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Low Level Arsenite Exposures Suppress the Development of Bone Marrow Erythroid Progenitors and Result in Anemia in Adult Male Mice

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Abstract

Epidemiological studies report an association between chronic arsenic (As) exposure and anemia in men, and women who are predisposed to anemia. The purpose of these studies was to determine whether a 60 d drinking water exposure of adult male C57BL/6J mice to 0, 100, and 500 ppb arsenite (As⁺³) results in anemia due to alterations in erythroid progenitor cell development in the bone marrow. Exposure to 500 ppb As⁺³ for 60 d resulted in a reduction of mean corpuscular hemoglobin (MCH) levels, but did not significantly alter red blood cell (RBC) counts, hemoglobin (Hgb) levels, mean corpuscular Hgb concentrations (MCHC), or mean corpuscular volumes (MCV). Attenuation of burst-forming unit-erythroid (BFU-E) colony formation was observed in bone marrow cells of mice exposed to 500 ppb As⁺³. The differentiation of late stage bone marrow erythroblasts were reduced with the 500 ppb As⁺³ exposure. Mice exposed to 500 ppb As⁺³ also had elevated serum levels of erythropoietin (EPO). Collectively, these results show that exposure to environmentally relevant levels of As⁺³ attenuates the development of early BFU-E cells and reduces the differentiation of later stage erythroblasts. This suppression of bone marrow erythropoiesis may be a contributing factor to the mild hypochromic anemia observed in 500 ppb As⁺³ exposed mice.

Keywords

arsenite; anemia; erythroid progenitor cells; erythropoiesis; bone marrow

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1. INTRODUCTION

As is a widespread environmental toxicant and common contaminant in food and drinking water (WHO 2011; Naujokas et al., 2013). Many people are chronically exposed to levels of As in their drinking water that exceed the World Health Organization and United States Environmental Protection Agency maximum contaminant level of 10 ppb (U.S. EPA 2012; WHO 2011). As occurs in the environment in organic and inorganic forms with multiple valence states (i.e. +3 or +5) that have differential toxicological profiles (Petrick et al., 2001; Styblo et al., 2000; Szyma ska-Chabowska et al., 2002). As⁺³ is commonly found in drinking water and is the most toxic inorganic form of As (Styblo et al., 2000; Naujokas et al., 2013). Exposure to elevated levels of As⁺³ has been documented to exert a multitude of detrimental health outcomes, including cancers, cardiovascular diseases, immunosuppression, and anemia (Hughes 2002; Heck et al., 2008; Naujokas et al., 2013; Ferrario et al., 2016).

Anemia is classified as a decrease in the number of RBCs and/or reduced Hgb levels in circulating RBCs (WHO 2015). Multiple epidemiological studies report an association between chronic As exposure and anemia (Heck et al., 2008; Surdu et al., 2015; Kile et al., 2016). Heck et al. (2008), found that low Hgb levels (<10 g/dL) were negatively associated with urinary As concentrations (>200 ppb) in men and women living in Bangladesh. Pregnant women exposed to elevated levels of As in their drinking water are particularly susceptible to developing anemia (Hopenhayn et al., 2006; Surdu et al., 2015). Findings from these studies emphasize the need to develop a clear understanding of the relationship between environmentally relevant As⁺³ exposures and anemia.

The bone marrow is very sensitive to As-induced toxicity (Szyma ska-Chabowska et al., 2002; Ezech et al., 2014; Ezech et al., 2016; Xu et al., 2016) and is the major site of erythropoiesis in adult humans and mice (Tsiftoglou et al., 2009; Dzierzak and Philipsen, 2013). Erythropoiesis is regulated by EPO released from the kidney in response to hypoxic conditions in the body (Hattangadi et al., 2011). Increased EPO levels stimulate the proliferation and differentiation of early erythroid progenitor cells in the bone marrow (Hattangadi et al., 2011). The first stage of erythroblast differentiation is BFU-E, which respond to increased EPO and other growth factors (e.g., SCF, IL-3, IL-6) to proliferate and mature to the highly EPO responsive colony-forming unit-erythroid (CFU-E) stage (Hattangadi et al., 2011). At the CFU-E stage, Hgb production is initiated, and the cells undergo four additional stages of differentiation (i.e. proerythroblast, basophilic, polychromatophilic, and orthochromatophilic), prior to enucleation and release from bone marrow into the circulation (Migliaccio, 2010; Elliott and Sinclair, 2012).

The purpose of this study was to determine whether a 60 d drinking water exposure of adult male C57BL/6J mice to environmentally relevant levels of As⁺³ (0, 100, and 500 ppb) results in anemia. As a potential target for As⁺³-induced toxicity with relevance to anemia, we evaluated the colony forming ability and differentiation of early erythroid progenitor cells in the bone marrow.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents.

Sodium meta9arsenite (CAS 774-46-5, Cat. No. S7400), Dulbecco's phosphate buffered saline w/o Ca^{+2} or Mg^{+2} (DPBS⁻), and Isocove's Modified Dulbecco's Medium were purchased from Sigma-Aldrich (St. Louis, MO). Hanks Balanced Salt Solution (HBSS) was purchased from Lonza (Walkersville, MD). Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA). Penicillin/Streptomycin 10,000 (mg/ml)/10,000 (U/ml) and 200 mM L-Glutamine was purchased from Life Technologies (Grand Island, NY). Serum-free methylcellulose-based medium containing EPO for culture of mouse erythroid cells (Cat. No. SF M3436) was purchased from STEMCELL Technologies (Cambridge, MA). FITC rat anti-mouse Ter119 clone Ter119 (Cat. No. 557915) and PE rat anti-mouse CD71 clone C2 (Cat. No. 553267) antibodies were purchased from BD Biosciences (San Jose, CA). Cellometer acridine orange/propidium iodide (AO/PI) staining solution in PBS (Cat. No. CS2-0106-5ML) was purchased from Nexcelom Bioscience (Manchester, UK). Mouse EPO Quantikine[®] ELISA kit (Cat. No. MEP00B) was purchased from R&D Systems (Minneapolis, MN).

2.2. Mouse drinking water exposures.

All experiments were performed in accordance with protocols approved by the Institutional Animal Use and Care Committee at the University of New Mexico Health Sciences Center. Male C57BL/6J mice were purchased at 8 weeks of age from Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate in our animal facility for one week prior to the onset of experiments. Mice were maintained on a 12:12 reverse light:dark cycle and were fed 2020X Teklad global soy protein-free rodent diet (Envigo, Indianapolis, IN) throughout the experiment. Mice were housed 2–3 individuals per cage and exposed to 0 (control), 100, or 500 ppb As^{+3} in their drinking water for 60 d ($n = 5$ mice/group). As^{+3} doses were prepared fresh weekly by weighing each water bag and determining the appropriate volume of stock As^{+3} to add into each bag to yield 100 or 500 ppb As^{+3} . Water bags were collected and weighed at the end of each week and the change in weight was used to estimate water consumption by mice in each cage.

2.3. Primary bone marrow cell isolation.

Bone marrow cells were isolated as previously described (Ezeh et al., 2014). Both femurs from each mouse were harvested and placed into cold HBSS. Femurs were then transferred into a 60 mm dish with HBSS to trim excess tissue from the bone. Trimmed femurs were placed into a 60 mm dish containing 5 mL cold colony-forming unit (CFU) medium (Isocove's Modified Dulbecco's Medium supplemented with 2% heat inactivated FBS, 20 mM L-glutamine, and 100 mg/ml streptomycin and 100 units/ml penicillin) and the ends of each femur were carefully cut to reveal the interior marrow shaft. Bone marrow cells were flushed from each femur by passing approximately 6–9 mL of CFU medium through the marrow shaft using a 1cc syringe and 25-G needle. The cell suspensions were then transferred to a 15 mL centrifuge tube, centrifuged at 200 $\times g$ for 10 mins, and resuspended in 5 mL of CFU medium. Cell viabilities and concentrations were determined using AO/PI staining and a Nexcelom Cellometer[®] Auto 2000 (Nexcelom Bioscience, Manchester, UK).

2.4. Blood collection, serum preparation, and hematological analysis.

Whole blood and blood for serum preparation was collected at the time of sacrifice by cardiac puncture into EDTA coated 250 μ L tubes or 1.5 mL microcentrifuge tubes, respectively. Hematological analysis of whole blood was performed using an Abaxis VetScan HM5 hematology analyzer (Abaxis, Union City, CA). For serum preparation, blood was clotted for 2 h at room temperature (RT). Clotted blood was centrifuged at 2000 $\times g$ for 30 mins and serum was carefully removed and transferred to a clean 1.5 mL microcentrifuge tube for storage at -80°C .

2.5. BFU-E assay.

Mouse BFU-E assays were setup following manufacturer's instructions described in version 3.4.0 of STEMCELL Technologies Technical Manual for Mouse Colony- Forming Unit Assays using MethoCultTM. Bone marrow cells from both femurs of each mouse were pooled and resuspended to 1×10^6 cells/mL in CFU medium. 400 μ L of the 1×10^6 cells/mL solution (4×10^5 cells) was transferred into 4 mL SF M3436 methylcellulose-based medium containing EPO to promote BFU-E development and expansion. Samples were then mixed thoroughly by vortexing and held for approximately 10 mins to allow bubbles to dissipate. 1 mL (1×10^5 cells) of each sample solution was then transferred in triplicate to treated 35 mm culture dishes (STEMCELL Technologies, Cambridge, MA) using a 5cc syringe and a 16-G blunt-end needle. Each plate was then gently rocked back and forth to evenly distribute the media across the surface of the dish. Two culture dishes and one uncovered 35 mm dish containing 3 mL of sterile water were placed into a covered 100 mm dish and incubated at 37°C in humidified incubator with 5% CO_2 for 14 d. After 14 d of culture, BFU-E colonies containing at least 30 cells were counted based on morphology using a dissecting microscope. Colony counts are reported as the number of BFU9E colonies per million bone marrow cells.

2.6. Flow cytometry.

Bone marrow erythroblast subsets were evaluated based on CD71 and Ter119 surface marker expression. 1×10^6 bone marrow cells from each mouse were transferred to 12 $\times$ 75 mm tubes and stained in 100 μ L of flow stain/wash buffer (DPBS⁻ with 2% heat inactivated FBS and 0.09% sodium azide) with 0.5 μ g of rat anti-mouse CD71-PE and rat anti- mouse Ter119-FITC monoclonal antibodies at RT for 30 mins. Samples were then washed twice with flow stain/wash buffer. After the final wash, samples were resuspended in 0.5 mL flow stain/wash buffer and analyzed using an AccuriTM C6 flow cytometer (BD Biosciences, San Jose, CA).

2.7. Mouse EPO ELISA.

Serum EPO levels were measured using the Mouse EPO Quantikine[®] ELISA kit according to manufacturer's instructions. Briefly, serum samples and EPO standards were diluted two-fold and 50 μ L of each sample was added in duplicate to the appropriate wells of a microplate pre-coated with an EPO specific monoclonal antibody. The plate was then covered and incubated at RT for 2 h on a microplate shaker. Following incubation, the plate was washed five times. After the last wash, the plate was emptied and 100 μ L of mouse EPO

monoclonal antibody conjugated to horse radish peroxidase was added to each well and incubated at RT for 2 h on a microplate shaker. The plate was washed again and 100 μ L of substrate solution (hydrogen peroxide and tetramethylbenzidine) was added to each well and incubated at RT for 30 mins. Colorimetric reactions were stopped by adding 100 μ L of 0.25 N hydrochloric acid to each well and the absorbance was read immediately at 450 nm and 540 nm using a SpectraMax® 340PC microplate reader (Molecular Devices, Sunnyvale, CA). Readings at 540 nm were subtracted from those at 450 nm to correct for optical imperfections in the plate and the corrected values were used for subsequent analysis. Sample concentrations were determined using a four-parameter logistic standard curve.

2.8. Statistics.

Data was analyzed using Sigma Plot 12.5 software. Five mice ($n = 5$) were assigned to each exposure group and unless otherwise specified in figure legends were utilized in statistical analysis. Differences between control and As^{+3} exposure groups were determined using a Student's t -test at a significance level of $p < 0.05$.

3. RESULTS

3.1. Hematological effects of drinking water exposure in adult mice.

Adult male C57BL/6J mice were exposed to 0, 100, and 500 ppb As^{+3} via their drinking water for 60 d. There were no differences in body weights in any of the As^{+3} exposure groups. A slight decrease in water consumption in the 100 ppb As^{+3} exposure group was observed, but this effect was not dose- dependent (Table 1). To determine whether As^{+3} exposure results in the development of anemia, RBC counts, Hgb levels, MCHCs, MCH levels, and MCVs were measured in whole blood using an Abaxis VetScan hematology analyzer. Mice exposed to 500 ppb As^{+3} had significantly lower MCH levels than control mice (Table 2). Although not statistically significant, a trend of decrease in MCHCs and Hgb levels was also noted in mice exposed to 500 ppb As^{+3} (Table 2). There were no changes in RBC counts or MCVs with either of the As^{+3} doses (Table 2). These results imply that exposure to 500 ppb As^{+3} for 60 d resulted in a moderate Hgb reduction consistent with hypochromic anemia.

3.2. Bone marrow BFU-E colony formation was attenuated by As^{+3} exposure.

To determine whether alterations in erythroid progenitor cell development is a target of As^{+3} toxicity, we evaluated the colony-forming ability of BFU-E cells from the bone marrow of mice exposed to 0, 100, and 500 ppb As^{+3} for 60 d. Bone marrow cells were cultured *ex vivo* in serum free methylcellulose-based medium containing EPO for 14 days and the number of BFU-E colonies was determined based on morphology using a dissecting microscope. A significant reduction in the number of BFU-E colonies was observed in mice exposed to 500 ppb As^{+3} (Figure 1A). Another interesting observation was BFU-E colonies from the bone marrow of 500 ppb As^{+3} exposed mice were not as dense as control colonies, which is indicative of suppressed proliferation, delayed differentiation, and/or reduced cell viability (Figure 1B). There were no statistically significant changes in BFU-E colony formation with the 100 ppb As^{+3} exposure (Figure 1A).

3.3. Suppressed differentiation of late stage erythroid progenitors in the bone marrow of mice exposed to As⁺³.

Based on the finding that As⁺³ attenuates BFU-E development, we investigated whether the downstream bone marrow erythroblast population subsets were also altered. Stages of erythroblast differentiation can be defined based on CD71 and Ter119 surface marker expression using flow cytometry (Socolovsky et al., 2001; Lau et al., 2012). Erythroblast subsets in bone marrow were defined as follows: (I) proerythroblast: CD71^{high}/Ter119^{med}, (II) basophilic erythroblast: CD71^{high}/Ter119^{high}, (III) = late basophilic and polychromatophilic erythroblasts CD71^{med}/Ter119^{high}, and (IV) = orthochromatophilic erythroblasts CD71^{low/9}/Ter119^{high} (Figure 2A). As⁺³ exposure at 500 ppb caused a significant decrease in the percentage of late stage orthochromatophilic erythroblasts (Figure 2B). No differences in other erythroblast subsets (I-III) were detected for either of the As⁺³ exposures (Figure 2B). These results indicate that As⁺³ also alters the differentiation of late stage erythroid progenitors in the bone marrow.

3.4. Serum EPO levels were elevated in As⁺³ exposed mice.

To evaluate whether disrupted erythropoiesis in the bone marrow (i.e. attenuated BFU-E colony-formation and suppressed differentiation of later stage erythroblasts) was accompanied by an increase in circulating EPO levels in As⁺³ exposed mice, the concentration of EPO in serum was analyzed using a mouse EPO ELISA kit. A significant increase in EPO concentrations was detected in serum from 500 ppb As⁺³ exposed mice (Figure 3). There was no difference in serum EPO levels in 100 ppb As⁺³ exposed mice (Figure 3). These findings suggest that suppressed erythropoiesis in the bone marrow of 500 ppb As⁺³ exposed mice stimulated EPO release from the kidneys.

4. DISCUSSION

In the present study, we evaluated whether a 60 d drinking water exposure of adult male C57BL/6J mice to environmentally relevant levels of As⁺³ (0, 100, and 500 ppb) would result in anemia. Mice exposed to 500 ppb As⁺³ had significantly lower MCH levels and also showed a non-statistically significant trend of decrease in MCHCs and Hgb levels (Table 2). RBC counts and MCVs were not altered with either of the As⁺³ exposures (Table 2). This is consistent with previous findings that As reduces heme metabolism and can bind to Hgb, which results in decreased Hgb concentrations in RBCs (Delnomdedieu et al., 1994; Hernández-Zavala et al., 1999; Lu et al., 2004). Acute high level As⁺³ exposure in humans and rodents has been reported to result in anemia as a result of bone marrow depression (Szyma ska-Chabowska et al., 2002). Morse et al. (1980), showed an inhibition of iron incorporation in circulating RBCs along with suppressed proliferation of early erythrocytes from the bone marrow of mice injected with high levels of As⁺³. These studies provide evidence that acute high level As exposures can cause anemia in mice.

The bone marrow is the major site of erythropoiesis in humans and mice (Tsiftoglou et al., 2009; Dzierzak and Philipsen, 2013). Several studies have shown that hematopoietic progenitor cells in the bone marrow are sensitive to low levels of As⁺³ *in vivo* and *in vitro* (Ferrario et al., 2008; Ezech et al., 2014; Ezech et al., 2016). As a potential target for As⁺³-

induced toxicity, we evaluated the development of BFU-E cells in the bone marrow. BFU-E cells are the earliest stage of erythroid cell development and they can be readily assessed based on colony formation in methylcellulose-based medium. BFU-E colony formation was only attenuated in bone marrow cells from 500 ppb As⁺³ exposed mice (Figure 1). BFU-E colonies from 500 ppb As⁺³ exposed mice were also less dense than control colonies, indicating compromised development of the cells likely as result of decreases in cell viability, proliferation, and/or differentiation capacity (Figure 1B).

To assess whether As⁺³ altered downstream erythroblast differentiation, we evaluated erythroblast subsets in bone marrow based on CD71 and Ter119 surface marker expression. The stages of erythroblast differentiation were identified based on the degree of CD71 and Ter119 surface marker expression using flow cytometry (Figure 2A). Interestingly, only the latest stage of erythroblast differentiation (i.e. stage IV, orthochromatophilic erythroblasts) was reduced in 500 ppb As⁺³ mice (Figure 2B). This provides evidence that As⁺³ not only attenuates the development of early BFU-E cells, but it also reduces the differentiation of later stage erythroblasts. Taken together, these results show that different stages of erythroid cell development may have differential sensitivities to As⁺³.

Our group previously reported that mice exposed to 500 ppb As⁺³ in their drinking water for 30 d had elevated As levels in the bone marrow (Xu et al., 2016). It has also been shown that As⁺³ and MMA⁺³ can inhibit the development of pre-B cells in the bone marrow by reducing STAT5 activation (Ezeh et al., 2016). Since the maturation of erythroid progenitor cells is also dependent on STAT5 signaling (Socolovsky et al., 1999; 2001), it is likely that the attenuation of BFU-E colony formation may be, at least in part, attributed to interference with this signaling pathway. As⁺³ has also been shown to inhibit the function of C3 and C4 zinc finger proteins by replacing zinc on the zinc fingers (Zhou et al., 2011; Zhou et al., 2014). Many of the transcription factors that regulate the expression of genes critical for development of early erythroid progenitors are zinc fingers with motifs that can be displaced by As⁺³ (Hattangadi et al., 2011). It is therefore possible that As⁺³ may be suppressing the development of early erythroid progenitors by inhibiting the function of these essential zinc finger transcription factors.

Mice exposed to 500 ppb As⁺³ also exhibited increased levels of EPO in their serum (Figure 3). Increased circulating EPO levels is likely a physiological attempt to cope with the loss of erythropoietic function in the bone marrow. In mice the spleen plays a critical role in maintaining erythropoiesis during stressful conditions or if the bone marrow is not capable of producing adequate amounts of RBCs to meet physiological demands (Dzierzak and Philipsen, 2013). Compensation from the spleen for the loss of erythropoietic capacity in the bone marrow is likely responsible for the stable RBC counts observed with the 60 d As⁺³ exposure. Taking findings from epidemiological studies into consideration, it is possible that a longer exposure duration, such as that experienced by chronically exposed human populations, may result in reduced RBC counts and further diminished Hgb levels.

Results from this study show that 60 d drinking water exposure to environmentally relevant levels of As⁺³ in adult male mice results in a moderate Hgb reduction that is consistent with hypochromic anemia. In addition, it was found that 500 ppb As⁺³ disrupts erythropoiesis in

the bone marrow by attenuating the colony-forming ability of early BFU-E cells and reducing the differentiation of later stage erythroblasts. Collectively, these findings provide initial insights into a possible means by which As⁺³ exposures can contribute to the development of anemia in chronically exposed individuals.

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HIGHLIGHTS

- Exposure to 500 ppb As⁺³ reduced MCH levels in adult male mice.
- BFU-E colony-formation was attenuated by exposure to 500 ppb As⁺³.
- Male mice exposed to 500 ppb As⁺³ had elevated serum levels of EPO.
- Erythropoiesis was impaired in 500 ppb As⁺³ exposed male mice.

Figure 1.

BFU-E colony formation in bone marrow of male C57BL/6J mice exposed to 0, 100, and 500 ppb As³⁺ for 60 d. A. Number of BFU-E colonies per million bone marrow cells cultured *ex vivo* in serum free methylcellulose-based medium containing EPO for 14 days.

B. Representative BFU-E colony images from 0, 100, and 500 ppb As³⁺ exposed mice. Data are expressed as mean $\pm$ SD of triplicate cultures per mouse ($n = 4-5$ mice/group).

*Statistically significant difference compared to control ($p < 0.05$).

Figure 2.

Erythroblast subsets in bone marrow of male C57BL/6J mice exposed to 0, 100, and 500 ppb As³⁺ in drinking water for 60 d. Bone marrow cells were stained with CD71 and Ter119 surface markers and analyzed on a flow cytometer. A. Flow cytometry gating strategy used to define erythroblast subsets (I-IV) in mouse bone marrow cells. B. % erythroblast subsets defined based on CD71 and Ter119 surface marker expression. Data are expressed as mean $\pm$ SD ($n = 5$ mice/group). *Statistically significant difference compared to control ($p < 0.05$).

Figure 3.

EPO concentrations (pg/mL) in serum from male C57BL/6J mice exposed to 0, 100, and 500 ppb As⁺³ via drinking water for 60 d. Serum EPO concentrations were determined using a mouse EPO ELISA (R&D Systems). Red line indicates the mean and black line indicates the median ($n = 4-5$ mice/group). *Statistically significant difference compared to control ($p < 0.05$).

Table 1

Body weights and drinking water consumption of male C57BL/6J mice exposed via drinking water to 0, 100, and 500 ppb As⁺³ for 60 d^a.

Treatment	Body Weight (g)	Water Intake (mL/day)
Control	34.42 ± 2.36	3.66 ± 0.29
100 ppb	32.76 ± 3.41	3.10 ± 0.13 *
500 ppb	33.30 ± 1.36	3.77 ± 0.57

^aMice were 9-week old at the start of the As⁺³ exposure. Water consumption was monitored weekly based on weight change of the water bags. Data are expressed as mean ± SD (*n* = 5 mice/group).

* Statistically significant difference compared to control (*p* < 0.05).

Table 2

RBC counts, Hgb levels, MCH levels, MCHCs, and MCVs in whole blood collected from mice exposed via drinking water to 0, 100, and 500 ppb As⁺³ for 60 d^a.

Parameter	Control	100 ppb As ⁺³	500 ppb As ⁺³
RBC (x10 ¹² /L)	10.85 ± 0.35	11.11 ± 0.27	10.99 ± 0.23
Hgb (g/dL)	17.16 ± 0.52	17.26 ± 0.18	16.76 ± 0.38
MCH (pg)	15.80 ± 0.16	15.56 ± 0.27	15.24 ± 0.46 [*]
MCHC (g/dL)	35.92 ± 0.36	35.26 ± 0.63	34.94 ± 1.20
MCV (fL)	43.80 ± 0.45	44.20 ± 0.45	43.80 ± 0.45

^aHematology analysis was performed using an Abaxis VetScan hematology analyzer. Data are expressed as mean ± SD (*n* = 5 mice/group).

^{*}Statistically significant difference compared to control (*p* < 0.05).