we observed a monotonic correlation between the two variables rather than a linear one. Also, monotonic correlation was observed between SOD and MDA patterns of variation \( (\rho = 0.361; p = 0.08) \) (graphically expressed in fig. 5). Therefore, it can be observed that the antioxidant enzymes pattern of variation is rather similar, whereas SOD and MDA variation is in an inverse correlation. In this way, while antioxidant defense promptly responses to OT administration leading to a decrease in lipid peroxidation process. However, a spike in MDA concentration was observed as a result to 33.2 ng/mL OT exposure to zebrafish and no significant variation in total protein content was obtained leading to the question that may OT be perceived as a harmful agent or these might actually be an over response to an overreaction to some abnormally enhanced physiological processes? Thus, it seems that oxytocin is cross-reacting and exerts clear effect on oxidative stress status main markers in zebrafish, perhaps in a similar way to what isotocin does (a future study regarding these aspects is under work by our research group).

Conclusions
This study provides consistent evidence of the possible influence of oxytocin in zebrafish oxidative stress status. Significant differences and correlations were found in the zebrafish exposed to a higher OT concentration, although lipid peroxidation tended to decrease as the OT concentration increased. However, OT-exposed zebrafish can be characterized by increased antioxidant enzyme response, but decreased lipid peroxidation. Thus, it seems that oxytocin is cross-reacting and exerts clear effect on oxidative stress status main markers in zebrafish.
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