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RESEARCH REPORT

Effects of fluoride on primary cultured haemocytes from the marine gastropod *Haliotis tuberculata*

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Abstract

As a consequence of human's activities, fluoride concentration in many aquatic ecosystems is significantly increasing. Nevertheless, little is known about fluoride toxicity to aquatic life. In this study the effect of exposure to different concentrations of sodium fluoride (2, 10, 50, 250 and 1,250 µg mL^{-1}) during 24 h on primary cultured haemocytes of the gastropod *Haliotis tuberculata* was realized. Results indicate no significant effect of NaF on cell viability, Lysosomal membrane stability, phagocytosis and ROS production at concentrations of 2, 10, 50 and 250 µg mL^{-1}. Nevertheless, lysosomal membrane alterations, a decrease of phagocytosis and morphological changes of *H. tuberculata* haemocytes were observed at concentration of 1,250 µg mL^{-1} NaF suggesting a potential impact of NaF at high concentration in the environment.

Key Words: Fluoride; Haemocytes; *Haliotis tuberculata*; Immune parameters; *In vitro*; Primary culture

Introduction

High exposure to fluoride may occur through a combination of natural and anthropogenic process as well as misuse of fluoride-containing dental care products (Borke and Whitford, 1999). The most obvious early toxic effects of fluoride on humans are dental and skeletal fluorosis (Barbier *et al*., 2010; Ullah *et al., 2017*). In mammalian cells, Agalakova and Gusev (2012a) showed that fluoride is an important modulator of the expression of genes implicated in apoptosis, amino acid phosphorylation, oxidative stress, cell cycle progression, chemotaxis, glycolysis, inflammation and signal transduction. Furthermore, fluoride acts as an inhibitor of the activity of a broad range of enzymes (Reddy *et al*., 2009; Barbier *et al*., 2010; Zuo *et al.,* 2018). In unpolluted seawater, fluoride concentrations generally range from 1.2 to 1.5 mg L^{-1}. However and as a consequence of human activities, these levels can increase more than 100 times (Camargo, 2003). Aquatic animals such as fish and invertebrates can take up fluoride directly from the water or via food(Hemens and Warwick, 1972; Nell and Livanos, 1988; Mondal and Nath, 2015) and the toxicity of this element was reported in the freshwater mussels like *Alasmidonta raveneliana* (Keller and Augspurger, 2005) and *Dreissena polymorpha* (Del Piero *et al*., 2012). In mollusks, the cellular immune system is represented by haemocytes due to their ability to interact with foreign materials and to develop immune responses (Galloway and Depledge, 2001; Hooper *et al*., 2007). A loss of haemocyte functionality due to pollutants like fluoride can be deleterious for the animal survival. Few data are available regarding the effects of fluoride on haemocyte parameters of mollusks and marine invertebrates in general. Thus, the aim of this work was to determine the *in vitro* effects of sodium fluoride on primary cultured haemocyte of the European abalone *Haliotis tuberculata*.

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Materials and methods

Specimens

Haliotis tuberculata were collected by France Haliotis (Plouguerneau, France), maintained in aerated seawater at 17 °C and fed *ad libitum* with a mixed algal diet (*Laminaria sp*. and *Palmaria sp*.). In the Centre de Recherche en Environnement Côtier (C.R.E.C., Luc -sur-Mer, Basse- Normandie, France), abalones were acclimated at least 2 weeks before the experiments began.

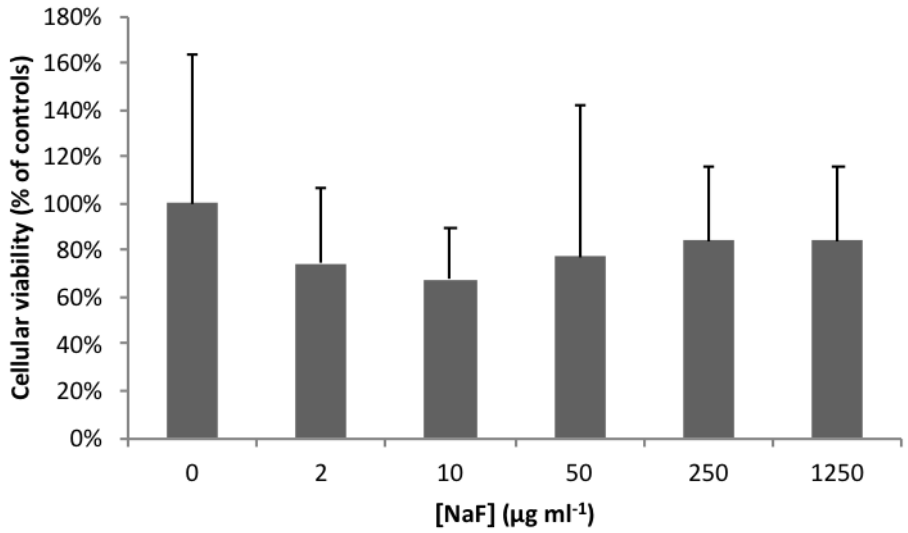

Fig. 1 Variations of haemocyte viability after exposure to 0, 2, 10, 50, 250 and 1,250 µg mL^{-1} of NaF for 24 h using the MTT reduction assay. Data shown are from three separate sets of experiments. Each experiment was realized in triplicate

Primary cell cultures

Haemolymph was extracted from abalone by inserting syringe needles into the pedal sinus in the middle of the foot. Haemocytes were counted with a hemocytometer and plated at a density of 300,000 cells per well in 24- well plates (neutral red assay) or 500,000 cells per well in 12-well plates (MTT, flow cytometry analysis). After addition of three volumes of sterile artificial seawater (ASSW) (436 mM NaCl, 53 mM $MgSO_4$, 20 mM Hepes, 10 mM $CaCl_2$, 10 mM KCl, final pH 7.4), the cultures were maintained at 17 °C for 90 min to allow cells to adhere onto the bottom of the culture well. Then, the ASSW was replaced by Hank's sterile 199 medium modified by the addition of 250 mM NaCl, 10 mM KCl, 25 mM $MgSO_4$, 2.5 mM $CaCl_2$ and 10 mM Hepes (final pH of 7.4) and supplemented with 2 mM L-glutamine, 100 µg mL^{-1} streptomycin, 60 µg mL^{-1} penicillin G and 2 mM concanavalin A. cells were incubated at 17 °C overnight (Lebel *et al*., 1996; Serpentini *et al*., 2000; Mottin *et al*., 2010; Ladhar-Chaabouni *et al*., 2017).

Sodium fluoride solution

A storage solution was prepared at a concentration of 40 mg mL^{-1} in Hank's sterile medium. Then, dilutions in Hank's sterile medium to obtain the working solutions (0, 2, 10, 50, 250 and 1,250 µg mL^{-1}) were realized. After cell cultures, the medium was aspirated and replaced by the NaF solutions for 24 h.

Haemocyte viability assay

Cellular viability was estimated using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay adapted to molluscan cell cultures as previously described (Domart-Coulon *et al*., 2000; Ladhar-Chaabouni *et al*., 2017). Briefly, after 24 h incubation of the cells (17 °C) with 10% (v/v) of MTT solution (5 mg mL^{-1} of PBS), resulting product (formazan) was dissolved using an equal volume of isopropanol containing 0.04 N HCl. Optical density was read at 570 nm and at 630 nm (reference)

Neutral red retention assay

Lysosomal membrane stability was assessed using Neutral red (NR) retention assay as previously described (Ladhar-Chaabouni *et al*., 2017). After 1 h incubation of the cells (17 °C) with 10% (v/v) of NR stock solution (0.5% in PBS 1X), wells were washed with Molluscan Physiological Saline (MPS) (0.4 M NaCl, 0.1 M $MgSO_4$, 20 mM HEPES, 10 mM $CaCl_2$ and 10 mM KCl) and NR was extracted from lysosomes using 1% acetic acid in 50% ethanol. Absorbance was estimated photometrically at 540 nm with 650 nm reference.

Phagocytosis and ROS assays

Phagocytosis and reactive oxygen species (ROS) production were determined using a flow cytometer (Gallios, Beckman Coulter®), as previously described by Mottin *et al*. (2010), Latire *et al*. (2012) and Ladhar-Chaabouni *et al*. (2017) on abalone haemocytes, and 10,000 events were counted for each sample. For phagocytosis assays, 7 µl of bead solution (carboxylate-modified FluoSpheres®, yellow-green fluorescence, 1 µm diameter, Molecular probes) was added to each well. After incubation at 17 °C in the dark during 60 min, the wells were scraped gently and the samples were centrifuged at 500 x*g* for 10 min at 4 °C. Then, 300 µL of 3% paraformaldehyde was added to the pellet. The percentage of phagocytic cells was evaluated as the percentage of haemocytes that had engulfed at least three beads (i.e. immunoefficiency). ROS production was evaluated using the 2'7'-dichlorofluorescein diacetate (DCFH-

DA, sigma) method. After haemocytes incubation for 60 min at 17 °C in the dark with DCFH-DA (final concentration of 100 µM), the wells were scraped gently and the samples were centrifuged at 500× g for 10 min at 4 °C. The resulting pellet was mixed with 300 µL of 3% paraformaldehyde (PFA). Samples were stored at 4 °C until analysis. The results were expressed as the percentage of cells exhibiting fluorescence.

Data analysis

Each experiment was repeated three times, and the means were calculated from triplicates for each experiment. The results were processed by SPSS software.

The statistical differences were assessed using the one-way ANOVA followed by a post hoc test (Tukey test) ($p < 0.05$ was considered significant). After statistical analysis the mean optical density (MTT and NR assays) in controls was assigned a value of 100%. For the phagocytosis and ROS production, the mean percentage in controls was assigned a value of 100%.

Results

Variations of haemocyte viability and morphology after NaF exposure

As shown in Figure 1, no significant difference in cell viability for haemocytes cultured for 24 h in the presence of different concentrations of NaF (2, 10, 50, 250 and 1,250 µg mL^{-1}) when using MTT assay.

In the absence of NaF, cells displayed many thin pseudopodia (Fig. 2A). At concentrations of 2, 10, 50 and 250 µg mL^{-1} NaF, the same shapes were observed (Fig. 2B-E). Nevertheless, after exposure to 1,250 µg mL^{-1} NaF, changes in cell morphology were observed (Fig. 2F) with the abundance of shrunk cells with no extensions.

Variations of haemocyte lysosomal membrane stability after NaF exposure

Figure 3 showed a significant decrease ($p < 0.05$) of NR staining of lysosomes after exposure to 1,250 µg ml^{-1} NaF compared to controls. This decrease was 79% compared to the 100% control.

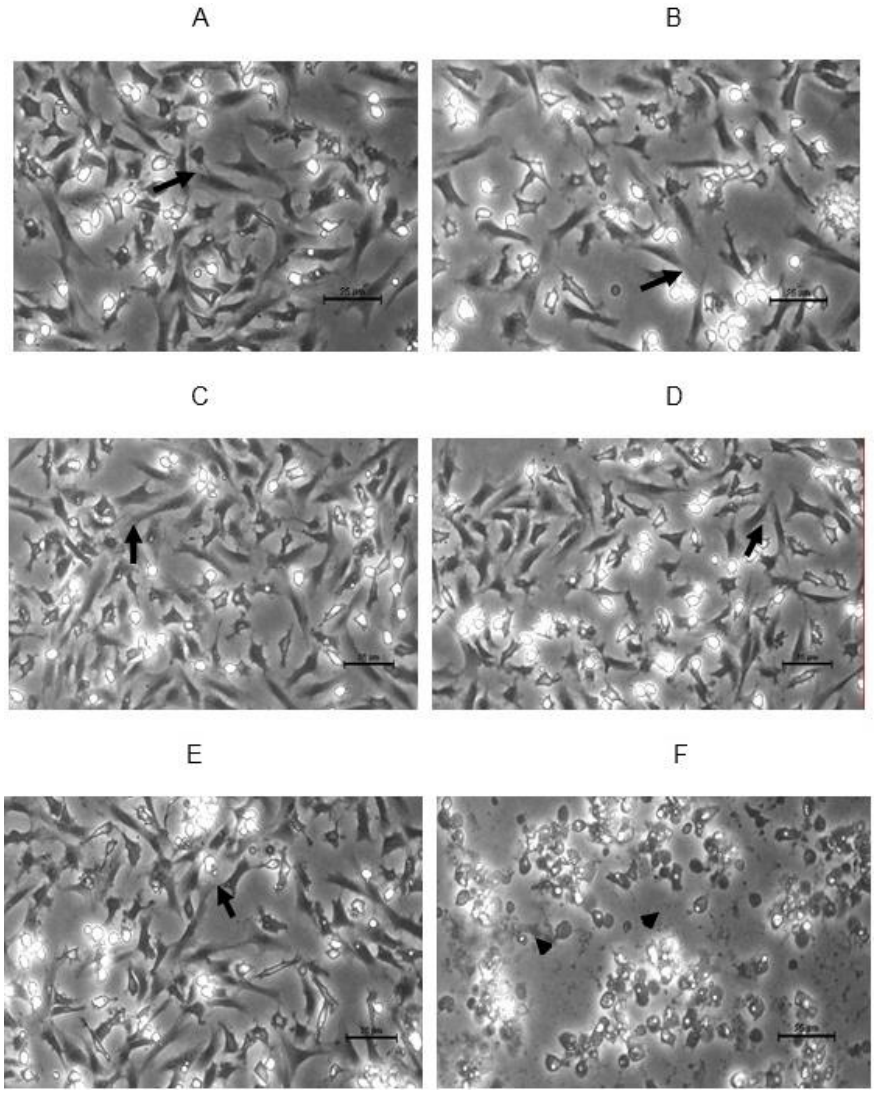

Fig. 2 Variations of *H. tuberculata* haemocytes morphology after exposure to 0 µg mL^{-1} NaF (A), 2 µg mL^{-1} NaF (B), 10 µg mL^{-1} NaF (C), 50 µg mL^{-1} NaF (D), 250 µg mL^{-1} NaF (E) and 1,250 µg mL^{-1} NaF (F) for 24 h using light microscopy. Arrow showed spreading cell with pseudopod; arrow head showed shrunk cells with no extension

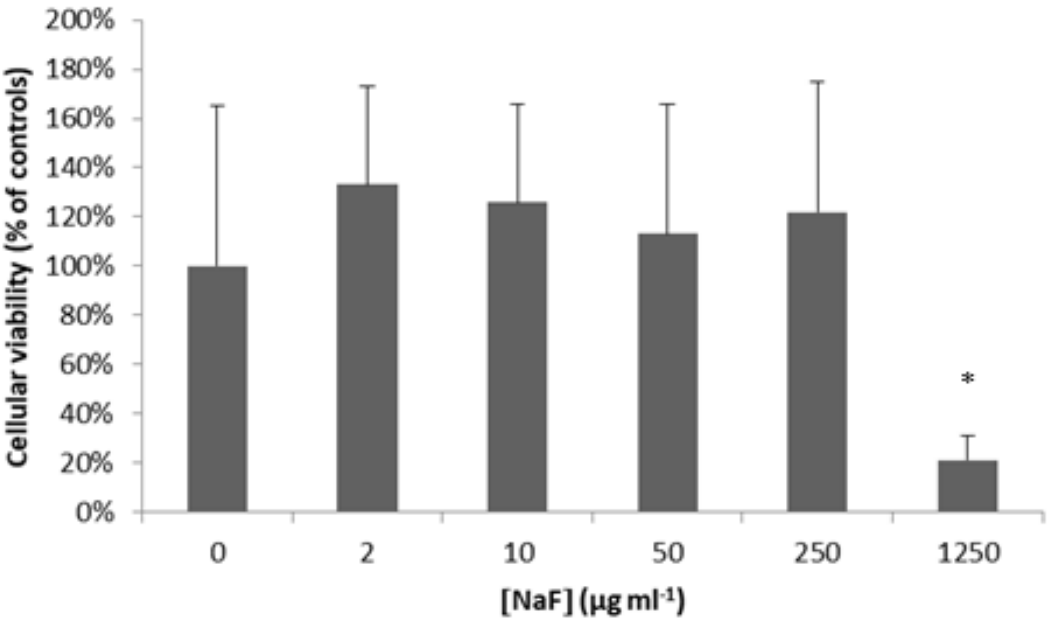

Fig. 3 Variations of haemocyte lysosomal membrane stability after exposure to 0, 2, 10, 50, 250 and 1,250 µg mL^{-1} of NaF for 24 h using the neutral red assay. Data shown are from three separate sets of experiments. Each experiment was made in triplicate. Significant differences compared to controls are marked by asterisk ($p < 0.05$)

Variations of immune parameters after NaF exposure

Figure 4 shows no significant variations of phagocytosis of abalone haemocytes after the exposure to 2, 10, 50 and 250 µg mL^{-1} of NaF. Nevertheless, the phagocytic activity was significantly inhibited ($p < 0.05$) when cells were exposed to 1,250 µg mL^{-1} NaF. This decrease was 24.25% compared to the 100% control. Concerning ROS production, Figure 5 shows no significant variations of ROS production by abalone haemocytes after exposure to different concentrations of NaF after a 24 h of exposure.

Discussion

In the present paper, we analyzed the effects of *in vitro* NaF exposure on the haemocytes of the European abalone *H. tuberculata*. The results showed no significant influence of exposure to 2, 10, 50 and 250 µg mL^{-1} of NaF on the viability, lysosomal membrane stability, morphology, phagocytic activity and ROS production of haemocytes after 24 h of exposure. However a significant decrease of lysosomal membrane stability and phagocytic activity was observed after an exposure to 1,250 µg mL^{-1} of NaF as well as changes in cell morphology. Concerning cell viability, our results were in disagreement with those observed by Ballarin *et al.*, (2014) who showed a significant increase of mortality index of *Venerupus philippinarum* haemocytes exposed to 10, 50 and 250 µg mL^{-1} of NaF during 60 min. The authors showed that 23% of cells exposed to 250 µg mL^{-1} of NaF stained positively with trypan blue indicating that cell membrane barrier function had been compromised. In the present study, cell viability was assessed using the MTT assay based on the assumption that MTT tetrazolium salt reduction to formazan occurs in the mitochondria of living cells due to the activity of mitochondrial dehydrogenases (in particular, succinate dehydrogenase). Therefore, it can be assumed that the increased MTT-tetrazolium salt reduction rate is an effect of elevated succinate dehydrogenase activity. According to Barbier *et al.*, (2010), who outlined disruption of enzymes activities (mostly inhibition) by fluoride by binding to functional amino acid groups that surround the active centre of an enzyme, we expected an inhibition of succinate dehydrogenase activity of abalone haemocytes exposed to NaF. Curiously, this inhibition was not detected with NaF concentrations used in this study. Thus, higher NaF concentrations should be tested to confirm the results of Mendoza-Shulz *et al.*, (2009) who indicated that fluoride at micromolar concentrations can act as an anabolic agent and promote cell proliferation, whereas at millimolar concentrations it acts as an enzyme inhibitor like phosphatases, which play an important role in the ATP production cycle and cellular respiration.

After 24 h exposure to 1,250 µg mL^{-1} NaF, morphological changes of *H. tuberculata* haemocytes were observed. In *V. philippinarum* the cell morphology changes were detected after an exposure to 250 µg mL^{-1} of NaF during only 60 min of exposure. Cell morphology changes in the presence of pollutants were also reported for other marine invertebrate species (Olabarrieta *et al.*, 2001;

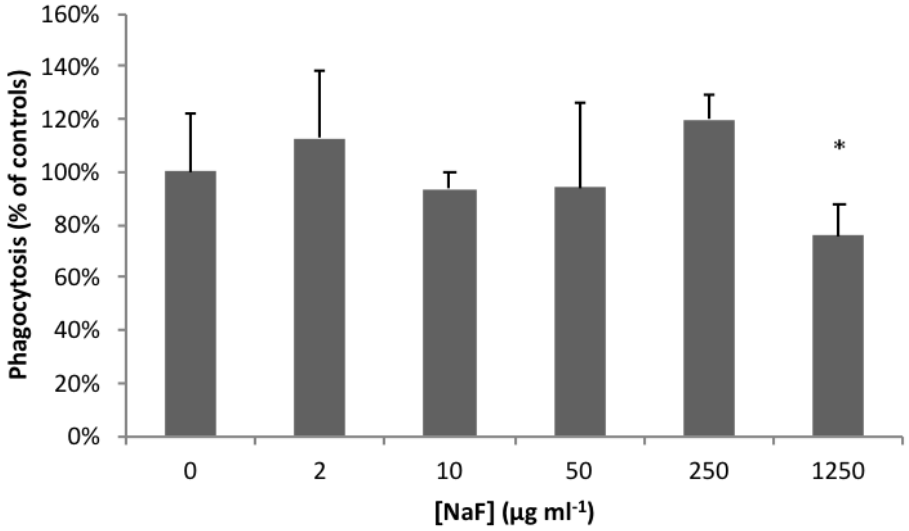

Fig. 4 Variations of phagocytic activity after exposure to 0, 2, 10, 50, 250 and 1,250 µg mL^{-1} of NaF for 24 h compared to the 100% control. Each data point represents the mean percentage ± standard deviation of triplicate cultures. Significant differences compared to controls are marked by asterisk ($p < 0.05$)

Gómez-Mendikute and Cajaraville, 2003; Menin *et al*., 2008) suggesting that some pollutants can alter the cytoskeletal organization (Cima *et al*., 1999). Further research is required to determine the effect of fluoride on glycolysis in molluscan cells (as it was shown for mammalian cells). Since glycolysis is a major ATP source and its inhibition can disrupt the membrane-cytoskeleton interactions.

Using NR assay, Ballarin *et al*., (2014) showed a decrease of lysosomal membrane stability of *V. philippinarum* haemocytes after exposure to 250 µg mL^{-1} of NaF during only 5 minutes. In the present study, such decrease was not observed when using the same concentration during 24 h. We had use a concentration 5 times greater to induce lysosomal membrane destabilization of *H. tuberculata* haemocytes. A loss of lysosomal integrity was also observed in *H. tuberculata* haemocytes exposed to cadmium chloride (Latire *et al*., 2012) and antidepressants (Minguez *et al*., 2014). It appears that lysosome alterations vary according to the species as well as to the nature of contaminant. In addition to lysosomal membrane destabilization, it was shown that fluoride induced plasma membrane alteration and cytoskeleton disorganization leading to cells morphological changes (Agalakova and Gusev, 2011). In mammalian cells, such phenomenon was explained by the direct inhibition of glycolysis and depletion of cellular ATP caused by fluoride (Otsuki *et al*., 2005). Indeed, fluoride induced accumulation of Na^+ and Ca^{2+} in the rat erythrocytes, accompanied by Ca^{2+} dependent K^+ loss and morphological changes of the cells, can be partly explained by inhibition of Na^+-K^+ and Ca^{2+}- pumps due to ATP depletion (Agalakova and Gusev, 2012b).

Flow cytometric evaluation of phagocytosis of fluorescent beads showed a decrease in the phagocytic activity of *H. tuberculata* haemocytes only at the highest concentration tested (1,250 µg mL^{-1} NaF). Ballarin *et al*., (2014) indicated that NaF reduces *V. philippinarum* cell phagocytosis in a dose-dependent way. Such results demonstrated that from species to species, the haemocyte phagocytic activity varies. Such suggestion was also determined by Sauvé *et al*., (2002) who showed that haemocyte phagocytosis varied with species after *in vitro* exposure to different metals (Ag, Cd, Hg and Zn). Mazur *et al*., (1977) showed that NaF markedly inhibited the macrophage phagocytosis due to interaction of this agent with cellular constituents, possibly contractile (and/or associated) proteins, which are direct or indirect determinants of cell deformability. Further studies are needed to elucidate the effect of pollutants like NaF on the function of contractile proteins and microtubules in molluscan haemocytes.

Concerning ROS production, no significant variations after exposure to different concentrations of NaF was observed in the present study although oxidative stress is a recognized mode of action of fluoride exposure that has been observed *in vitro* in several types of cells (Ghosh *et al*., 2002; Zhang *et al*., 2007; Garcia- Montalvo *et al*., 2009). Fluoride is thought to inhibit the activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and catalase (Nobes *et al*., 1995; Garcia-Montalvo *et al*., 2009; Miranda *et al.,* 2018). The excessive production of ROS leads to macromolecule oxidation, mitochondrial membrane depolarization, and apoptosis (Barbier *et al*., 2010; Giri *et al., 2016*). The last phenomenon was observed in *V. philippinarum* haemocytes exposed to 50 and 250 µg mL^{-1} NaF during 60 min (Ballarin *et al*., 2014). The authors suggest that apoptosis could be a consequence of the oxidative stress caused by the exposure to NaF. Suchocki *et al*., (2010) showed that the presence of ROS leads to

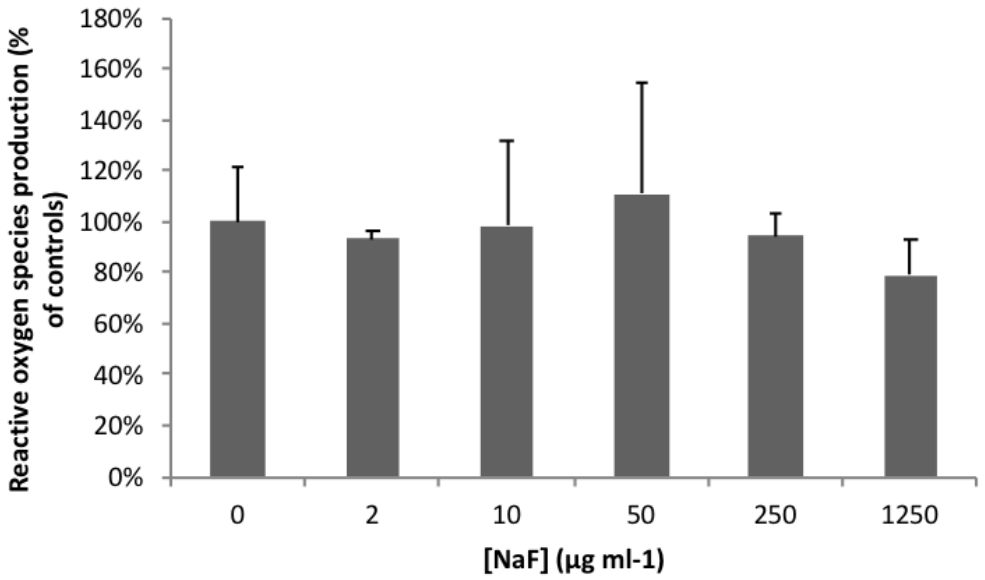

Fig. 5 Variations of ROS production after exposure to 0, 2, 10, 50, 250 and 1,250 µg mL^{-1} of NaF for 24 h compared to the 100% control. Each data point represents the mean percentage ± standard deviation of triplicate cultures

the disruption of mitochondrial enzyme activity. Scatena *et al*., (2004) showed that under increased ROS level conditions, the activity of mitochondrial succinate, an enzyme responsible for MTT salt reduction, is inhibited. Hence, the absence of significant increase of ROS production in the present study could explain the stability of MTT-tetrazolium salt reducing mitochondrial enzymes activities in abalone haemocytes exposed to different concentrations of NaF.

In summary, our study has shed some light on the effect of NaF on the primary cultured haemocytes of *H. tuberculate.* The results showed that NaF at concentrations of 2, 10, 50 and 250 µg mL^{-1} didn't have immunotoxic effects. Nevertheless, a reduction of immune functions was observed at concentration of 1,250 µg mL^{-1} NaF. Further studies concerning molecular mechanisms of NaF toxicity such the inhibition of glycolysis thus causing the depletion of cellular ATP in *H. tuberculate* haemocytes are required.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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