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Thyrotoxicity of Sodium Arsenate, Sodium Perchlorate, and Their Mixture in Zebrafish *Danio rerio*

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Both perchlorate and arsenate are environmental contaminants. Perchlorate is a definitive thyroid disruptor, and arsenic may disrupt thyroid homeostasis via multiple pathways. To evaluate the effects of sodium perchlorate and sodium arsenate on thyroid function and possible interactions between them, zebrafish (*Danio rerio*) were exposed to sodium perchlorate (10 and 100 mg/L), sodium arsenate (1 and 10 mg/L), and the mixture sodium perchlorate + sodium arsenate (10 + 1 and 100 + 10 mg/L) for up to 90 days. At day 10, 30, 60, and 90, fish were sampled and analyzed for thyroid histopathological end points including follicular cell height, follicle size, colloid size, colloid depletion, hyperplasia, and angiogenesis. Effects on epithelial cell height (hypertrophy) were seen as early as 10 days after exposure. Perchlorate induced changes in all parameters staring at 30 days of exposure. Prolonged perchlorate exposure induced angiogenesis, a relatively new marker of thyroid disruption. Sodium arsenate was less effective than sodium perchlorate in causing thyroid histopathologies, but transient responses were seen for hypertrophy, hyperplasia, and colloid depletion (% colloid). This is the first report of arsenate-induced effects on thyroid histopathology. However, because statistically significant effects were not consistently seen in all end points, evidence for arsenate as a thyroid disruptor remains equivocal. In general, the sensitivity of the following histopathological indicators for indicating thyroid perturbations is, in descending order: follicular cell height > percent of colloid area/follicle area > colloid area/follicular cell height > hyperplasia > angiogenesis > colloid area >follicle area = fish growth.

Introduction

Thyroid hormones (TH) have been implicated in a variety of critical fitness-related processes such as growth, reproduction, development, and mediation of other hormone activities and physiological functions (*1*). Environmentally induced thyroid dysfunction has been an increasing concern for some time. Indeed, a variety of organic and inorganic chemicals have been found to be thyroid disruptors (*2*). Evaluation of the effects and elucidation of the actions of mode by these chemicals contribute to mitigation of thyroid disruption and diseases by xenobiotic chemicals.

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Perchlorate is a well-known thyroid disruptor and a goitrogen, via inhibition of thyroidal iodine uptake (*3*). Perchlorate contamination is widespread in the United States and has been reported to occur in many states (*4*). Perchlorate concentrations up to 3700 and 480 mg/L have been detected in groundwaters and surface waters, respectively, in several U.S. states (*4*, *5*). Consequently, aquatic organisms may be at risk of perchlorate exposure.

Like perchlorate, arsenic is a widespread environmental contaminant as a consequence of both anthropogenic and natural processes. Arsenic is widely distributed in surface water, groundwater, and drinking water in the United States. Up to 276 and 48 mg/L arsenic have been reported in surface water and groundwater, respectively (*6*). Arsenic may exist in a variety of forms in aqueous environments, although arsenate is the dominant form in aerobic waters (*7*). As a result, aquatic organisms may be exposed to arsenate. To date, arsenic has not been unambiguously established as a thyroid disruptor, although it may alter plasma T_3 and T_4 concentration and deiodinase (a selenoenzyme) activity (*8*, *9*).

Perchlorate has been proven as a definitive thyroid disruptor, and arsenate has been found to alter thyroid hormone status and was hypothesized to be a thyroid disruptor (*3*, *8*, *10)*. Due to their disruption on thyroid hormone homeostasis, we hypothesized that an additive joint action existed between these two anions. These two chemicals have been documented as coexisting in aquatic ecosystems (*11*). Unfortunately, there has been little attention paid to interactions among such chemicals. In a previous study, we have found an additive interaction between these two anions indicated as 96-h LC_{50} (*12*).

To examine thyrotoxicity, various types of end points have been used in the past. Assays of thyroid hormones in the plasma or whole-body animal is probably most commonly used for examining thyrotoxicity of perchlorate (*2*, *13*−*18*). The second type of end point that has been used as an indicator of thyrotoxicity is histopathological end points, including follicle size, follicular cell vacuolization, colloid size, follicular cell height, colloid depletion, hypertrophy, hyperplasia, active follicle number per unit of area, and nuclear vesiculation (*15*−*17*, *19*−*21*). Angiogenesis (the formation of new blood vessels) in thyroid follicles is a relatively new index of thyroid disruption (*17*) and is induced by the thyroid stimulating hormone (TSH; *22*).

The aim of the current study is to evaluate the thyrotoxicity of perchlorate and arsenate and to determine the degree to which these two chemicals interact to produce thyrotoxicity, using male zebrafish as a model animal. The hypotheses were as follows: (i) both arsenate and perchlorate cause thyrotoxicity in zebrafish, which will be manifested by the end points mentioned above, in response to the exposure and (ii) there will be an additive interaction between arsenate and perchlorate in causing thyrotoxicity.

Experimental Section

Test Chemicals. Dibasic 7-hydrate sodium arsenate (SA, 99.0% purity) was purchased from J. T. Baker (Phillipsburg, NJ), and anhydrous sodium perchlorate (SP, 99% purity) was from EM Science (Gibbstown, NJ). Stock solutions were prepared by adding appropriate amount of chemicals to 18.3 MΩ Milli-Q water. The sodium perchlorate concentration was reported as $NaClO_4$ and that of sodium arsenate as Na_2HASO_4.

Animals. Juvenile male zebrafish (*Danio rerio*) were used to avoid the confounding factors of sex and ovulation cycle

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(about 2.5 months). Zebrafish were purchased from Ekkwill Waterlife Resources (Gibsonton, FL). Fish were allowed to acclimate to laboratory conditions for 2 weeks prior to the initiation of the experiment. Fish were fed formulated flake food (AquatoxFlake, Zeigler Bros. Corps., Gardners, PA) twice daily ad libitum. The food contained 9 mg/kg iodine (data supplied by Zeigler Bros. Corps., Gardners, PA). Fish were cultured and tested in reconstituted water, consisting of reverse osmosis water mixed with 60 mg·L^{-1} Instant Ocean artificial sea salts (Aquarium Systems, Inc., Mentor, OH). On the basis of this formulation and the amount of iodine in Instant Ocean (data supplied by Aquarium Systems, Inc.), the amount of iodine in the test solution was estimated to be approximately 0.4 μg/L of iodide anion. This is within the range of iodine concentrations in surface water (0.3−5 μg/L; *23*). Based on morphological characteristics of zebrafish, male fish (smaller in body size and slimmer relative to the female) were chosen for the experiment. Those male fish free of any deformities, disease, or lesions were used in the experiment. Average fish wet weight used in the experiment was 0.28 ± 0.03 g (mean ± SD).

Experimental Design and Fish Sampling. Fish were exposed to different concentrations of individual chemicals and the mixtures, including 0, 10, and 100 mg/L SP, 1 and 10 mg/L SA, 10 + 1 mg/L SP + SA, and 100 + 10 mg/L SP+SA. The experiments were conducted in 20-gal glass aquaria filled with 60 L of test solutions. Fish were randomly assigned to each aquarium. There were triplicates for each treatment. The test solutions consisted of reconstituted water with the desired amount of stock solution of either chemical. The water temperature in the aquaria was maintained at approximately 24 °C. During the experiments, the photoperiod was set at 14-h light/10-h dark.

During the exposure, one-third of the test solutions were changed twice per week, and the appropriate amount of water and stock solution was added to maintain the desired chemical concentrations. Prior to water changes, dissolved oxygen, salinity, specific conductivity, and temperature were measured with an YSI model 85 m (Yellow Springs Instrument Co., Yellow Springs, OH); the pH was measured using an Oakton pH meter (Gresham, OR); and un-ionized ammonia was determined with a Hach spectrophotometer model DR/2000 (Loveland, CO). Water samples were obtained before water changes for chemical analysis. Water samples for perchlorate analysis were stored at 4 °C and analyzed within 2 weeks. The sampling, storage, and analysis for arsenic in water samples were conducted following the U.S. EPA method 200.9 (*24*). Briefly, a 10-mL water sample was acidified with 30 μL of 35% nitric acid to reach a final pH of less than 2 and then stored at 4 °C until analysis.

The exposure lasted for 90 days. At day 10, 30, 60, and 90, three fish were sampled from each replicate (i.e., 9 fish for each treatment at each sampling time point). At the time of sampling, fish were removed from the aquaria, rinsed with deionized water, euthanized in MS-222 (0.5 g/L), weighed, and subjected to histological processing.

Analysis of Chemicals. Arsenic analyses were conducted using a Thermo model M series atomic absorption spectrometer (Thermo Electron Corporation, Cambridge, U.K.) equipped with a GF95 graphite furnace atomizer. The analysis of total recoverable arsenic in water samples, the quality control, and the arsenic detection limit in water samples followed U.S. EPA method 200.9 (*24*). The detection limit for arsenic in water was 0.65 μg/L. For perchlorate analyses, water samples were analyzed following a method similar to U.S. EPA method 314.0 (*25*) by using a Dionex DX-500 ion chromatography system equipped with a GP50 gradient pump and a CD20 conductivity detector (Dionex Corp., Sunnyvale, CA). A Dionex IonPac AS16 (250 mm × 4.0 mm) analytical column was used for ion separation. The detection limit of perchlorate in water was determined similarly as the method for arsenic, and it was 1.0 μg/L.

Thyroid Histological Processing. Fish heads were fixed in Bouin's fixative for 24 h and then decalcified using 5% trichloroacetic acid, washed in 70% ethanol, and embedded in paraffin. Serial 5-μm sections were prepared, mounted on slides, and stained with hematoxylin-eosin. Serial sections examined until 8−16 follicles were found. Care was taken to avoid one follicle from being chosen and counted repeatedly.

All histopathological end points were assayed using these follicles. Because an accurate estimation of follicle volume is hard to conduct, the area was used as a substitute for follicle size. To quantify follicle area, colloid area, follicular cell height, and angiogenesis, a photograph of each follicle was taken at the largest follicle diameter (determined by observing serial sections) with an Olympus digital camera (BX51; Tokyo, Japan). Follicle area, colloid area, and follicular cell height on the pictures were quantified using SimplePCI (version 4.01.1605, Compix Inc., Imaging system, 1996−2001). For determination of follicular cell height, five measurements along the follicle perimeter were made at regular intervals (i.e., 40−80 follicular cell heights for each fish and 360−720 for each treatment). Hypertrophy was evaluated by follicular cell height. Angiogenesis was evaluated by counting the blood vessels within the follicle basement membrane and between follicular cells. A grading system was applied for the blood vessel evaluation: small size blood vessels were assigned grade 1, medium size were grade 2, large size were grade 3, and very large size were grade 4. The sum of the number of blood vessels (per 10 follicles) multiplied by the corresponding grade was calculated.

The number of thyroid follicles with characteristics of hyperplasia were counted in these chosen follicles. In the current study, we found that focal hyperplasia (i.e., stratification of follicular cells) was primary hyperplasia and that the severity of the hyperplasia was similar across all treatments. Therefore, we did not use a hyperplasia grading system, which reports the severity of hyperplasia. Care was taken not to score tangential sections along the follicle wall, which appeared morphologically hyperplastic.

In addition to the end points above, the percent of the follicle area represented by colloid area was calculated ([colloid area/follicle area] × 100). Because the thyroid gland may still be experiencing growth in the juvenile stage and the zebrafish used in the current study were juvenile at the initiation of experiment, colloid area as a percent of follicle area may be a more appropriate end point than colloid or follicle area in evaluating thyrotoxicity.

Statistical Analyses. All statistical analysis was performed using SAS software (SAS, version 9.1, Cary, NC). Normality within groups was tested by Shapiro−Wilk test and homogeneity of variance across groups by Levene's test. If the data passed normality and homogeneity tests, the data analysis was conducted using ANOVA with Duncan's multiple comparison. If the data did not pass normality and/or homogeneity tests, a Kruskal−Wallis test was conducted with a Tukey's type nonparametric multiple comparison if the difference was significant. Nested ANOVA analyses followed by multiple comparison tests were used to test differences among: (i) treatments for each exposure interval or (ii) exposure intervals for the same treatment and chemical concentration. The significance level was set at 0.05.

Results and Discussion

Water Quality, Chemical Concentration, and Experimental Animals. The water characteristics (mean ± SD) were as follows: temperature, 23.4 ± 0.8 °C; dissolved oxygen, 7.2 ± 0.3 mg/L; pH, 6.8 ± 0.2; specific conductivity, 122.3 ± 11.7 to 224.7 ± 13.1 mS/cm; salinity, 60.0 ± 4.0 mg/L; DO, 7.2 ± 0.3; nonionized ammonia concentration, 3.9 ± 0.5 μg/L.

TABLE 1. Wet Weight (mean ± SE) of Zebrafish Exposed to Sodium Arsenate (mg/L), Sodium Perchlorate (mg/L), or the Mixture (mg/L) at Various Time Intervals[a]

	exposure concentration (mg/L) and fish wet weight (g; mean ± SE; $n = 9$)						
exposure period (day)	0	SP10	SP100	SA1	SA10	10 + 1	100 + 10
10	0.32 ± 0.01	0.28 ± 0.01	0.32 ± 0.01	0.29 ± 0.01	0.32 ± 0.01	0.31 ± 0.01	0.28 ± 0.01
30	0.32 ± 0.01	0.34 ± 0.01	0.35 ± 0.35	0.34 ± 0.01	0.33 ± 0.01	0.34 ± 0.01	0.32 ± 0.01
60	0.36 ± 0.01	0.39 ± 0.01	0.4 ± 0.01	0.40 ± 0.01	0.38 ± 0.01	0.36 ± 0.01	0.37 ± 0.01
90	0.42 ± 0.01	0.42 ± 0.01	0.41 ± 0.01	0.47 ± 0.02	0.39 ± 0.02	0.43 ± 0.01	0.39 ± 0.01

[a] SP, sodium perchlorate; SA, sodium arsenate; 10 + 1, SP + SA; 100 + 10, SP + SA.

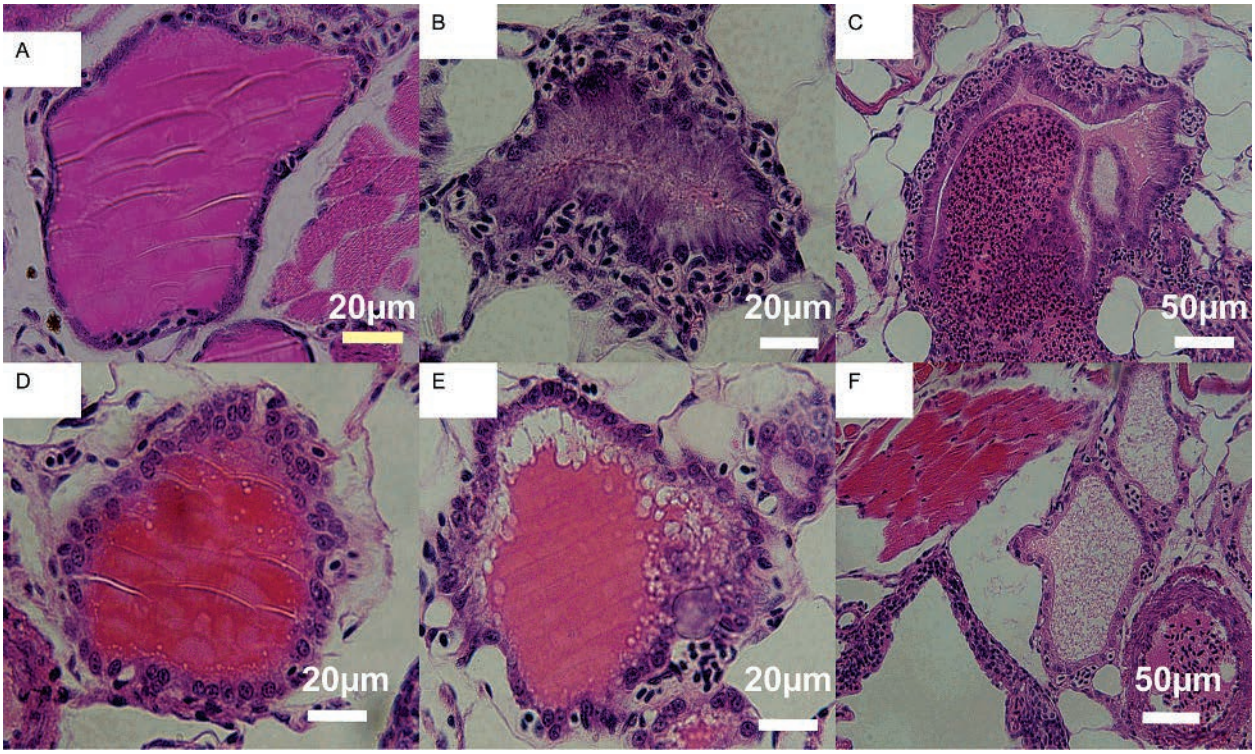

FIGURE 1. Representative photomicrographs of structure of thyroid follicles. (A) Control fish follicle with squamous to cuboidal follicular epithelium. (B) Significant hypertrophy with little colloid in the lumen in fish exposed to 100 mg/L sodium perchlorate. Angiogenesis was apparent (compare A and B). (C) Diffuse hyperplasia with hypertrophy in fish exposed to 10 + 1 mg/L mixture. The enlarged epithelial cells projected into the lumen of the follicles accompanying significant angiogenesis. (D) Focal hyperplasia with increased number of epithelial cells in fish exposed to 100 + 10 mg/L sodium perchlorate; (E) Colloid depletion indicated by the scalloped appearance at the edges of the colloid and reduced colloid area in fish exposed to 100 + 10 mg/L mixture. (F) Dispersed and reticular colloid (compare F to the other pictures) in fish exposed to 10 + 1 mg/L mixture. Bar = 20 μm.

Actual concentrations for sodium perchlorate and sodium arsenate were 103.4 ± 11.7% ($n = 48$) and 104.0 ± 11.6% ($n = 48$) of the nominal concentrations, respectively.

One or two fish from all chemical exposures showed intoxication symptoms indicated by partial loss of equilibrium and lack of feeding behavior. However, these fish eventually recovered to normal behavior. Mortality occurred in the control and the 10 + 1 mg/L treatment, and one fish died in either treatment. The fish wet weight was 0.27−0.33 at the initiation of experiment g and 0.38−0.48 g at the end of the experiment (see Table S1 in the Supporting Information).

Fish growth (as indicated by increase in wet weight) was not statistically significantly different between controls and any of the treatments ($p > 0.05$, ANOVA; Table 1). This is in agreement with some studies (*13*, *17*, *18*, *20*) but contrary to others (*15*, *19*, *26*, *27*) regarding the perchlorate effect. The lack of effect of arsenate on fish growth was in accordance with some studies (*10*, *28*). It seemed that chemical effect on fish growth depends on species, the stage of development, or exposure duration.

Thyroid Histopathology. The representative histopathological abnormalities were illustrated in Figure 1, including increased follicular cell (Figure 1B,C,E,F), angiogenesis (Figure 1B,C,D,E,F), focal hyperplasia (Figure 1E), and diffuse hyperplasia (Figure 1C). Focal hyperplasia was the primary hyperplasia occurrence in exposed fish. Colloid depletion was observed in the SP treatment (Figure 1C). A dispersed and reticular colloidal pattern in the lumen was also noted in a 10 + 1 mg/L treatment, which has not yet been reported in the literature (Figure 1F).

There was no statistically significant difference in thyroid follicle area among treatments at any exposure interval (Figure 2A). Thyroid follicle area increased over time for all treatments, which may be a result of general fish growth (Figure 2A). This may have been a confounding factor obfuscating any potential effects.

Severity of colloid depletion is a routinely employed marker for thyroid disruption. Colloid area was not significantly different among treatments at exposure days 10 and 30 (Figure 2B). There was a general decrease pattern of colloid

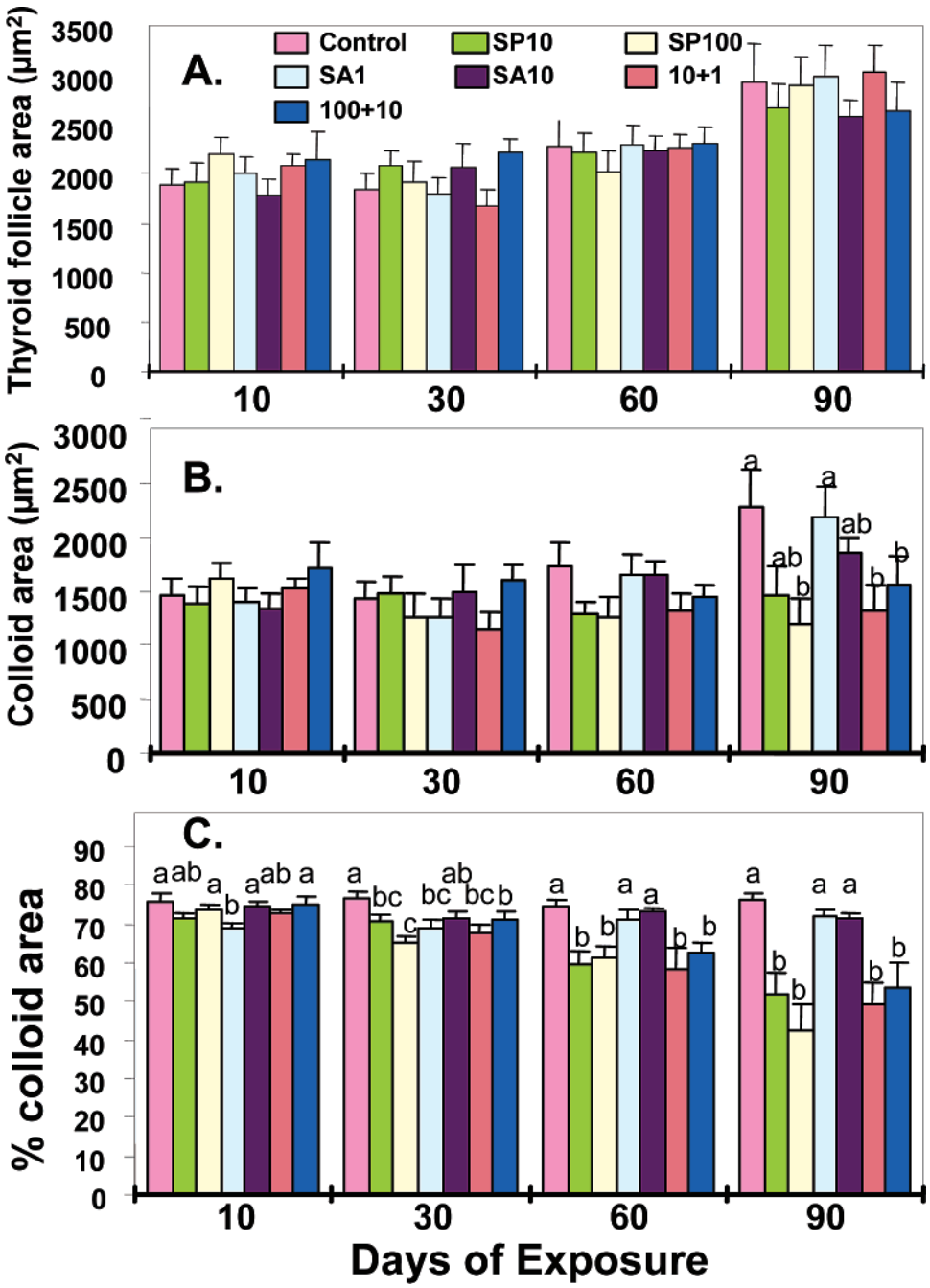

FIGURE 2. Thyroid follicle area (A), colloid area (B), or colloid area expressed as a percentage of follicle area (% colloid area; C) in zebrafish exposed to sodium arsenate (SA, 1 or 10 mg/L), sodium perchlorate (SP, 10 or 100 mg/L), or the mixture (SP + SA, 1 + 10 or 10 + 100 mg/L) at exposure day 10, 30, 60, and 90. (A) Values are mean ± SE, $n = 8-9$/treatment. Bars with different letters denote statistically significant differences ($p < 0.05$) among treatments within the same time interval.

area in chemical treatments, and this increase was not statistically significant until 90 days in the 100 mg/L SP and 10 + 1 mg/L treatments. That the decrease was significant in the 10 + 1 but not in 10 mg/L SP or 1 mg/L SA indicated that SP and SA had a joint action in reducing colloid area at the low concentration mixture (Figure 2B).

Significant colloid depletion was manifested only in 100 mg/L SP and the mixture treatments at exposure day 90. This is in contrast to previous studies with zebrafish (*17*, *20*) that found effects on the colloid size after 30 days. It appears that juvenile zebrafish are less sensitive to perchlorate-induced thyroid disruption (in terms of colloid depletion) than adult zebrafish. The different assay methods may also contribute to different conclusions.

In the current study, we also measured colloid area as a percentage of follicle area ("% colloid area", Figure 2C) in order to control for any effect of increase in colloid area over time due solely to growth of the fish. The % colloid area to total follicle area is displayed in Figure 2B. Sodium arsenate at 1 mg/L significantly decreased this ratio at day 10 and 30, but 10 mg/L SA did not have this effect (Figure 2C). Both SP and the mixtures significantly reduced the % colloid area at days 30−90.

Hypertrophy may be the most commonly used and most sensitive histopathological end point in evaluating thyroid disruption by thyroid-disrupting chemicals (*15*, *16*, *19*, *21*, *29*, *30*). The findings from the current study were in agreement with these previous studies. Epithelial cell height was sensitive

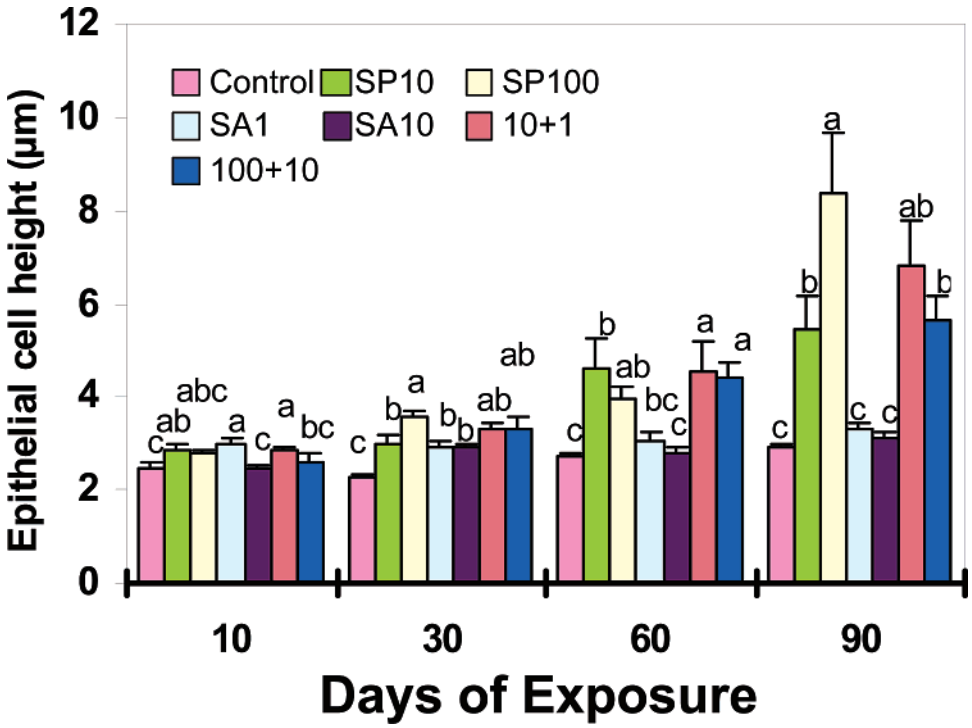

FIGURE 3. Thyroid follicle epithelial cell height area in zebrafish exposed to sodium arsenate (SA, 1 or 10 mg/L), sodium perchlorate (SP, 10 or 100 mg/L), or the mixture (SP + SA, 1 + 10 or 10 + 100 mg/L) at exposure day 10, 30, 60, and 90. Values are mean ± SE, $n = 8-9$/treatment. Bars with different letters denote statistically significant differences ($p < 0.05$) among treatments within the same time interval.

TABLE 2. Summary of Different End Points Statistically Significantly Different from Control as Markers for Thyroid Response to Sodium Perchlorate and Sodium Arsenate as Single Chemical or Mixtures in Zebrafish[a]

	exposure duration (days)			
end point	10	30	60	90
FA				
CA				SP, SP + SA
% colloid area	SA1	SA1, SP, SA + SP	SP, SP + SA	SP, SP + SA
height	SA1, SP10, and 10 + 1	SA, SP, SA + SP	SP, SP + SA	SP, SP + SA
angiogenesis		SP100	SP	SP, SP + SA
hyperplasia		SP100, 100 + 10	SA10, 100 + 10	SP100, 10 + 1

[a] SA includes both 1 and 10 mg/L sodium arsenate except for specifically mentioned (e.g., SA1 = 1 mg/L sodium arsenate). SP includes both 10 and 100 mg/L sodium perchlorate except for specifically mentioned (e.g., SP100 = 100 mg/L sodium perchlorate). SP + SA include 10 + 1 and 100 + 10 mg/L sodium perchlorate + sodium perchlorate except for specifically mentioned (e.g., 10 + 1 = 10 mg/L SP + 1 mg/L SA). Only those data showing significant difference from the control was summarized in the table ($p < 0.05$, ANOVA + Duncan's test). FA, follicle area (μm^2). CA, colloid area (μm^2). height, follicular cell height (μm).

to both SA and SP exposure (Figure 3). The increase of follicular cell height was observed at day 10 at low concentrations (SP10, SA1, and 10 + 1) but not at high concentrations (SP100, SA10, and 100 + 10). At day 30, all treatments showed significant increase ($p < 0.05$, ANOVA). A concentration-dependent increase in follicular cell height was observed in most SP treatments but not in the SA and mixture treatments. In the current study, thyroid responses to SA and SP displayed different patterns. SP caused hypertrophy across all exposure intervals whereas SA induced statistically significant hypertrophy only after the shorter exposures (days 10 and 30). The 1 mg/L SA treatment seemed to be more effective at inducing epithelial hypertrophy than 10 mg/L SA in short-time exposure (day 10) (Figure 3). Although there was a clear concentration-dependent increase in epithelial cell height for perchlorate, there was no such pattern for the mixture (Figure 3). This indicates that arsenate may affect the dose dependency of perchlorate-induced hypertrophy.

Another significant finding was that hypertrophy was induced as early as 10 days of exposure to sodium perchlorate (10 mg/L) and sodium arsenate (1 mg/L; Figure 3). This is in contrast to the findings of Mukhi et al. (*17*) and Patiño et al. (*20*) but in accordance with Bradford et al. (*16*), who found early thyrotoxic effects of perchlorate in mosquitofish (*Gambusia holbrooki*). Although the hypertrophic effects of perchlorate persisted and even increased throughout the exposure regime, the effects of 1 mg/L arsenate were only seen at days 10 and 30 (Figure 3).

In terms of angiogenesis, no significant effects were observed for any treatment at day 10 (Figure 4). At day 30, the 100 mg/L SP exposure caused a statistically significant increase of the angiogenesis. The significant increase was also observed in SP at day 60 and in both SP and the mixture treatments at day 90. In the current study, SA did not cause significant alteration of angiogenesis (Figure 4). Addition of SA seemed to mitigate the dose-dependent tendency for SP-induced angiogenesis (Figure 4), particularly in 10 mg/L SA at day 90.

An increased level of hyperplasia was observed at day 30 in the 100 mg/L SP and 100 + 10 mg/L SP + SA treatments and at day 60 in the 10 mg/L SA treatment. A 90-day exposure induced hyperplasia in the 100 mg/L SP and 10 + 1 mg/L mixture treatments whereas this did not occur in either the 10 mg/L SP treatment or the 1 mg/L SA treatment (Figure

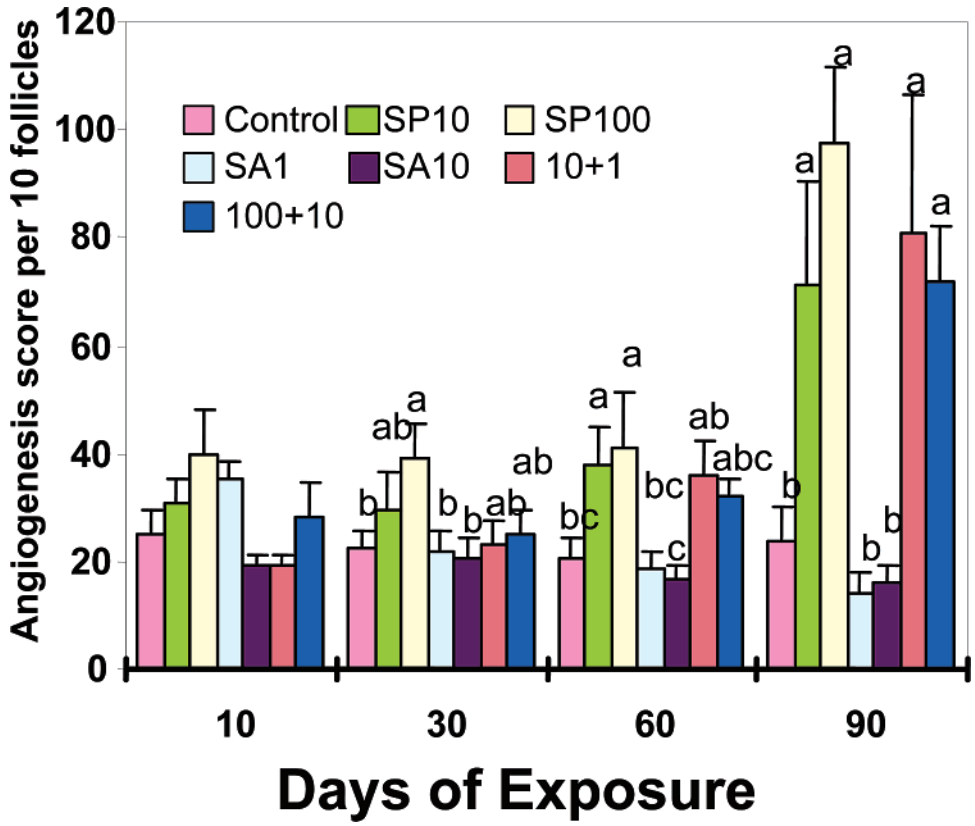

FIGURE 4. Thyroid follicle angiogenesis score in zebrafish exposed to sodium arsenate (SA, 1 or 10 mg/L), sodium perchlorate (SP, 10 or 100 mg/L), or the mixture (SP + SA, 1 + 10 or 10 + 100 mg/L) at exposure day 10, 30, 60, and 90. Angiogenesis score was evaluated by counting the blood vessels within the follicle basement membrane and between follicular cells. A grading system was applied for the blood vessel evaluation: small size blood vessels were assigned grade 1, medium size were grade 2, large size were grade 3, and very large size were grade 4. The sum of the number of blood vessels (per 10 follicles) multiplied by the corresponding grade was used to calculate the angiogenesis score. Values are mean ± SE, $n = 8-9$/treatment. Bars with different letters denote statistically significant differences ($p < 0.05$) among treatments within the same time interval.

5). This indicated an interaction between SA and SP at their low levels in inducing hyperplasia at this time point (Figure 5). Although the effect was statistically significant at the 0.05 level only for day 60, there was a tendency for an increased level of hyperplasia in both arsenate treatments at all time points.

When we compared the patterns among end points, some interesting patterns were found. When comparing the time-course patterns of hypertrophy (Figure 3) and hyperplasia (Figure 5), it can be seen that hyperplasia occurred later than hypertrophy and at higher concentrations of either chemical. This was in agreement with another study (*29*), suggesting that hypertrophy was an early response to thyroid hormone disruption while hyperplasia occurs mainly due to prolonged exposure. Alternatively, because the exposure started at the juvenile stage and continued through the adult stage, it could be that these are merely age-related differences in response to thyroid disruption. Angiogenesis may also accompany hypertrophy and/or hyperplasia (*17*). The angiogenic response (Figure 4) was generally parallel to the hyperplasia response (Figure 5, Table 2) but lagged behind the hypertrophy response when zebrafish were exposed to SP (Figure 3, Table 2). Our results are different from a previous study (*17*) that found angiogenesis was significantly induced in zebrafish thyroid as early as 2 weeks and at ammonium perchlorate concentrations as low as 90 μg/L, indicating that angiogenesis was a more sensitive indicator than hypertrophy (*17*).

The main differences between Mukhi et al. (*17*) and the present study are the zebrafish sex (we used male only and they used both male and female), perchlorate salts (we used sodium salt and they used ammonium salt), and quantification methods (we used a method considering both blood vessels size and number and Mukhi et al. (*17*) accounted for the number only). The different conclusions from the present study and Mukhi et al. (*17*) may be attributable to these factors.

The hypothesis that there is a thyrotoxic interaction between these two chemicals was not unequivocally shown. For some end points (e.g., hyperplasia) there are indications of an interaction between these two chemicals, as evidenced by the fact that there was no statistically significant effect at 10 mg/L perchlorate or 1 mg/L arsenate, but there was a significant effect in the 10 + 1 mg/L mixture (Figure 5). Statistically significant interactions between these two chemicals were not observed for all end points and at all exposure intervals. In addition, there were indications of a concentration-dependent response for perchlorate for most end points, especially for the 90-day exposures, but this pattern was mitigated by the addition of arsenate (Figures 2−5). This may be related to the finding that arsenate affects uptake and/or elimination of perchlorate in these same zebrafish (*31*). The weak interaction for some end points may be attributable to the fact that perchlorate is a much more effective than arsenate in affecting these end points and lower concentrations of arsenate were used compared to those of perchlorate.

In conclusion, the current study indicated that sodium perchlorate was more effective in inducing histopathological changes than sodium arsenate, with equivocal evidence for thyroid-disrupting ability of arsenate. In general, all end points except for the thyroid follicle area were influenced to

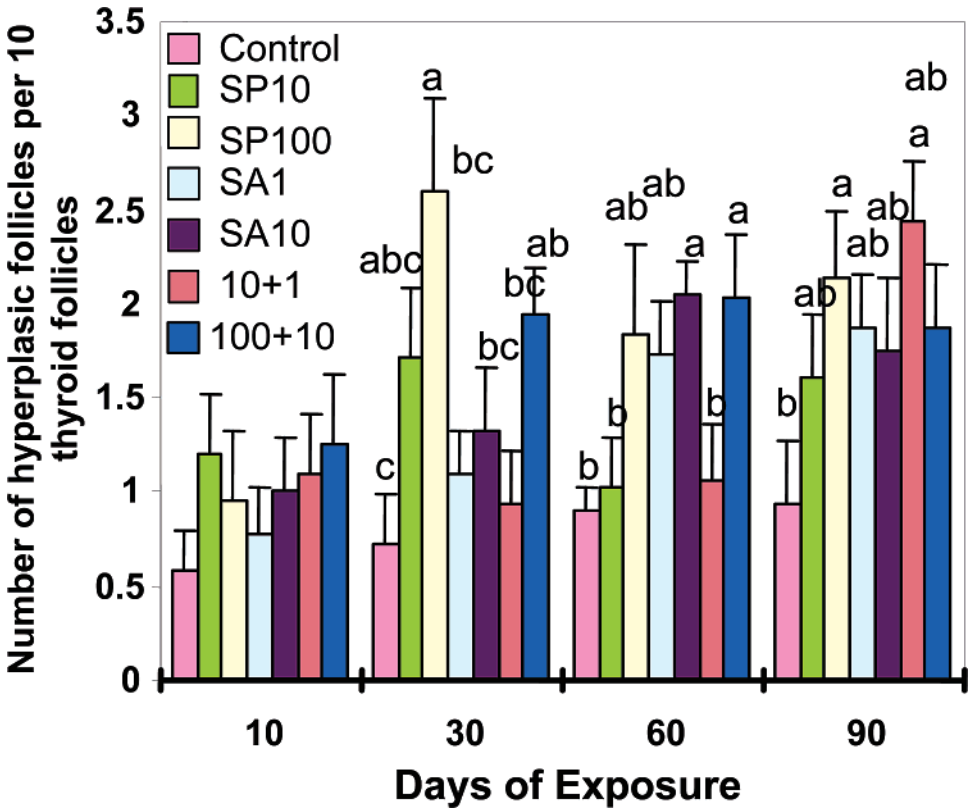

FIGURE 5. Percent hyperplasic follicles in zebrafish exposed to sodium arsenate (SA, 1 or 10 mg/L), sodium perchlorate (SP, 10 or 100 mg/L), or the mixture (SP + SA, 1 + 10 or 10 + 100 mg/L) at exposure day 10, 30, 60, and 90. Values are mean ± SE, $n = 8-9$/treatment. Bars with different letters denote statistically significant differences ($p < 0.05$) among treatments within the same time interval.

different extents at various time intervals. Zebrafish growth was not affected by prolonged exposure to either chemical or their mixtures at concentrations tested. There were thyrotoxic interactions between these two chemicals with respect to some end points (hyperplasia and colloid area). Among the thyroid histopathological end points applied in the current study, the most sensitive thyroid histopathological end point was hypertrophy (epithelial cell height). The significant induction was observed as early as day 10 at lower concentrations of both chemical alone and the corresponding mixture. Follicle area was not responsive and therefore inappropriate as a marker in revealing thyroid disruption by these two chemicals in zebrafish. This is the first report of arsenate-induced effects in thyroid histopathology (Table 2). However, because statistically significant effects were not consistently seen in all end points, evidence for arsenate as a thyroid disruptor remains equivocal. The sensitivity of the following histopathological indicators for indicating thyroid perturbations is, in descending order: follicular cell height > percent of colloid area/follicle area > colloid area/follicular cell height > hyperplasia > angiogenesis>colloid area > follicle area (Table 2). Because the concentrations used in the current study are environmentally relevant, epithelial cell height (hypertrophy) can be used as an indicator of possible perchlorate and/or arsenate exposure.

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Supporting Information Available

One figure and two tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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