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Original Article

Hypochlorous acid induces a redox-dependent growth of C2C12 myoblasts

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Abstract

Hypochlorous acid (HOCl) is a reactive chlorine species generated by the enzyme myeloperoxidase present in phagocytes. HOCl plays a vital role in inflammation and has been linked to tissue regeneration through redox signalling, however, the relevant evidence is rather scarce. The present investigation aimed to study the effects of HOCl on the growth of C2C12 myoblasts and its association with alterations of cellular redox profile. C2C12 cells were incubated for 10 min, 1 h and 24 h with a wide range of HOCl concentrations (628 pM - 4 M). Cell survival was increased when cells were incubated with HOCl concentrations between 6.28 μM and 628 μM, which are encountered in biological systems. Intriguingly, after a 10 min-incubation with 3 mM of HOCl, the highest cell growth was observed through a redox-related mechanism, as indicated by the decrease of the levels of reactive oxygen species and the enhanced levels of reduced glutathione measured by flow cytometry. The in vitro model created herein simulates the in vivo inflammatory and regeneration response of muscle cells and can putatively give mechanistic answers about the contribution of HOCl to muscle regeneration.

Keywords: Hypochlorous acid, Muscle regeneration, Inflammation, Reactive oxygen species, Glutathione, reactive chlorine species.

1. Introduction

Skeletal muscle is a tissue capable of sustained regeneration. Muscle repair and maintenance rely on a population of muscle stem cells, known as satellite cells, which have been extensively studied [1-4]. Muscle stem cells remain quiescent under normal conditions [5,6]. However, in response to different stimuli (i.e., injury, exercise) they become activated and proliferate extensively to give rise to mononuclear myoblasts, which can repair the damaged muscle, either by directly fusing with pre-existing myofibers or by generating new myofibers through fusion with one another [2,7-9]. Skeletal muscle repair is a highly synchronized process involving the activation of various cellular and molecular responses, where the coordination between inflammation and regeneration is crucial for the beneficial outcome of the repair process following muscle damage [10-12].

Inflammation is a biological phenomenon that is inextricably linked to reactive species generation. Reactive species are implicated in diverse physiological processes, such as exercise adaptations and signal transduction but also in inflammation, aging and disease onset [13-18]. Oxidative stress, which is defined as the excessive production of reactive oxygen and nitrogen species leading to disruption of redox signalling is a well-studied phenomenon both *in vitro* and *in vivo* [16-23]. However, chlorinative stress caused by reactive chlorine species (RCS), especially hypochlorous acid (HOCl), is rather overlooked.

HOCl is generated by the enzyme myeloperoxidase (MPO) which is present in neutrophils and macrophages and stimulates phagocytes during inflammation [24, 25]. This enzyme exerts a strong bactericidal and antiviral action, which is mediated by the HOCl production via the reaction of hydrogen peroxide with chloride ions. Its role in inflammatory response is crucial, as indicated by the enhanced MPO levels in acute and chronic inflammatory conditions, such as cardiovascular and neurodegenerative diseases, as well as specific cancer types [26-28]. HOCl is a powerful, metal-independent oxidative agent *in vivo*, as it oxidizes biomolecules including DNA, proteins, and lipids [13]. However, its most common target is proteins and, hence, it induces oxidative modifications to amino acids and peptides [29]. Specifically, HOCl oxidizes methionine and cysteine but the oxidized products of tyrosine, namely 3-chlorotyrosine and 3,5-dichlorotyrosine are the most promising biomarkers of HOCl-induced chlorination [30]. A characteristic example of the harmful role of HOCl due to protein oxidation is its contribution to atherosclerosis as it oxidizes apolipoprotein A-I, whose normal role is to remove cholesterol from macrophages, thus, impair-

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ing its physiological function [31]. Furthermore, it contributes to the pathogenesis of other inflammation-related conditions, such as senescence and muscle atrophy via activation of several transcription factors, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) that regulates the most fundamental antioxidant pathways [32].

Muscle regeneration through inflammatory process is well examined [10-12,33]. However, the role of HOCl, an RCS that has been poorly studied, in this biological procedure has not yet been elucidated. To our knowledge, the effects of HOCl on skeletal muscle cells have never been studied before. We anticipate that HOCl, like any reactive species, will decrease cell viability but we also hypothesize that HOCl concentrations with physiological significance which will promote C2C12 cell growth will be identified. Thus, the model created herein appears to mimic the first step of muscle regeneration, i.e., the proliferation of muscle cells in response to HOCl production by myeloperoxidase-expressing immune cells. Furthermore, another important goal was to analyse whether the effect of HOCl on muscle cell growth is regulated by a redox-related mechanism.

2. Materials and Methods

2.1. Evaluation of the concentrations of the tested HOCl solutions

The optical absorbance of the HOCl solutions diluted in phosphate-buffered saline (PBS) was measured immediately before use at 293 nm and their concentrations were evaluated through the law of Lambert-Beer using the HOCl molar extinction coefficient (i.e., 350 $M^{-1}cm^{-1}$) at pH = 10-12.

2.2. Cell culture conditions

The mouse-derived C2C12 myoblast cell line was purchased from the European Collection of Authenticated Cell Cultures, Catalogue No. 91031101. The cells were maintained in Dulbecco's modified eagle medium (DMEM containing 4.5 g/l glucose and L-glutamine) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in the presence of antibiotics (PS: 100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified chamber at 37 °C and 5% CO_2. The cells used in all experiments did not exceed 10 population doublings. DNA samples were routinely extracted from the cultured cells and were subjected to PCR amplification with specific mycoplasma primers to verify that cells used in all experiments were mycoplasma-free.

2.3. Cell viability measurements

Cell viability and proliferation were measured by the XTT colorimetric assay. Briefly, 5×10^3 C2C12 cells were seeded in 96-well flat-bottomed microplate in full culture media (DMEM+FBS+PS) and incubated for 24 h at 37 °C under humidified 5% CO_2. Then, different HOCl solutions (with concentrations ranging from 628 pM up to 4 M diluted in DMEM and PS) were added and the cells were incubated for additional 10 min, 1 h or 24 h. Then, the XTT reagent (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) was added, and each sample was additionally incubated for 4 h at 37 °C. The wells containing C2C12 cells and culture media without the addition of HOCl were used as the control samples. The optical density was measured using a microplate reader (Biotek Instruments, Inc.) at 450 nm with a reference wavelength of 650 nm. Cell survival (viability) was calculated as a percentage relative to the control samples. In each experiment, all samples were tested in duplicates. At least 4 independent experiments were performed.

2.4. Evaluation of the effect of HOCl on the intracellular ROS and GSH levels using flow cytometry

In order to evaluate the levels of reactive oxygen species (ROS) and reduced glutathione (GSH) produced after the treatment of C2C12 cells with HOCl, the cells were incubated with selected concentrations of HOCl for different time periods. Briefly, the cells were cultured overnight in T_{25} flasks in full culture media (DMEM+FBS+PS) at a density of 5.3×10^5 cells/flask. Then, the cells were washed with PBS and were incubated with different selected concentrations of HOCl (diluted in DMEM+PS) for 10 min, 1 h or 24 h at 37 °C in a humidified atmosphere of 5 % CO_2. Control samples containing only medium DMEM in the presence of antibiotics were also prepared for the same time periods. Following the end of incubation, the cells were washed in PBS, incubated with trypsin and transferred in falcon tubes containing full media. The samples were centrifuged (160 rpm, 10 min, 28 °C), the supernatant was discarded, and the cells were washed in PBS and centrifuged again at the same conditions. Finally, the cell pellet was stored at -80 °C until preparation of analysis using flow cytometry.

The levels of ROS and GSH in C2C12 cells were evaluated using their ability to interact with 2,7-dichlorofluorescein diacetate (DCF-DA) and mercury orange respectively. Inside the cellular milieu, DCF-DA loses its acetate groups by esterases and following oxidation by ROS, it is converted to a fluorescence dye. On the contrary, mercury orange is a fluorescence dye that binds directly to the molecule of GSH. In detail, the cell pellets were re-suspended in PBS and incubated with DCF-DA (10 μM) for ROS and mercury orange (40 μM) for GSH in the dark (30 min, 37 °C). Then, PBS was added, the samples were centrifuged (300 g, 5 min, 5 °C) and the supernatant was discarded. Subsequently, the cells were re-suspended in PBS and introduced into the flow cytometer (FACScan flow cytometer, Becton-Dickinson, Franklin Lakes NJ, USA). The excitation and emission wavelengths were 488 and 530 nm for ROS and 488 and 580 nm for GSH. The analyses were performed on 10,000 cells for each sample. Data was analyzed with the BD Cell Quest software (Becton-Dickinson). All experiments were performed in triplicate.

2.5. Statistical analysis

All data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test for multiple pairwise comparisons. Data are presented as mean ± standard deviation (SD). The level of significance was set at $P<0.05$. For the statistical analysis, the SPSS software version 21.0 (SPSS Inc., Chicago, IL, USA) was used.

3. Results

3.1. Effect of HOCl on C2C12 cell viability

C2C12 viability was statistically significantly reduced to 84% and 15% compared to the control (untreated) sample after 10 min of incubation with HOCl at the lowest (i.e., 628 pM and 6.29 nm) and at the highest (i.e., 4 M) concentrations, respectively (Figure 1). On the contrary,

cell viability was increased when cells were treated with 628 µM and 3 mM HOCl at the same time point (Figure 1). After 1 h of incubation, HOCl inhibited cell growth at the majority of the tested concentrations (i.e., 628 pM, 6.29 nm, 6.28 nM, 6,28 nM, 3 mM and 4 M) (Figure 1). Finally, C2C12 growth was inhibited when 24 h-incubations were performed with all the tested concentrations of HOCl (except for 3 mM) (Figure 1).

3.2. Effect of HOCl on ROS levels of C2C12 cells after 10 min of incubation

The ROS levels were decreased by 38% compared to the control sample after the 10-min incubation of the cells with 3 mM HOCl, whereas they were not affected by the rest of the tested HOCl concentrations (i.e., 628 pM, 628 nM and 628 µM) (Figure 2).

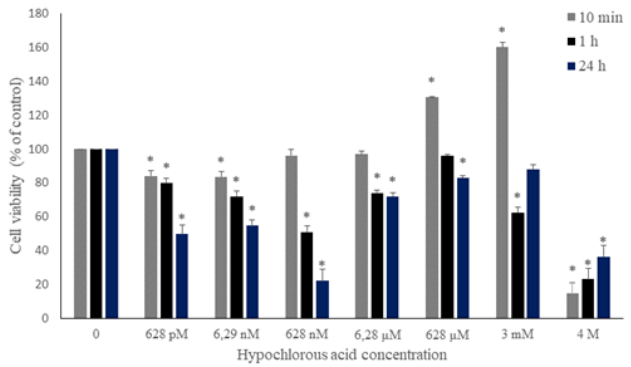

Fig. 1. The effect of HOCl treatment on the viability of proliferating C2C12 cells. C2C12 cells were incubated with the indicated HOCl concentrations for 10 min, 1 h and 24 h and the cell viability was determined by the XTT assay. Viability is expressed as % of the control sample (i.e., C2C12 cells incubated with culture media without HOCl). *: Statistically significant values compared to the control sample.

3.3. Effect of HOCl on ROS levels of C2C12 cells after 1 h of incubation

After 1 h of incubation with the tested range of HOCl concentrations, the ROS levels were decreased at 628 nM, 628 µM and 3 mM by 25%, 15% and 75% respectively, compared to the control sample (Figure 3).

3.4. Effect of HOCl on GSH levels of C2C12 cells after 10 min of incubation

GSH levels were decreased by 28%, 33% and 37% compared to the control sample, at the HOCl concentrations of 628 pM, 628 nM and 628 µM respectively (Figure 4). In contrast, HOCl increased the GSH levels at the highest tested concentration (i.e., 3 mM) by 23% compared to the control sample (Figure 4).

3.5. Effect of HOCl on GSH levels of C2C12 cells after 1 h of incubation

The levels of GSH were significantly increased after 1 h of incubation with HOCl at 628 pM by 48% and at 3 mM by 315% and they were decreased at 628 nM by 17% (Figure 5).

3.6. Effect of HOCl on ROS and GSH levels of C2C12 cells after 24 h of incubation

The ROS levels of C2C12 cells were markedly decreased after 24 h of incubation with 3 mM HOCl by 97%, whilst a significant elevation (i.e., 33%) was observed at the GSH levels following incubation with the same HOCl concentration for 24 h (Figure 6).

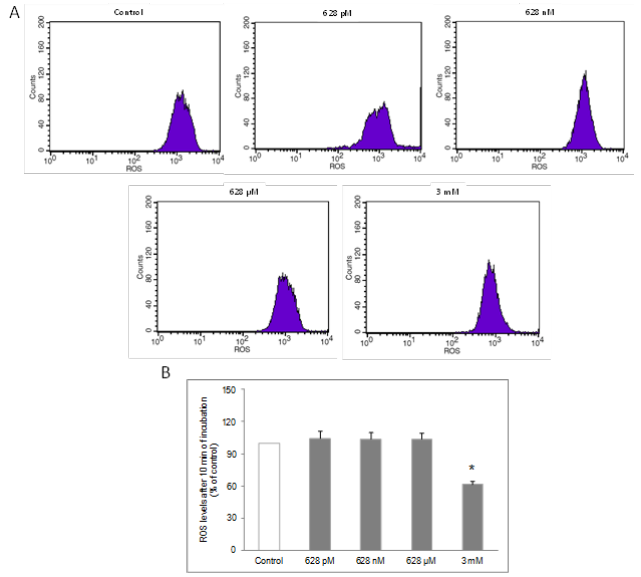

Fig. 2. The effects of the incubation of C2C12 cells with HOCl in the tested concentrations (i.e., 628 pM, 628 nM, 628 µM and 3 mM) for 10 min on ROS levels evaluated using flow cytometry. (A): The respective histograms of cell counts (n=10,000) vs fluorescence. (B): The bar chart that illustrates the alterations of ROS levels induced by the tested HOCl concentrations expressed as % of the control sample (i.e., C2C12 cells incubated with PBS without HOCl). *: Statistically significant values compared to the control sample; ROS: reactive oxygen species; PBS: phosphate- buffered saline.

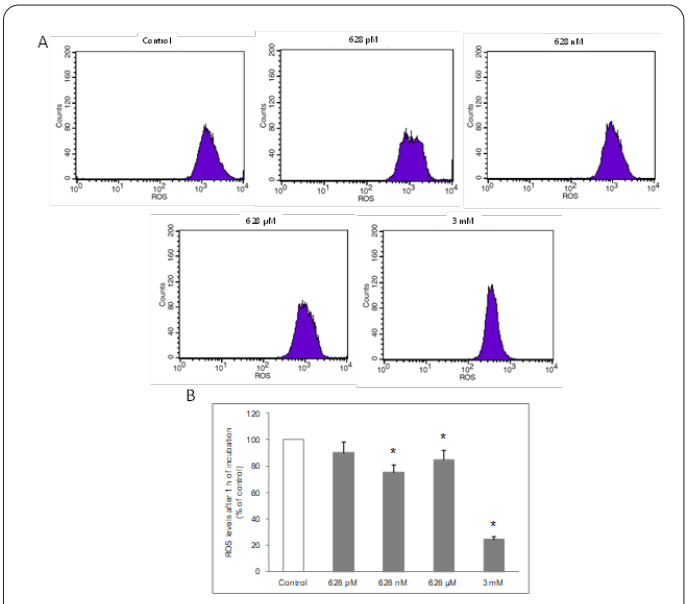

Fig. 3. The effects of the incubation of C2C12 cells with HOCl in the tested concentrations (i.e., 628 pM, 628 nM, 628 µM and 3 mM) for 1 h on ROS levels evaluated using flow cytometry. (A): The respective histograms of cell counts (n=10,000) vs fluorescence. (B): The bar chart that illustrates the alterations of ROS levels induced by the tested HOCl concentrations expressed as % of the control sample (i.e., C2C12 cells incubated with PBS without HOCl). *: Statistically significant values compared to the control sample; ROS: reactive oxygen species; PBS: phosphate-buffered saline.

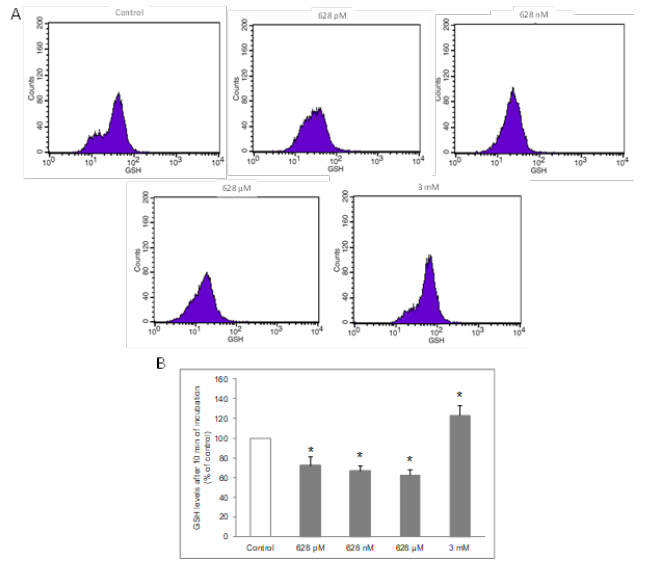

Fig. 4. The effects of the incubation of C2C12 cells with HOCl in the tested concentrations (i.e., 628 pM, 628 nM, 628 µM and 3 mM) for 10 min on GSH levels evaluated using flow cytometry. (A): The respective histograms of cell counts (n=10,000) vs fluorescence. (B): The bar chart that illustrates the alterations of GSH levels induced by the tested HOCl concentrations expressed as % of the control sample (i.e., C2C12 cells incubated with PBS without HOCl). *: Statistically significant values compared to the control sample; GSH: reduced form of glutathione; PBS: phosphate-buffered saline.

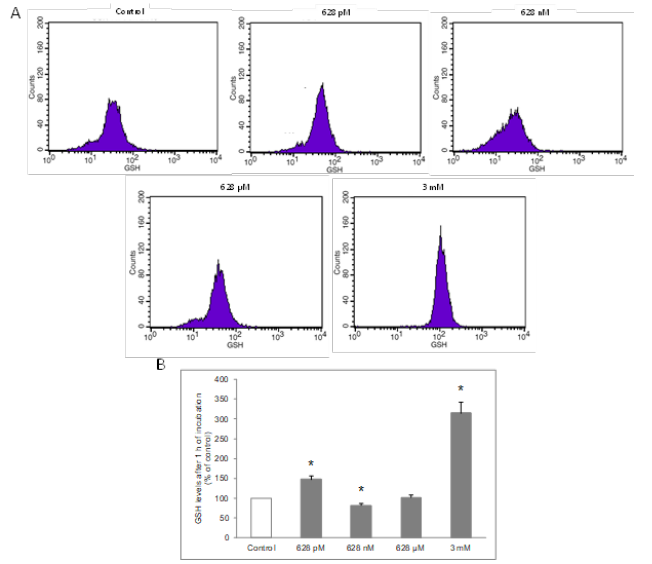

Fig. 5. The effects of the incubation of C2C12 cells with HOCl in the tested concentrations (i.e., 628 pM, 628 nM, 628 µM and 3 mM) for 1 h on GSH levels evaluated using flow cytometry. (A): The respective histograms of cell counts (n=10,000) vs fluorescence. (B): The bar chart that illustrates the alterations of GSH levels induced by the tested HOCl concentrations expressed as % of the control sample (i.e., C2C12 cells incubated with PBS without HOCl). *: Statistically significant values compared to the control sample; GSH: reduced form of glutathione; PBS: phosphate-buffered saline.

4. Discussion

The present study reports for the first time the creation of an *in vitro* (*in vivo*-like) model that simulates the inflammatory response of skeletal muscle (i.e., myoblasts treated with HOCl). Furthermore, it proposes a redox-dependent mechanism for the induction of cell survival caused by

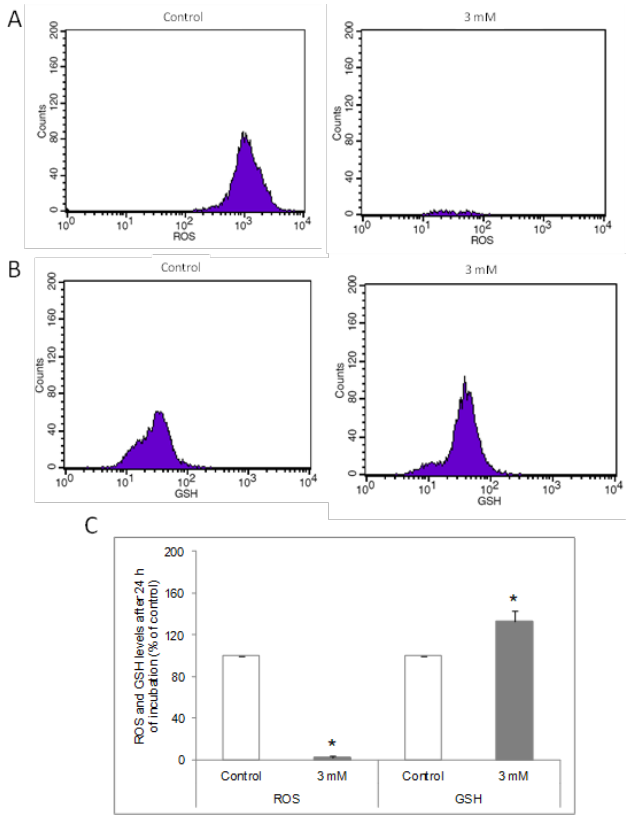

Fig. 6. The effects of the incubation of C2C12 cells with 3 mM of HOCl for 24 h on ROS and GSH levels evaluated using flow cytometry. (A): The respective histograms of cell counts (n=10,000) vs fluorescence. (B): The bar chart that illustrates the alterations of ROS and GSH levels induced by the tested HOCl concentration expressed as % of the control sample (i.e., C2C12 cells incubated with PBS without HOCl). *: Statistically significant values compared to the control sample; ROS: reactive oxygen species; GSH: reduced form of glutathione; PBS: phosphate buffered saline.

HOCl, which under the appropriate conditions leads to muscle regeneration. According to the results obtained, the incubation of C2C12 cells with HOCl caused an increase in cellular GSH levels, accompanied by a decrease of ROS levels. This finding was observed when C2C12 cells were incubated with 3 mM of HOCl at all incubation times tested (i.e., 10 min, 1 h and 24 h), indicating a redox-dependent mechanism of action for HOCl at this concentration. Interestingly, this is the only HOCl concentration that did not affect cell viability following the longest 24-h incubation period.

The role of inflammation in muscle damage and regeneration has been well established. Indeed, it has been demonstrated that inflammation promotes muscle damage through excessive generation of ROS by neutrophils during phagocytosis and, of note, this molecular mechanism can also impair pre-existing muscle injury *in vivo* [33,34]. On the other hand, muscle regeneration is a process whose precondition is inflammation as well. The process of muscle regeneration includes several stages involving activation of muscle stem cells, proliferation of the activated muscle stem cells, and differentiation of the muscle stem cells which ultimately fuse with each other and existing fibers to restore injured tissue [2,7,8]. Acute inflammation and immune cells play critical roles in almost all stages of muscle regeneration process. The immune cells, especially macrophages, can trigger a cascade of cellular responses

to regulate muscle stem cell activation, proliferation, and differentiation and, in this aspect, they serve as important mediators to orchestrate muscle repair [10-12]. *In vivo* investigations which include myeloid depletion experiments and the use of chemokine/chemokine receptor-deficient mice (Ccl2 and Ccr2 knockout) have shown the requirement of monocyte-derived macrophages in skeletal muscle regeneration [35-37]. The mechanisms of muscle regeneration have been studied in the context of exercise, which is a model that induces inflammatory response [38,39] as well as in the context of cell-mediated therapy of degenerating diseases, such as muscular dystrophies [40-43]. A major mediator of inflammation is HOCl, a highly RCS that is produced by neutrophils and macrophages at the site of inflammation and exerts mostly detrimental action on muscle cell integrity and normal function [44]. Interestingly, HOCl, through its inflammation-promoting action, is implicated in aging and aging-related conditions [29,45], as well as in cardiovascular and in rheumatoid pathologies [46,47].

To that end, it has been shown that at sites of inflammation, the *in vivo* HOCl concentration may be equal to 200 μM or even up to 340 μM [48,49]. These experimental observations strengthen the undermentioned noteworthy finding of the present study. Following the 10 min incubation period, the HOCl concentration that equals to 6.28 μM did not affect cell growth, however the next tested concentration (i.e., 628 μM) increased cell survival. This means that muscle cell survival might follow an increasing trend following incubations with HOCl concentrations between 6.28 μM and 628 μM, indicating a physiological meaning of this evidence. After 1 h and 24 h of HOCl incubation, cell growth was inhibited, obviously due to the long duration of the incubation.

The effect of oxidative stress, mainly in the form of H_2O_2 exposure, on C2C12 survival has been previously investigated [50-56]. The majority of these studies reported an EC50 of 1.33 mM following 6 h of incubation with H_2O_2. However, it is worth mentioning that previous studies have shown large variation in reported cytotoxic concentrations of H_2O_2 in cell cultures, which has been attributed to differences in the experimental conditions applied, specifically cell type, cell concentration and incubation times [57]. It is generally accepted that in proliferating mammalian cells very low levels (3 to 15 μM) of H_2O_2 cause growth stimulation (possibly due to adaptations), higher levels (120 to 400 μM) induce growth arrest, and high concentrations (>1 mM) produce necrotic cell death [58,59]. Compared to other cell types, C2C12 cells appear to be more robust to exposure to oxidative stress. This may be due to the fact that muscle precursor cells are exposed repeatedly to high levels of ROS. As this is the first study of the effect of HOCl on skeletal muscle cells, we can assume that the effect of HOCl on C2C12 cell survival differs considerably compared to that of H_2O_2. Alternatively, as seen with H_2O_2, the effect of HOCl varies depending on the incubation time, as seen by the clearly demonstrated differential effects of short and long (i.e., 10 min versus 1 h) incubation times.

Apart from the role of HOCl on cell survival, the redox-based mechanism that regulates its action is of great interest. At 628 pM, cell growth was inhibited both after 10 min and 1 h of incubation. GSH levels, although decreased after 10 min, were enhanced after 1 h. Given that after 1 h of incubation cell viability was affected to a greater extent compared to the 10 min incubation, it can be deduced that the activation of the antioxidant potential of the cells is a defensive mechanism against the detrimental impact of cell death. At 628 nM, the decreased GSH after the 10 min-incubation with HOCl indicates that there is no need for activation since cell growth is not affected. Nevertheless, after 1 h the decrease in GSH implies that its levels have been consumed to act towards the reduction of ROS levels, a finding that is depicted in Figure 3. At 628 μM after 10 min, cell growth is promoted and GSH is reduced probably due to homeostatic mechanisms. Of note, previous studies have also reported non-redox-related mechanisms for the action of HOCl. Indeed, it has been observed that HOCl attacks specific sites on and within the cells triggering a variety of cell death mechanisms depending on the cell type [60-65].

The most interesting redox-based results are those observed at 3 mM of HOCl at all incubation times. In detail, after 10 min, cell survival increased but declined after 1 h, as anticipated. The redox pattern was consistent, though, since GSH and ROS levels were enhanced and decreased, respectively after 10 min, 1 h and 24 h of incubation. The intensification of the cellular antioxidant defence and the scavenging of ROS appears to be crucial reasons for the induction of cell growth after 10 min of incubation with HOCl. Furthermore, the enhanced levels of GSH could putatively be a crucial mechanism for cell survival. Indeed, the only concentration of HOCl where the cell growth remained unaffected after 24 h was 3 mM. In conclusion, although HOCl has not been measured at the concentration of 3 mM *in vivo*, it appears that the *in vivo*-like model created herein can putatively give mechanistic answers about the role of this intriguing RCS on inflammation and cell growth. Interestingly, a similar finding has been recently reported about the effect of HOCl on smooth muscle cells. Specifically, pre-treatment of matrices with HOCl induced chemical and structural changes of extracellular matrix proteins and these modifications contributed, amongst other behaviour changes, to enhanced proliferation of human coronary artery smooth muscle cells [66].

It must be stated that this investigation touches upon one of the most fundamental queries of biology, that is whether *in vivo*-like (i.e., *in vitro*) simulation models are able to offer mechanistic answers to questions concerning *in vivo* settings. In other words, the creation of quantitative *in vitro* models that promote the understanding of cellular behaviour *in vivo* is a major challenge for modern science [23,67]. The correct creation of simulation *in vitro* models with the potential to promote the comprehension of *in vivo* biological redox pathways and to face the reasonable discrepancies between them is undoubtedly a reliable tool for redox biology [23,68].

In conclusion, the present investigation reports for the first time the effect of HOCl, a rather neglected RCS, on the viability of skeletal muscle cells. Furthermore, we present preliminary findings for creating an *in vitro* model, alternatively characterized as an *in vivo*-like setting (simulation of *in vivo* conditions) of HOCl-induced inflammation and proliferation on muscle cells, which is the first step of muscle regeneration. Finally, it appears that HOCl promotes muscle cell growth in a redox-dependent manner, offering a notion concerning a putative role of this molecule on muscle regeneration.

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Interest conflict
The authors declare no conflict of interest.

Author's contribution
Kalliopi Liadaki and Aristidis S. Veskoukis: Conceptualization. Writing-Original draft, Methodology, Writing-Review and Editing, Supervision. Zoi Skaperda, Christina Christodoulou and Demetrios Kouretas: Investigation. Christina Christodoulou and Aristidis S. Veskoukis: Formal Analysis. Kalliopi Liadaki and Demetrios Kouretas: Funding Acquisition. All authors have read and agreed to the published version of the manuscript.

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