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Additional Information

DISCRIMINATION OF THE EFFECTS ON ZEBRAFISH REPRODUCTION FROM POLLUTANTS IN DRINKING WATER VIA FEMALE, VIA MALE AND/OR VIA FECUNDATION WATER.

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"EFFECTS ON ZEBRAFISH REPRODUCTION".

ABSTRACT

The lack of preventive policy legislation and the low removal rate of organic pollutants in conventional potabilization treatments lead to some of them being present in drinking water. The problem arises because some of these substances have detrimental effects on human reproduction health, via females, via males or even both. In this work, we established the zebrafish as a bioindicator of these types of substances with the goal of discriminating the effects through three different pathways: male, female or water where the fertilization took place.

For this purpose, four parameters were analysed: fertility rate, hatching rate and survival and abnormalities rates. So, for each parameter two groups were formed, according to whether adult males or females were reared in bottled spring water (Z) or tap water (B) and if the in vitro fertilization took place in water Z or B.

Results revealed a decline in the fertility and hatching rate in water B, due to a water effect. The most plausible explanation could be the presence of substances which affect the micropyle and chorion. Moreover, a decrease in the fertility rate due to an effect over the female was also observed, but in this case by an alteration of the oocyte quality.

Keywords: organic pollutants; reproductive effects; drinking water; bioindicator; zebrafish.

1. INTRODUCTION

Several emerging organic pollutants (endocrine disruptors, pharmaceutical substances and personal care products) are released mostly through urban wastewater and many of them can spread through the water cycle, even reaching drinking water, due to their low removal rate (Rodil et al., 2012). The problem is exacerbated by the fact that many emerging pollutants are non-regulated (Richardson and Ternes 2011) or newly introduced, or have only recently been regulated, as is the case with some pharmaceutical substances. Furthermore, although concentrations are generally low (ng/l) and some individual chemicals are not dangerous to human health (Schriks et al., 2010), there are worries about the potential and unknown risks of exposure to mixtures (Silva et al., 2002), especially in human reproduction, where the alteration could be via female or male or even both.

The detection of organic pollutants in drinking water through the study of the most sensitive developmental and reproductive parameters in zebrafish, particularly the latter, was the aim of our last work (Martínez-Sales et at., 2015). In our current work, we attempt to elucidate the origin of the effects on survival, abnormality, hatching and fertility rate in zebrafish adults reared in two waters (Z and B) also tested in our previous works, from three different pathways: male origin, female origin or the water where the in vitro fertilization took place, with the aim of establishing the zebrafish as a bioindicator in water quality studies.

2. MATERIAL AND METHODS

Zebrafish maintenance

The F1 colony was reared in the laboratory following the protocol described in Westerfield (1995). Briefly, adult zebrafish were kept in 20 L tanks at 28.5ºC, in a 3:2 ratio (females: males) (Westerfield, 2007) and fed on granular food supplemented with recently defrosted hen egg yolk and shrimp meat (Simão et al. 2010) twice a day. The light cycle was regulated at 14h light/ 10h dark (Matthews et al. 2002; Brand et al. 2002). The aquariums had water recirculation systems but without active carbon filters. According to the Westerfield (2007) recommendations, a quarter of the total aquarium water was removed weekly and replaced by clean water to avoid ammonium concentrations.

It must be stated that all environmental conditions were identical to all aquariums and the spatial distribution of the aquariums was randomized.

The experimental procedures and animal care in the present work fully comply with the standards for use of animals laid down by the Ethical Committee of the Polytechnic University of Valencia, which specifically approved this study.

Water origin

Two different waters were used in this work. Bottled spring water (Z) that was used as control in our previous works, and water B also tested in previous works from the tap water distribution network of a medium-sized city, supplied from the Túria and

Xúquer rivers. Water B was selected to manifest the most harmful effects on the sensitive parameters studied in our previous works (Martínez-Sales et al., 2015).

It should be noted that water B is potable and also that the chemical parameters set forth for tap water for human consumption in Royal Decree 140/2003 of 7 February, whereby the health criteria for the quality of water intended for human consumption are established, are suitable for zebrafish breeding and maintenance (Westerfield 2007). Furthermore, the drinking waters used meet the physical and chemical requirements set by this Royal Decree.

Obtaining inactivated gametes

Gametes extraction was carried out following the method describe by Westerfield (2007). Zebrafish adults (5 months post fertilization) were carefully selected and separated from the colony after having manifested courtship behaviour at dawn. Before any extraction, specimens were sedated in a clove oil solution (100µl oil in 1L of decalcified and dechlorinated water: system water) for a few minutes, then were cleaned in clear water. Eggs were extracted and deposited in a plastic spoon after gentle but firm pressure with plastic forceps on the belly previously dried. Only good eggs (yellow and translucent colour) were kept in Hanks' buffered salt solution supplemented with 1.5% (v/v) of BSA (Bovine serum albumin) and 0.1 g of NaCl/100 cc of Hanks' medium (egg medium (F_1); ph: 7.4; osmolarity: 310-320 mOsm) in a 35 mm Petri dish.

For semen extraction, males were placed belly up in a slit of a damp sponge. The genital pore was gently dried to avoid sperm activation. The sides of the fish were gently but firmly pressured with plastic forceps to collect the sperm with a microcapillary (1 x 90 mm, Narishige Scientific Instrument Lab.), which were kept on ice until use. Sperm from 2-3 males was diluted in 100µl of F_1 and kept inactivated in a Petri dish of 35 mm.

In vitro fertilization (IVF)

The IVF and solutions used were at room temperature. Non activated eggs and sperm were mixed in F_1 for a couple of minutes. Two groups were carried out depending on the water (Z or B) used to activate both gametes. So, 1 mL of Z or B water, depending on the experimental group, was added to the egg-sperm mixture. After 2-3 min, the time required for fertilization in zebrafish, the 35 mm Petri dish was fully filled with the corresponding water. The Petri dish was left in the incubator at 28.5ºC until the 5^{th} day post fertilization.

Experimental design

The following combinations were carried out:

- Sperm from males reared in water B were mixed with oocytes from females reared in water B, and the egg-sperm mixture (fertilization) was cultured in water B.

- Sperm from males reared in water B were mixed with oocytes from females reared in water B, and the egg-sperm mixture was cultured in water Z.
- Sperm from males reared in water B were mixed with oocytes from females reared in water Z, and the egg-sperm mixture was cultured in water B.
- Sperm from males reared in water B were mixed with oocytes from females reared in water Z, and the egg-sperm mixture was cultured in water Z.
- Sperm from males reared in water Z were mixed with oocytes from females reared in water B, and the egg-sperm mixture was cultured in water B.
- Sperm from males reared in water Z were mixed with oocytes from females reared in water B, and the egg-sperm mixture was cultured in water Z.
- Sperm from males reared in water Z were mixed with oocytes from females reared in water Z, and the egg-sperm mixture was cultured in water B.
- Sperm from males reared in water Z were mixed with oocytes from females reared in water Z, and the egg-sperm mixture was cultured in water Z.

All these combinations are summarized in the following diagram:

WATER ♂	WATER ♀	WATER IVF
B	B	B
B	B	Z
B	Z	B
B	Z	Z
Z	B	B
Z	B	Z
Z	Z	B
Z	Z	Z

In each of these combinations we analysed the following parameters: fertility rate at mid blastula transition (MBT) stage, hatching rate at 72 hours post fertilization (hpf) and survival and abnormalities rates at 5 days post fertilization (dpf). Results were grouped according to the water origin, B or Z, when male effect, female effect or water effect were studied.

Statistical analysis

Results were analysed using Chi-square test (Statgraphics Plus 5.1). The Yates correction for continuity was used when a single degree of freedom was involved. Values were considered statistically different at $P<0.05$.

3. RESULTS

3.1.- Fertility rate at MBT stage

Significant differences ($p<0.05$) appeared between all groups regardless of the effect analysed (see table 1). When the male effect was analysed, water B presented better rates than water Z (64.87% vs. 57.51%). However, when the female effect and the water where the in vitro fertilization took place were analysed, the worst result was obtained in water B.

3.2.- Hatching rate at 72 hours post fertilization

Embryo hatching rates were evaluated at 72 hpf (Martínez-Sales et al., 2015). No statistically significant differences appeared when the male or female effects were assessed. However, significant differences (p<0.05) appeared when the water effect, where the in vitro fertilization took place, was analysed (see table 2). Water B presented the worst result (7.83%).

3.3.- Survival and abnormality rate at five days post fertilization

Embryo survival rates evaluated at 5 dpf were high in all groups, with no statistically significant differences (p<0.05) between waters, except when the female effect was studied, where significant differences appeared (p=0.0342) (see table 3). Water B obtained the worst result (89.11%) compared to water Z (94.68%).

In the case of abnormalities at 5 dpf, pericardial edema, curled tails and skeletal deformities (lordosis, scoliosis, and abnormal skeletal development) were the main malformations observed. No differences were observed in the abnormality rate evaluated at 5 dpf in any group (see table 4).

4. DISCUSSION

Based upon results obtained in the current work, it can be stated that the effects of pollutants on the sensitive parameters are caused by three different non-exclusive routes: affecting oogenesis in females, spermatogenesis in males and even by a direct effect of the water during the fertilization process. As these effects operate by different pathways and have also been demonstrated in mammals and in humans,

especially via sperm (Toft et al., 2006; Vested et al., 2014), the value of zebrafish as a bioindicator is confirmed.

As mentioned in material and methods, in our previous work (Martínez-Sales et al., 2015) water B manifested the most harmful effects on reproductive parameters, which is the reason we have focused on this water in the present work.

Regarding hatching rate, the male or female direct effects were not the origin of the decrease with respect to the results obtained in the control water. However, this effect was exclusively observed in water B when it was used in the in vitro fertilization process. Pollutants with effects on chorion seem to be the source of this decrease without an alteration of the female gametes or male gametes. Certainly, some substances found in drinking waters have decreased or even inhibited the hatching process in zebrafish, such as ibuprofen or acetaminophen (Galus et al. 2013). In our case it has not been determinate if the reduction in the hatching rate has been due to an effect on embryo or on chorion structure, or even both.

Survival rates at 5dpf were high in all cases studied. In many toxicological studies, a delay in the hatching process entails a decrease in the survival rate (Shi et al., 2008; Zhu et al., 2008), related with the toxic concentration used. The lower the concentration, the lower the mortality (Powers et al., 2010). In our work, the waters employed are drinkable, so the concentration levels (ng/l or µg/l) of emerging contaminants expected are low (Khetan and Collins, 2007; Rodil et al., 2012) and thus a high survival rate is predictable.

With respect to fertility rate, there was a decrease when the in vitro fertilization took place in water B, but in this parameter there was also an effect on the quality of oocytes through the oogenesis from female adults reared in water B. However, sperm fertility from male adults reared in water B was not affected. So, despite the water effect, a female effect in this parameter also seems to be the origin of this decrease. The female effect could be explained by the possible presence in water of substances like endocrine disruptors (17α-ethinylestradiol) which could decrease the number and quality of the female gametes produced (Santos et al., 2007) and/or pharmaceutical substances (carbamazepine and gemfibrozil) which have been shown to reduce fecundity (total embryos produced) (Galus et al. 2014). Regarding the water effect, this decrease could be explained by the presence of substances which affect the chorion structure in the micropyle, altering the sperm entry through it. Moreover, this effect can also alter the overall structure of the chorion, which could explain the decrease in the hatching rate previously described. No references to the possible substances which alter the chorion structure were found in the literature reviewed. However, it could be substances that affect directly the chorion in the same way that the bleach.

It is known that human reproduction can be affected by a wide variety of pollutants (Sharpe and Irvine, 2004; Vested et al., 2014) via male or female or even both, due to the continual occurrence of emerging or newly identified contaminants in the water resources (Bolong et al., 2009) and the lack of preventive policy legislation (Braw-Tal, 2010). For this reason, due to the complex detection and removal of these substances, in our current work and with the support to our previous works (Martínez-Sales et al., 2014; Martínez-Sales et al., 2015), we verify the use of the

zebrafish as a bioindicator of emerging contaminants in drinking water with the possibility, in this case, of discriminating the effects through three different pathways: male, female or water where the in vitro fertilization took place.

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7. DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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382

383 TABLES

384

385 Table 1: Fertility rate of zebrafish (*Danio rerio*) embryos, from adult males reared
386 in water Z and in water B, adult females also reared in these two waters and the
387 water (Z or B) where the in vitro fertilization (IVF) took place.

388

Fertility rate	Z	B
Water ♂	180/313 (57.51%) [b]	314/484 (64.87%) [a]
Water ♀	301/446 (67.48%) [a]	193/351 (54.98%) [b]
Water IVF	291/408 (71.32%) [a]	203/389 (52.18%) [b]

389 *Columns with different superscripts are statistically different (p<0.05)*

390

391 Table 2: Hatching rate of zebrafish (*Danio rerio*) embryos at 72 hpf, from adult
392 males reared in water Z and in water B, adult females also reared in these two
393 waters and the water (Z or B) where the in vitro fertilization (IVF) took place.

394

Hatching rate	Z	B
Water ♂	109/166 (65.66%)	180/299 (60.20%)
Water ♀	184/287 (64.11%)	105/178 (58.98%)
Water IVF	232/252 (92.06%) [a]	13/166 (7.83%) [b]

395 *Columns with different superscripts are statistically different (p<0.05)*

396

Table 3: Survival rate of zebrafish (*Danio rerio*) embryos at 5dpf from adult males reared in water Z and in water B, adult females also reared in these two waters and the water (Z or B) where the in vitro fertilization (IVF) took place.

Survival rate	Z	B
Water ♂	164/180 (91.11%)	293/314 (93.31%)
Water ♀	285/301 (94.68%) [a]	172/193 (89.11%) [b]
Water IVF	273/291 (93.81%)	184/203 (90.64%)

Columns with different superscripts are statistically different (p<0.05)

Table 4: Abnormality rate of zebrafish (*Danio rerio*) embryos at 5dpf, from adult males reared in water Z and in water B, adult females also reared in these two waters and the water (Z or B) where the in vitro fertilization (IVF) took place.

Abnormality rate	Z	B
Water ♂	5/164 (3.05%)	3/293 (1.02%)
Water ♀	5/285 (1.75%)	3/172 (1.74%)
Water IVF	6/273 (2.19%)	2/184 (1.08%)

Columns with different superscripts are statistically different (p<0.05)