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The Effect of Selective Cyclooxygenase (COX) Inhibitors on Japanese Medaka (*Oryzias latipes*) Reproduction Parameters

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***Abstract*—**Our results showed that treatment with both cyclooxygenase (COX1 or COX2) inhibitors impair reproduction parameters of the medaka. Resveratrol (COX1 inhibitor) caused an decrease in the number of spawning females at the first week of feeding fish with experimental diets. In the group treated with NS-398 (COX2 inhibitor) we found the lowest sperm velocity parameters and decreased linearity of movement. The ovaries of the medaka fed feed supplemented with Resveratrol or NS-398 were confirmed to have a lower share of matured oocytes however during the experiment (four weeks) the number of eggs spawned by females was similar. Both inhibitors in fish diet (20 mg/kg body weight/day) caused a decrease in the embryo survival. Our results revealed that for the medaka female reproduction, activity of both COX enzymes might be necessary whereas males reproduction competence, as expressed by sperm motility parameters, might be related to COX2 activity.

***Keywords*—**COX innibitors, medaka, reproduction parameters

I. Introduction

THE medaka fish (*Oryzias latipes*) is an popular aquatic model organism in reproduction researches [16]. Their manageable cyclicity of reproduction behavior is an important factor for conducting the research in the field of reproduction and development [12]. The spawning behavior is easy to control by temperature and light regime [11]. Moreover, transparent eggs and relatively easy management of broodstock as well as offspring make the medaka a suitable model for researchers.

Cyclooxygenase (COX1 and COX2) are the enzymes responsible for the conversion of arachidonic acid (ARA) to eicosanoids, e.i. prostaglandins (PGs) [18]. These compounds are highly biologically active and have a wide range of reproduction actions [13]. The presence of pharmaceutical products affecting COX activity have been detected in aquatic environment and possibly might disturb spawning process [10]. Literature concerning the effect of COX inhibitors on live organisms indicates that changes in the function of several physiological systems are possible with long term exposure [4].

The Japanese medaka has two COX1 genes (ptgs1a and ptgs1b) and one COX2 gene (ptgs2) [5]. The expression of ptgsa1 was found in testis whereas ptgs2 was reported to be major COX gene actively expressed in ovary [5]. It was found that COX2 activity in the ovary may be involved in ovulation of medaka [5]. The role of COX in fish male reproductive system remain unclear. It is known that non selective COX inhibitors (NSAIDs – non-steroidal anti inflammatory drugs) can disrupt the medaka reproduction [10]. However effect of selective COX inhibition on fish reproduction was not yet reported.

The aim of this study was to evaluate COX1 and COX2 inhibitor treatment on the reproductive parameters of Japanese medaka. The reproduction analysis consists of female fecundity, sperm quality, histological investigation of gonads, quantity of mature oocytes in ovaries and embryo survival.

II. Materials and Methods

A. Fish culture

The experimental material was comprised of broodstock medaka (body weight BW, ~0.40 g; total length TL, ~33mm; age, three months post-hatch) originally stocked with fish from the National Institute of Natural Science (Japan). The adult medaka were placed in six glass aquaria each with a volume of 20 dm^3 (13 individuals per aquarium; 7 male and 6 female in each aquarium). Each aquarium was equipped with indoor filter with impeller pump (Head 750, Aqua Szut, Wrocław, Poland), heater (AQ 25, Aquael, Warsaw, Poland) and external light (Palm Light, AZOO, California, USA). Water quality parameters for the culture average pH 6.63, >5.0 mg/dm^3 dissolved oxygen. Ammonia and nitrite were generally undetectable. The experimental animals were maintained on a 14:10 light:dark regime at 27°C to induce spawning. The light intensity measured at the surface of the rearing aquarium was 650 lux. The animals were maintained in a balanced salt solution (BSS) [16].

B. Fish diet

The feed used to prepare the experimental diets was commercial diet recommended for medaka (TetraMin, Germany). The feed was supplemented with one of two COX inhibitors: Resveratrol (R, COX1 inhibitor) or NS-398 (NS, COX2 inhibitor). We tested effect of Resveratrol or NS-398 at dose: 20 mg/kg body weight/day. Administration method was:

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with dry food, reagents were dissolved in DMSO mixed with ethanol (1:2 v/v). Dissolved inhibitors were sprayed on Petri dish and immediately dry food was placed on it and well mixed with use of pipette tip. After mixing food was kept 3 hour in room temperature for evaporation of ethanol. Dose of DMSO in dry food was about 0.8%. Control diet (C) were feed with diet formulated with use of dissolvent (DMSO+ethanol 1:2 v/v) addition. Feed was prepared and delivered twice daily. The daily ration was 4% of the fish biomass. The experiment was conducted for four weeks.

In the beginning (period I: the 1st and 2nd week of experiment) all fish were fed a commercial diet recommended for medaka (TetraMin, Germany). During period II: the 3th and the 4th week of experiment, the fish were fed control feed (group C) or feed supplemented with either inhibitor COX1, Resveratrol (group R) or inhibitor COX2, NS-398 (group NS). The fish were divided into three feeding treatment groups, each in two replicates.

C. Fish reproduction characteristics

Spawning was watched everyday. Eggs were collected everyday during four weeks of experiment. Spawned eggs were collected from the abdomen of the females of each species 2-3 hr after the start of the light period. The eggs were incubated in an embryo culture medium at 23°C with a plastic plate. Number of female spawning and fertilization rate, numbers of eggs and survival rate of embryos were calculated.

D. Sperm motility parameters

At the end of experiment 6 males from each group (3 fish from each aquarium) were sacrificed. Male fish were anesthetized on ice for 1 min after that time testis were dissected, and leaking sperm were collected. The testes were removed and transferred to 1.5-ml tubes contained 10µl of Hanks' balanced salt solution (HBSS; 0.137 M NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, and 5.55 mM glucose, pH = 7.2) supplemented with 50mM of trehalose and 0.5% of BSA was used as the extender [20]. Sperm motility were observed 1 hour after samples collection. Activated sperm was placed on Teflon-coated 12 wells slide glasses (Tekdon, Inc., Myakka City, FL) and covered with standard cover slips. The mixture of sperm and Hanks' balanced salt solution of 1 µl were placed on a slide and sperm movement was recorded six seconds after activation. Video recordings for CASA (computer assay sperm analysis) were made using a microscope with a 20x negative phase. Recordings were made with a Basler a202K digital camera integrated with a Olympus BX51 microscope. The recording speed was 47 frames per second. The first 200 frames from each recording were analyzed using the program, Image House CRISMAS Company Ltd. From fifteen motility parameters straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), linearity (LIN = 100 x VSL/VCL, %), beat cross frequency (BCF, Hz), amplitude of lateral head displacement (ALH, µm) and percentage of motile sperm (MOT, %) were chosen for further analysis.

E. Histology

Histological analyses of ovary were performed on a sample of 10 individuals females from each group (5 fish from each aquarium) on the last days of the experiment. The fish were fixed in Bouin's solution, dehydrated in ethanol, cleared in xylene, embedded in paraffin blocks, cut into 5-µm slices with a rotating micro-tome (Leica, Bensheim, Germany), and then stained with H&E. A Nikon E600 (Japan) light microscope was used for the histological observations, and NIS-Elements BR 3.2 (Nikon, Japan) programs were used for analyses.
The histological analyses of gonad maturation stages included all cells in each of the ovary samples analyzed from each individual. All reproductive cells were identified and counted in each female. The oocyte development were identified as described for medaka and was determined on a three-degree scale: previtellogenic phase (PV), vitellogenic phase (V) and postvitellogenic phase with oocytes in maturation stage (M) [12]. Oogonias were counted from digitalized images at 4× (for M) and at 20× (for V) and at 40× (for PV).

F. Statistical analysis

The results of all the measurements and calculations were subjected to statistical analysis with the GraphPad Prism program (Soft. Inc., Avenida de la Playa la Jolla, CA, USA). The means were compared with single factor analysis of variance (ANOVA). When statistically significant differences were confirmed among dietary treatments ($P \leq 0.05$), further statistical analysis was performed with Tukey's test (for the analysis of female spawning and fecundity; PV, P, M oocytes quantity; embryo survival). The Kruskal-Wallis test was applied to analyze the sperm quality parameters. All values expressed as percentages were transformed with arcsine before statistical processing.

III. Results

The share of spawning female was similar in all aquaria at the beginning of the experiment (period I). Resveratrol caused an decrease in the number of spawning females at the beginning of feeding fish with experimental diets (the 3rd week of rearing, period II) ($P<0.05$; Fig. 1). This effect was also observed in the 4th week of the experiment in both R and NS groups. During the experiment the number of eggs spawned by females was similar ($P>0.05$) in all the groups. However, while the share of previtellogenic and vitellogenic oocyte in ovary was the same in all the dietary treatments, the ovaries of the fish fed feed supplemented with Resveratrol and NS were confirmed to have a lower share of matured oocytes ($P<0.05$; Fig. 2 and Fig. 3). We also found a difference in sperm motility parameters among experimental groups. The lowest value of VCL, VSL, and LIN, was recorded for sperm obtained from fish in group NS ($P<0.05$; Fig. 4). Parameters BCF, ALH and MOT remain unaffected in all group of fish ($P>0.05$, Fig. 4). The survival rate of embryos was approximately 80% during period I of the experiment (Fig. 5).

Addition of COX1 inhibitor in medaka broodstock feed resulted in a decrease in survival embryo (53% in group R compared 81% in group C) in the 3rd week of the experiment (P<0.05; Fig 5). In the 4th week of the experiment, the survival rate of embryos in the both groups R and NS (57 and 53% respectively) was lower compared to that of group C (84%) (P<0.05; Fig. 5).

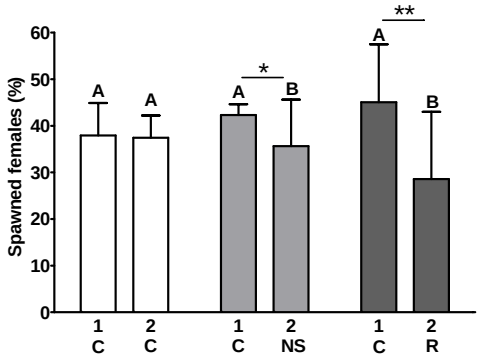

Fig. 1 Percentage of spawning females at the beginning and the end of experiment;1 – first two weeks; fish fed control feed (C), 2 – last two weeks, fish fed control feed or feed supplemented with NS-398 (NS) or Resveratrol (R). Groups with the same superscript do not differ significantly statistically (P>0.05)

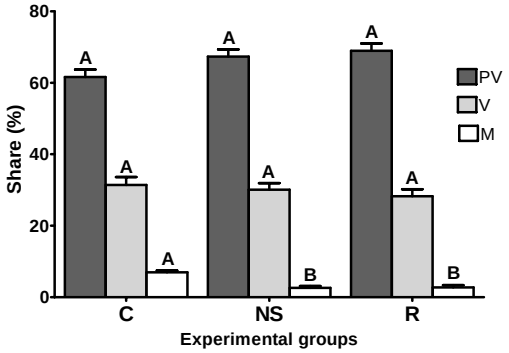

Fig. 2 Share of previtelogenic (PV), vitelogenic (V) and mature (M) oocyte in female gonads of medaka fed control feed (C) and feed supplemented with inhibitor of COX2 (NS) or COX1 (R). Groups with the same superscript do not differ significantly statistically (P>0.05)

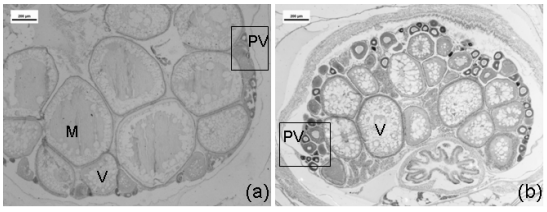

Fig. 3 Gonad histological picture of female fed control feed (a) and feed supplemented with inhibitor of COX1 (b); PV-previtellogenic oocytes, V-vitrllogrnic oocyytes, M-mature oocytes

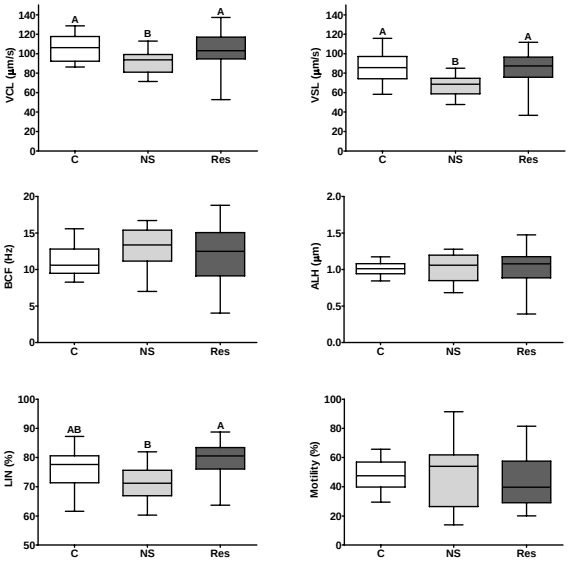

Fig. 4 Sperm motility parameters of medaka after 2 weeks of COX inhibitor treatments. Groups with the same superscript do not differ significantly statistically (P>0.05)

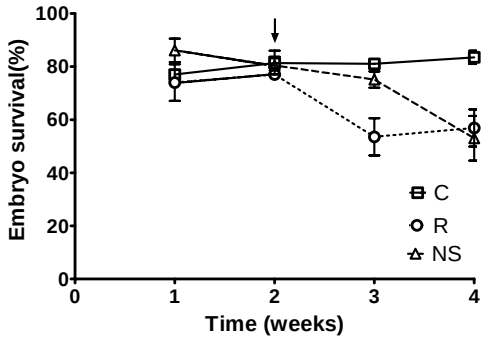

Fig. 5 Embryo survival rate from the eggs collected at the 1, 2, 3 and 4 weeks of experiment; arrow indicate start of feeding experimental diets

IV. Discussion

Treatment with both, COX1 and COX2 inhibitors can alter reproduction of medaka females and males. For female related reproduction parameters more serve effect had an COX1 inhibitor (Resveratrol) while males sperm motility was negatively affected only by COX2 inhibitor (NS-398). Both COX inhibitors had a negative effect on embryo survival rate while NS-398 produce this effect after 2 weeks of feeding and Resveratrol after 1 weeks. It is assumed that function of both COX enzymes (COX1 and COX2) are necessary for maintain optimal reproduction parameters in medaka.

It was shown that the Japanese medaka exposed to ibuprofen (COX inhibitor) spawn less frequently producing greater number of eggs [4]. In our study this after 2 weeks of feeding with both, Resveratrol and NS-398 we observed the lower number of spawning female. The effect of Resveratrol

on number of spawning female was greater ($P<0.01$) than effect of NS-398 ($P<0.05$). It might indicate that for female spawning behavior COX1 is an enzyme of greater importance than COX2. This can be an interesting funding as we know already that medaka posses two COX1 genes (ptgs1a and ptgs1b) and one COX2 gene (ptgs2) [9]. However *in vitro* study regarding medaka ovulation showed that COX2 are enzyme which are expressed in ovary at much higher rate compare to both form of COX1. Disparity between our results and cited *in vitro* study might resulted from unexpected side effects of used reagents. However, it is possible that in our study other than ovary endocrine system were affected where COX1 was an major enzyme.

The number of released eggs did not differ between the all group. Neither COX1 nor COX2 inhibitor at the selected dose was capable to change the numbers of ovulated eggs. In the study with use of ibuprofen (non selective COX inhibitor) was shown that in concentration of drug equal 100µg/l medaka tend to spawn less frequently with higher numbers of eggs [4]. It was also reported that diclofenac (nonselective COX inhibitor) can induce vitellogenesis in medaka [10]. In our study we applied same dose of Resveratrol and NS-398 which was equal to 20mg/kg/day as their effective concentration for COX inhibition are similar [15]. It is possible that effective concentration of COX inhibitors in our experiments was lower than achieved with use of immersion [4]. It is also possible that only combined action of both inhibitors COX1 and COX2 are capable to increase number of ovulated eggs per spawning event.

Impaired COX1 expression in zebrafish (*Danio rerio*) resulted with disrupted development of embryos [3], [7]. In our study embryo survival was strongly related with inhibitors used and time after start of treatment. We found that eggs collected from the fish feed with COX1 inhibitor during 3th week of experiment showed low embryo survival (53%). Moreover, eggs collected during 4th week of experiment from both Resveratrol and NS-398 treated group showed similar, low level of embryo survival (56 and 53% respectively). At the same time control group showed higher embryo survival rate (83%). Histology examination revealed that fish treated with both COX inhibitors had lower share of mature oocytes in ovary. It might be possible that medaka treated with COX inhibitors ovulated not fully mature oocytes which resulted in lower embryo survival rate.

Our data shown that COX activity might be not only related to fecundity and spawning behavior but also to quality of produced gametes. References [4], [8] indicate that exposure of medaka on diclofenac did not change fertility and hatching rate of their offspring. It is not clear whatever observation made by us are result of used feed regime (no brine shrimp in medaka diet) resulting with possible lower level of essential fatty acids and thus feasibility to impair their overall reproduction performance, especially when COX inhibitors were applied.

Sperm motility in vertebrate might be impaired by the presence of COX inhibitors [15]. In our study we found that sperm obtained from the group of fish treated with NS-398 showed lower sperm velocity compare to both, control and Resveratrol treated group. Also linearity of sperm movements was lower in the NS-398 treated fish. This changes in sperm motility might resulted with lower fertility as sperm velocity is the parameter affecting fertilization success in fish [6]. Our results indicate that COX2 activity might be related with medaka spermatozoa velocity and trajectory.

Changes in medaka reproduction performance reported herein might result from direct toxic effect of used reagents. However toxicity of NS-398 was reported, their occurrence was connected with dose greatly exceeded those necessary for COX inhibition [17]. Dose used in our study was similar to doses used for other vertebrates [2]. Therefore it is plausible that effects observed in our study was rather resulted from impaired COX activity and alternation in eicosanoids production than from their non specific toxic effect.

Resveratrol was found to be responsible for reduction of medaka fertility. Interestingly, Resveratrol were previously found to prolong fish lifespan [19]. It might be related to suppression of prostaglandin E2 production which was already reported for rat microglia [1]. Prostaglandine E2 was found to be an important PG of medaka ovary [5]. However in medaka ovary COX1 activity is scarce therefore Resveratrol action in ovary might be related to antioxidative function of these polyphenol which may lead to overall reduction in whole eicosanoids production and consequently to changes in reproduction physiology [14]. Reduction of reproduction efforts after administration of Resveratrol observed herein might be one of the factor which can lead to prolongation of the fish lifespan.

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