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Full Length Research Paper

Thyroidal angiogenesis in zebrafish (*Danio rerio*) exposed to high perchlorate concentration

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As a well known environmental contaminant, perchlorate inhibits thyroidal iodide uptake and reduces thyroid hormone levels. In zebrafish (*Danio rerio*) exposed to high concentrations of sodium perchlorate (200, 350 and 500 mg/L) for 10 days, remarkable angiogenesis was identified, not only histopathologically but also statistically by counting of the small, medium and large sized (grades 1 to 3) vessels per unit area. Angiogenic response is concluded as the most sensitive parameter for rating of high concentrations of perchlorate exposure.

Key words: Perchlorate, zebrafish, thyroid, angiogenesis.

INTRODUCTION

The mediator functions of teleost thyroid have always been a great concern in the context of aquatic contamination. Being a thyroid disrupter, perchlorate causes hypothyroidism, which arises in many histopathological determinants that include follicle size, colloid size and depletion, active follicle number per unit of area, follicular cell height, hyperplasia, hypertrophy and angiogenesis (Liu et al., 2006). Although various types of physiological markers such as thyroid hormone levels in the plasma or whole-body animal are used widely (Brown et al., 2004; Crane et al., 2005; Mukhi et al., 2005), histopathological indicators have never lost their value for determination of thyroidal disruption. Angiogenesis is traditionally used as a marker in evaluation of thyroid tumor occurrence (Rzeszutko et al., 2004); however, it is reported as a sensitive biological marker for perchlorate exposure in recent years (Mukhi and Patino, 2003; Mukhi et al., 2005; Liu et al., 2006). The aim of this study is to evaluate the angiogenic effects of high dose of sodium perchlorate on the thyroid of adult, male zebrafish (*Danio rerio*).

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MATERIALS AND METHODS

Adult male fishes were purchased from commercial dealers and allowed to acclimate in 20 L aquaria for 2 weeks. Fishes were fed once daily with commercial fish food (Sera-San). The water temperature was maintained at 27 ± 0.3ºC and the photoperiod was set at 14 L/10D. The water quality parameters were 32.0-33.0 FrH (French hardness degree), 17.1 ± 0.9 ppm free chlorine, 69.3 ± 0.5 ppm calcium, 37.2 ± 0.4 ppm magnesium, 20.8 ± 0.4 ppm sodium, 2.8 ± 0.2 ppm potassium, 0.03 ± 0.02 ppm aluminium, 0.06 ± 0.05 ppm iron. Nominal concentrations (200, 350 and 500 mg/L) of sodium perchlorate 1-hydrate (Panreac Quimica SA-PA 134387, CAS Number: 7791-07-3; Eksper Ltd. İzmir, Turkey) were prepared by diluting the stock solution (50 g/L) in dechlorinated water. Experimental animals were divided randomly into one control (CG) and three test groups (TG 1-3), each containing 5 fishes. Test groups were exposed to 200, 350 and 500 mg/L of sodium perchlorate 1- hydrate (SP) for 10 days. Being anaesthetized with MS222, all fishes were sacrificed and the tissues were fixed in Bouin's solution at room temperature for 48 h. Tissue samples were embedded into paraffin and serial sections (5 µm) were obtained. Sections were stained with hematoxylin-eosin, mounted and examined with light microscope.

In order to select the regions where the blood vessels would be counted, serial sections of all of the fishes were screened at X40 magnification and five representative fields in each of the three slides prepared from each specimen were identified. Excluding the larger one, which are aortic branches, the vessels located around and between the follicles were graded according to their sizes. The

Table 1. Mean values ± SD of the number of small (grade1), medium (grade 2) and large (grade 3) sized vessels in control (CG) and test groups (TG).

Vessels / groups	CG	TG-1	TG-2	TG-3
Grade 1	5.20±1.930	10.00±2.928	9.46±1.330	9.18±1.811
Grade 2	1.20±0.775	3.00±0.756	2.77±0.725	3.53±1.068
Grade 3	0	3.00±1.000	3.38±0.506	2.47±1.007
Average	2.13±2.497	5.33±3.790	5.13±3.072	5.11±3.372

vessels, with diameters measuring as 15.4 ± 4.1 µm (mean ± SD), are considered as grade 1 (small), 45.2 ± 6.3 µm as grade 2 (medium) and 105.1 ± 7.2 µm as grade 3 (large). They were then counted twice at X100 magnification. Kruskal-Wallis test was applied for the comparison of the control and test groups. All of the analyses were evaluated at a 95% confidence interval.

RESULTS

Quantitative analysis

In order to evaluate angiogenesis statistically, mean values of the number of small, medium and large (grade 1 to 3) sized vessels in control and test groups are given in Table 1 as well as the compartments (Tables 2, 3 and 4) for each group. This statistical summary revealed that, despite a slight decrease for the total number of vessels in TG-3 based on TG-1 and TG-2, a significant increase is seen in all test groups when compared to the controls (for all compartments, p<0.0001).

Grade 1 vessels (Table 2) are found to significantly increase in number in TG-1, TG-2 and TG-3 when compared to the control group (for all, p<0.0001). Although the rate of increase is calculated to be lowered in TG-2 and TG-3, compartments were not statistically significant. The numbers of grades 2 (Table 3) and 3 (Table 4) vessels also increased prominently in TGs when compared with CG (for all, p<0.0001); however, there were some differences among TGs. While there were no significant differences between the num-bers of grades 2 and 3 vessels when TG-1 vs TG-2 and TG-1 vs TG-3 compartments were made, there were statistically significant increase of grade 2 and decrease of grade 3 vessels in TG-3 according to TG-2 (p=0.016 and p=0.003, respectively).

Thyroid histopathology

In CG, elliptical or round follicles encircled by cuboidal or flattened epithelial cells are loosely distributed around the ventral aorta and in the vicinity of branchial arterioles. Colloid is expressed homogenously (Figure 1).

The expansion of the vessels is very striking at TG-1 (Figure 2). Moreover, some of the large follicles are seemed to be embedded in the walls of highly enlarged vessels (grade 3), which are never seen in CG (Figure 3, Table 1). It is clearly observed that, the follicles of the specimens at TG-1 are increased in number, and are arranged into two groups: the first ones, small, round or ovoid; the second ones are larger and ellipsoid. Colloid is heterogeneous in appearance.

In TG-2, enlargement of vessels (Figure 4) is retained while their number is increased (Table 1). The follicles surrounded by hyperplasic epithelial cells are frequently

Table 2. Compartment of the number of small-sized (grade 1) vessels in control and test groups according to p value.

Groups	CG	TG-1	TG-2	TG-3
CG	X	<0.0001	<0.0001	**<0.0001**
TG-1	**<0.0001**	X	0.325	0.461
TG-2	**<0.0001**	0.325	X	0.744
TG-3	**<0.0001**	0.461	0.744	X

Statistically significant results are shown in bold characters.

Table 3. Compartment of the number of medium-sized (grade 2) vessels in control and test groups according to p value.

Groups	CG	TG-1	TG-2	TG-3
CG	X	**<0.0001**	**<0.0001**	**<0.0001**
TG-1	**<0.0001**	X	0.267	0.106
TG-2	**<0.0001**	0.267	X	**0.016**
TG-3	**<0.0001**	0.106	**0.016**	X

Statistically significant results are shown in bold characters.

Table 4. Compartment of the number of large-sized (grade 3) vessels in control and test groups according to p value.

Groups	CG	TG-1	TG-2	TG-3
CG	X	**<0.0001**	**<0.0001**	**<0.0001**
TG-1	**<0.0001**	X	0.106	0.116
TG-2	**<0.0001**	0.106	X	**0.003**
TG-3	**<0.0001**	0.116	**0.016**	X

Statistically significant results are shown in bold characters.

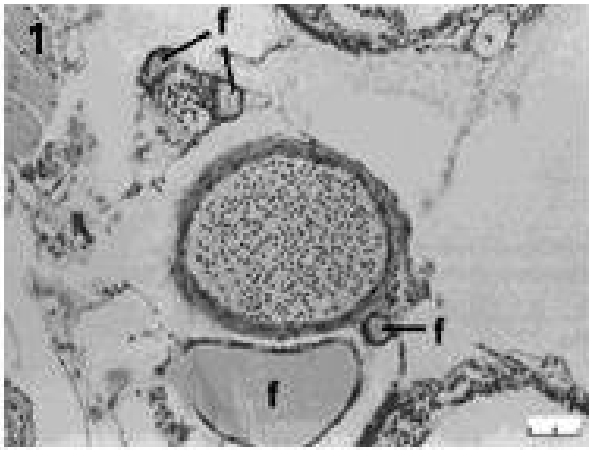

Figure 1. Thyroid follicles (f) loosely distributed around branchial arterioles in CG; bar = 20µ.

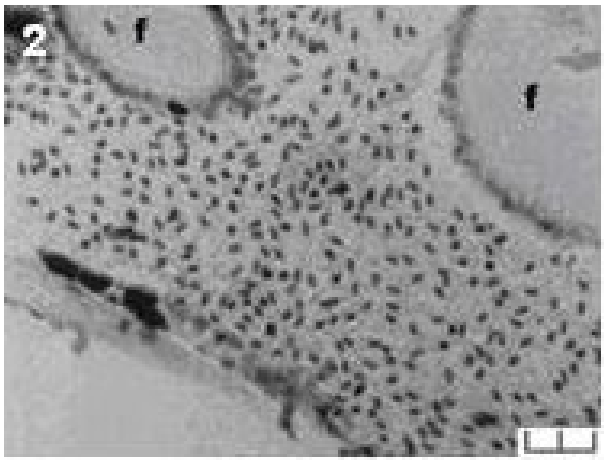

Figure 2. Two follicles (f) surrounded by an expanding vessel in TG-1; bar = 20µm.

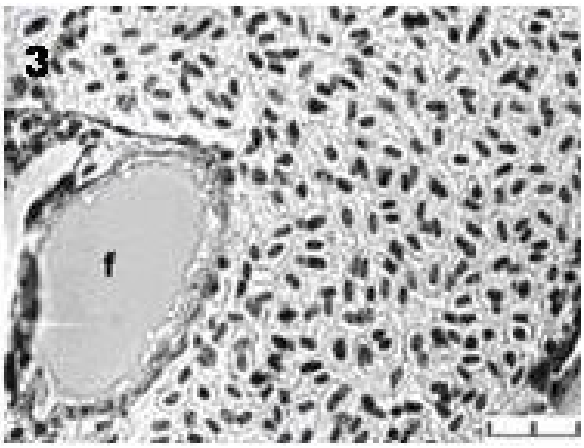

Figure 3. A large follicle (f) embedded in the vessel in TG-1; bar = 20µm.

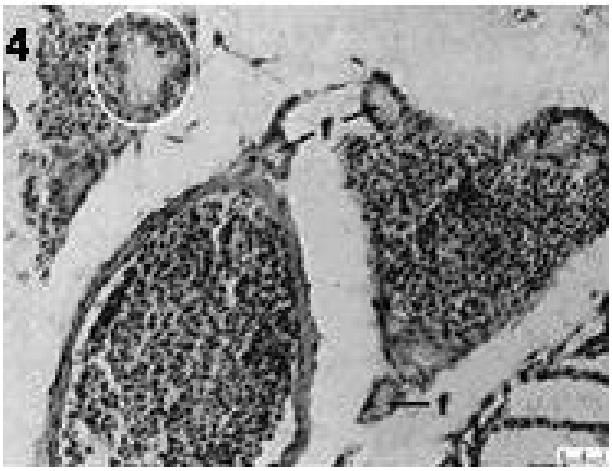

Figure 4. Enlarged vessels and irregulary shaped follicles (f) in TG-2. Note the embedding follicles (encircled); bar = 20µm.

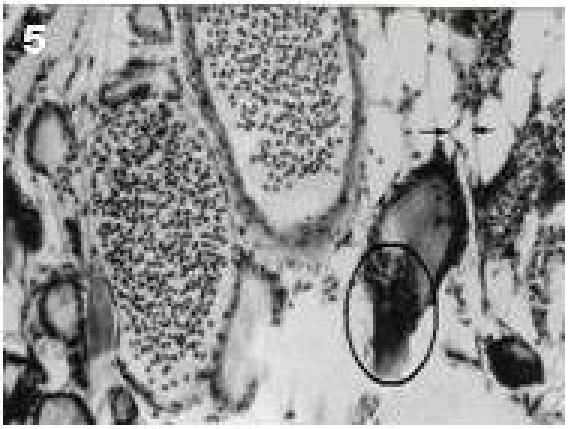

Figure 5. The anastomoses (arrows) between non-enlarged vessels nearby collapsing follicle (encircled) in TG-3; bar = 20µm.

irregular in shape. Similar to TG-1, some of the follicles seemed to be embedded in the wall of vessels.

Most of the follicles in TG-3 have some dramatic deformations characterized by degrading colloid and collapsed follicle layer (Figure 5). There is a relative decrease in the number of the vessels when compared with TG-1 and 2 (Table 1).

The non-enlarged vessels in TG-3 have some anastomoses (Figure 5). Although no follicles embedded in the wall of the vessels were observed, follicular hyperplasia is prominent (Figure 6).

DISCUSSION

Perchlorate acts on hypothalamic-pituitary-thyroidal axis

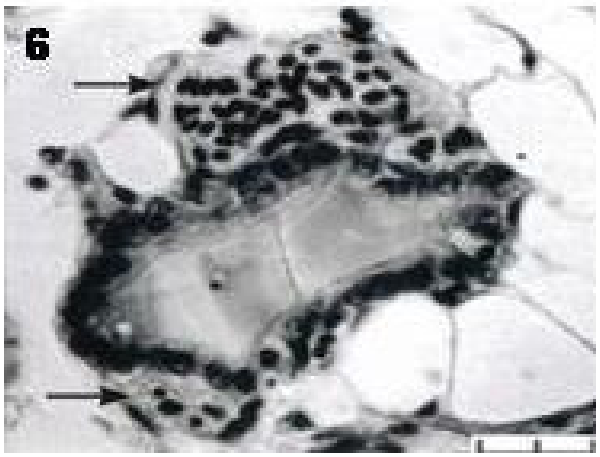

Figure 6. A hyperplastic follicle surrounded by vessels (arrows) in TG-3; bar = 20 µm.

via hypersecretion of TSH, which was found to induce hyperplasia and angiogenesis (Mitchell and Parangi, 2005). Although the inhibitory effects of perchlorate on thyroidal iodide uptake is a well documented process in mammals (Wolff, 1998; Ben Hamida et al., 2001; US EPA, 2002), there are only few reports regarding its effects on fish. The histopathological profile of the mode of action of perchlorate in teleosts could not fully compare with mammals due to the differences in thyroidal structure. In teleosts, thyroid follicles do not form a compact gland; on the contrary, they are widely dispersed around branchial arterioles. Furthermore, data on the biomarkers, which could be used mostly to detect the acute effects of perchlorate on adult fish, are limited (Liu et al., 2006; Mukhi and Patino, 2007). Among the biological end-points that can be used for detection of thyrotoxical effects of perchlorate, angiogenesis is suggested as a novel index (Mukhi and Patino, 2003; Mukhi et al., 2005).

Angiogenesis has been recognized as a complex process contributing to the pathophysiology of many benign and malignant diseases of thyroid (Mitchell and Parangi, 2005). As mentioned by same authors, adult vasculature is quiescent except for some complex circumstances such as ovulation, implantation, pregnancy and wound healing. Once the balance between stimulator and inhibitor factors are originated from the endothelial cells, surrounding matrix, stroma, and epithelial tissue are disturbed, and then angiogenesis would begin.

In classical terms, this multi-stage phenomenon involves formation of new blood vessels and capillary limb from formerly existing vascular bed (Rzeszutko et al., 2004). However, not only remodeling, branching and anastomosing, but also enlargement of the vessels via the proliferation and migration of endothelial cells are included in the context of angiogenesis. The latter type of angiogenesis was concluded as an effector for proliferation and metastasing potential of tumoral cells (Mitchell and Parangi, 2005).

Angiogenesis in perchlorate-exposed teleosts was first reported in *Salmo gairdneri* by Van Putten et al. (1983), as an increase in blood circulation around the follicles in 0.03% potassium perchlorate exposed immature rainbow trout. Hereafter, remarkably few studies have been performed on zebrafish in order to determine the effects of perchlorate by the context of angiogenesis. Mukhi and Patino (2003), Patino et al. (2003), Mukhi et al. (2005) and Liu et al. (2006) concluded the both types of angiogenesis in *D. rerio.*

Mukhi and Patino (2003) determined angiogenesis at two weeks in 0.1 - 10 ppm ammonium perchlorate exposed zebrafish. Patino et al. (2003) concluded that vascularization around the follicles in zebrafish, which was exposed to 677 ppm ammonium perchlorate appeared in fourth week. The authors also reported that in fishes kept in 18 ppm for eight weeks, angiogenesis was more prominent. Mukhi et al. (2005) noted that angiogenesis arose as a result of exposure of ammonium perchlorate in two weeks to 90 ppb concentration. More recently, Liu et al. (2006) reported a significant increase for angiogenesis only at 100 mg/L sodium perchlorate concentration, not at day 10, but at days 30, 60 and 90.

However, comparison of the time-course patterns of histopathological markers indicated an important difference: a prominent angiogenesis occurred at day 10 in current study, and it is early than previously noted. This is strongly due to the high concentrations used in our study. Indeed, the concentrations used in present study are extremely high and not within the range of values reported in surface and ground waters of highly contaminated sites in the United States (Urbansky, 1998). Someone could ask if it is logical to use environmentally irrelevant concentrations. The reason for this is to attract the attention of researchers to the study of contamination, which is never documented in our country.

Recently, Mukhi and Patino (2007) concluded that the inconsistent observations concerning the effects of perchlorate might be at least partly due to the differences in the concentrations of perchlorate used and, perhaps more importantly, the length of the treatment. At least for angiogenesis, it is clear that the determining parameter in the same exposure length is chemical concentration.

In addition to extremely high dose-relatively short time exposure used in current investigation, some other methodologic differences had been certainly noted. Mukhi et al. (2005) used male and female zebrafish while only males were used in this study. Besides, the different conclusions on time-course of the present study and the study of Liu et al. (2006) may be regarded as age-related as only adult specimen was used in this study, whereas Liu et al. (2006) exposed zebrafish at the juvenile stage and continued through the adult stage. While ammonium

perchlorate was used by different researchers such as Mukhi and Patino (2003), Patino et al. (2003) and Mukhi et al. (2005); we and Liu et al. (2006) preferred sodium salts. Probable discrepancy between the mode of action of ammonium and sodium salts of perchlorate must always be taken into consideration.

The sites of angiogenesis are also a controversial point. Consistent with Patino et al. (2003), we demonstrated the striking vascularization only in extrafollicular space. However, Mukhi et al. (2005) reported an increase of vessels penetrated to follicular epithelium. We did not observe this finding. On the contrary, some of the follicles at TG-1 and TG-2 were seen to be embedded in the wall of the vessels.

Counting of the micro-vessels per unit area by light microscopy is the routine method for determination of angiogenesis. Computer-assisted image analysis was also used for this purpose (Rzeszutko et al*.,* 2004). We did not use image analysis method and preferred using an index combining both the number and size of micro-vessels, in parallel with Liu et al. (2006). Our data indicate that significant and satisfactory evidences of angiogenesis are found in all test groups, most prominently in TG-1. Regarding both the increase in number (hyperplasia) and enlargement (hypertrophia) of vessels, the severity of angiogenic response is found to be slightly reduced in TG-2 and TG-3. Moreover, the statistical data concerning the number of grade 2 and 3 vessels in TG-2 and TG-3 varied endogenously in opposite ways, seeming some discordant at the first glance. In our opinion, these kinds of discrepancies should not be interpreted as signs of methodological inaccuracy, since they may due to the unknown adaptive processes as well as methodological differences. Newly forming and branching vessels which are observed in either TG-2 or TG-3 support this idea.

Finally it is noted that, not only remodeling, branching and anastomosing, but also enlargement of the vessels should be included in the context of angiogenesis. Our results and comments are in concordance with Mukhi et al. (2005), reporting that angiogenesis is a more sensitive indicator than hypertrophy, but disagree with Liu et al. (2006) who claimed that the most sensitive marker is hypertrophy in environmentally relevant doses.

As expected, histological observations support the statistical analyses. Angiogenesis accompanied with hyperplasia and hypertrophy of epithelial cells occurred significantly at TG-1. These findings became more prominent in TG-2 and then relatively reduced in TG-3. In parallel with this finding, number of grade 3 vessels reached its maximum at TG-2 and reduced at TG-3.

Although vascular supply for the follicles is evaluated as a parameter for homeostasis, it is interesting that no one had reported vascular wall embedded-like follicles observed mostly in TG-1 and rarely in TG-2 specimens of this study. We strongly believe that, this appearance is a dramatic indicator for urgent homeostatic response to the shocking effects of high-dose treatment. Surprisingly, such follicles are not demonstrated in TG-3 fishes. This unexpected finding may hypothetically be due to other, probably more complex homeostatic processes.

Conclusions

Our study strongly suggests that, angiogenesis may be the golden key in revealing not all, but at least acute effects of high doses of perchlorates on teleost thyroid, and evaluation of angiogenesis can be made by a simple, reproducible and reliable method based on solely histopathology. Further studies oriented to homeostatic mechanisms would certainly give rise to new approaches.

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