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Effect of methionine feeding on oxidative stress, intracellular calcium and contractility in cardiomyocytes isolated from male and female rats

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Abstract

Homocysteine (Hcy) is a breakdown product of methionine metabolism. The risk of cardiovascular disease correlates with an increase in plasma Hcy levels. The aim of this study was to investigate whether 1% methionine supplementation of adult rats altered intracellular reactive oxygen species (ROS) generation, intracellular Ca^{++} content and contractile activity in freshly isolated cardiomyocytes. This was measured under normal conditions and during oxidative stress in freshly isolated cardiomyocytes. Single rat cardiomyocytes from both sexes were isolated by enzymatic and mechanical dispersion techniques. Fluorescence microscopy was used to measure ROS production and intracellular Ca^{++} concentration. Cell contraction was measured using a video camera. During exposure to 200μM H_2O_2 female cardiomyocytes produced significantly fewer ROS and had a higher intracellular Ca^{++} concentration compared to male cardiomyocytes in control and methionine fed conditions. The contractility of cardiomyocytes isolated from male rats was insignificantly decreased after methionine feeding compared to control, whilst the contractility of cardiomyocytes from female rats insignificantly reduced after methionine feeding and acute exposure to oxidative stress. These findings provide evidence that during exposure to 200μM H_2O_2 cardiomyocytes from female rats produce less ROS and have higher intracellular Ca^{++} levels. There were no significant effects on contractility in cardiomyocytes from either gender and under any of the different conditions.

Key Words: Freshly isolated cardiomyocytes; reactive oxygen species; calcium; contractility

1. Introduction

The cardiomyocyte is the contractile unit of the heart. Therefore anything detrimental which reduces the contractility of the cardiomyocytes will adversely affect cardiac output. Such harmful conditions arise during ischaemia (e.g. during a myocardial infarction) or ischaemia reperfusion (e.g. during open heart surgery where cardioplegia has been used to stop the heart). Two of the major pathogenic mechanisms during ischaemia and ischaemia reperfusion are oxidative stress [1] and calcium overload [2]. In previous work we have shown that oxidative stress leads to reactive oxygen species formation and changes to contractile activity [3]. However these experiments have been exclusively carried out on cardiomyocytes isolated from males with little known about effects on cardiomyocytes from females. It is important to know this because it is known that whilst cardiovascular disease results in 1 in 7 deaths in males, it also causes 1 in 12 deaths in females [4]. However at a young age, the risk of coronary heart disease is higher in men than women [5].

Homocysteine (Hcy) has been implicated in the onset of cardiovascular diseases (CVD). Over the last decade, studies have shown that elevated plasma Hcy levels are associated with an increased risk of CVD. Hcy (2-Amino-4-mercaptobutyric acid), is a sulphur containing amino acid biosynthesized naturally in organisms from methionine in the methylation cycle [6-8]. It has been shown that hyperhomocysteinemia (Hhcy) in isolated adult rat cardiomyocytes resulted in an increase in ROS production, a reduction in cardiomyocyte contractility and the development of apoptosis [9]. In addition, a study by Almashhadany *et al* [10] reported that acute exposure to 0.1mM Hcy affects the functional recovery of the isolated and perfused rat heart during ischemia-

reperfusion (IR), disrupts the contractile activity of freshly isolated cardiomyocytes under oxidative stress and reduces the calcium transient under normal conditions.

The aim of the current study was to investigate whether the protection afforded to women against coronary heart disease is reflected in a greater resistance of their cardiomyocytes to oxidative stress and hyperhomocysteinaemia. In order to achieve this we investigated the effect of 1% L-methionine supplementation in young adult rats on intracellular ROS production, $[Ca^{++}]_i$ and cell contractility in freshly isolated cardiomyocytes under normal and oxidative stress conditions in both female and male rats.

2. Materials and Methods

2.1. Materials

Fluorescent dyes were from Molecular Probes (Invitrogen, Sydney NSW, Australia). All other chemicals were from Sigma-Aldrich (Sydney NSW, Australia), Scharlau (Sydney NSW, Australia) and Chem-Supply (Sydney NSW, Australia). Parts for the perfusion system were from ADInstruments (Bella Vista, NSW, Australia).

2.2. Experimental Animals

Young post-weaned Wistar rats (6 weeks old) (body weight, 130 - 190 gm) (n = 24) of both sexes were divided into 2 sets of two groups (n=6 in each group) according to sex and diet. The control groups received standard rat chow and water ad libitum while the other groups received standard rat chow and water supplemented with 1% L-methionine [11] for 10 weeks [12]. Regular checks

were made on the rats' weight, general health and well-being. At the end of this time the rats were sacrificed by stunning and cervical dislocation. We have already reported on the effects of methionine feeding on cardiomyocyte Hcy and reduced glutathione levels [20]. This study was approved by the Animal Ethics committee of the University of New England (Approval Number: 13-083) and followed international guidelines.

2.3. Isolation of heart cells

Calcium tolerant ventricular cardiomyocytes were isolated from Wistar rats by enzymatic digestion and mechanical dispersion using Langendorff techniques as described previously [3, 14-15]. In brief, male and female Wistar rats were killed by cervical dislocation, the heart dissected and rinsed in cold solution A containing (in mM): 137 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 20 N-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), 16 glucose, 5 Na pyruvate and 1.8 $MgCl_2$ (pH 7.25 with NaOH) + 0.75 mM $CaCl_2$. The heart was cannulated via the aorta and perfused for 4 min with solution A + 0.75 mM $CaCl_2$ (all perfusing solutions were oxygenated and maintained at 37°C). This was followed by a 4-min perfusion with solution A + 0.09 mM ethylene glycol-bis (b-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA). Next the heart was digested with 50 ml of enzyme solution containing: solution A + 0.09 mM EGTA, 50 mg collagenase, 5 mg protease until the tissue felt soft. There was a final 4-min perfusion with solution A + 0.15 mM $CaCl_2$ before the ventricles were cut down and sliced. The sliced ventricles were suspended in approximately 20 ml solution A+ 0.15mM $CaCl_2$ and shaken for 6 min at 37 °C. After filtration, cells were allowed to sediment, the supernatant was discarded, and the remaining cell layer suspended in solution A + 0.5mM$CaCl_2$. This sedimentation, removal of supernatant and resuspension step was repeated, but this time the cells were suspended in solution A + 1 mM$CaCl_2$.

This technique typically produced a yield of over 80% rod-shaped cells with the ability to exclude Trypan Blue (not shown).

2.4. Measurement of intracellular ROS production

Intracellular ROS generation in isolated rat cardiomyocytes were examined using the 5-(and-6)-chloromethyl-2′,7′ dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) probe [3, 16]. A stock solution was prepared containing 10 mM CM-H_2DCFDA in dimethyl sulphoxide (DMSO). 2 ml of buffer containing cells was pipetted into an Eppendorf tube. The cells were then loaded with 2 µl (10µM) CM-H_2DCFDA and incubated in the dark at room temperature for 15 minutes. The cells were centrifuged at 400 rpm for 1 minute. At the end of the centrifugation, the supernatant was removed and cells were suspended in fresh buffer solution (2 ml Tyrode + 1 mM $CaCl_2$). The cells were then placed in a microscope chamber with parallel platinum wires for field stimulation at room temperature. The cells were electrically stimulated at a frequency of 0.2 Hz, with 2ms voltage pulses using an HSE Stimulator (HARVARD, version 1.5, type 223, Hugstetten, Germany) and maintained at 37°C. Contraction of the cells was recorded using a color cooled DXM1200C digital camera (Nikon). Fluorescence was recorded using a bandpass excitation filter centred at 470 ± 20 nm, a dichroic mirror at 500 nm and a 520 nm long pass filter. A single cell was selected, which was rod-shaped and contracting rhythmically in response to stimulation with no spontaneous contractions (see Figure 1).

After collection of baseline data for 1 minute, the cardiomyocytes were exposed to oxidative stress for 15 minutes (using H_2O_2, final concentration 30 and 200 µM). For this study cardiomyocytes were exposed to mild oxidative stress using 30 µM H_2O_2. This micromolar range of ROS was found in both *in vivo* and *in vitro* in the ischemic-reperfused myocardium [17-19]. In addition

cardiomyocytes were also exposed to toxic oxidative stress with 200 µM H_2O_2. This concentration has previously been shown to elicit significant ROS production, contractile dysfunction and cell death [3]. Fluorescent images were collected using 40x Plan Fluor ELWD N.A. 0.6 W.D.3.7mm on a NIKON ECLIPSE Ti. Video recordings were made for 6 to 15 minutes. The video data acquired were analysed using NIS - Elements AR 3.10 software (Frame time: 0.5 second, gain: 2X). Experimental data were obtained on 6 to 8 cardiac cells per rat and 3 rats per group. Since the total fluorescence can vary with the amount of dye loaded in the cell, the data were first normalised to the initial fluorescence intensity and then the changes were measured as a function of time.

Evaluation of the microscope capacity to record the changes in fluorescence due to the generation of ROS was achieved using negative and positive controls according to the manufacturer's instructions (Invitrogen). Two Negative controls were obtained by examining (1) unstained cells for autofluorescence in the green emission range in high calcium (1 mM $CaCl_2$) and low calcium (0.15 mM $CaCl_2$) buffer. (2) Cells loaded with 10 µM dye (CM-H_2DCFDA), in high calcium (1 mM $CaCl_2$) and low calcium (0.15 mM $CaCl_2$) buffer. Positive controls were obtained using cells loaded with 10 µM dye and exposed to 100 µM H_2O_2 in high and low calcium buffer solution.

2.5. Measurement of $[Ca^{++}]_i$

Intracellular calcium $[Ca^{++}]_i$ was measured using Fura Red™, AM, cell permeant according to the manufacturer's instructions (Invitrogen). 2 ml of buffer containing cells was pipetted into an Eppendorf tube. Then loaded with 1.6 µl (4 µM) Fura Red™, AM and incubated in the dark at room temperature for 15 minutes. The cells were centrifuged at 400 rpm for 1 minute. At the end

of the centrifugation, the supernatant was discarded and cells were suspended in fresh buffer solution (2 ml Tyrode + 1 mM $CaCl_2$).

The heart cells were used as soon as possible after isolation. Fluorescent intensities were measured using the NIKON ECLIPSE Ti fluorescent microscope. The filter set used was the same as for the ROS measurements (see section above). A change in fluorescence level is correlated to $[Ca^{++}]_i$, which is recorded using NIS - Elements AR 3.10 software as described in section 2.4 above. Fura Red™, AM, provides capacity for ratiometric measurement of Ca^{++} in single cells [20].

The calcium dye responsiveness has been assessed following the manufacturer's instructions (Invitrogen). Fifty µg of Fura Red™ were dissolved in 50 µL DMSO. Fifty µL of methanol was added to the previous dye solution. 25 µL of 2 M KOH/water was added. The solution was allowed to stand for an hour and then the pH was adjusted to ~7 with HCl. The fluorescence response was tested by diluting 5 µL of the dye solution into 100 µL of Milli-Q water. Then this was added separately to high calcium 1 mM $CaCl_2$, low calcium 0.15 mM $CaCl_2$ and no calcium buffer solutions in a final volume of 3 ml, respectively.

The NIS element software separates the wavelength based on their RGB colours into blue, green and red pixels but with no indication of the relationships between wavelength and RGB colours. Therefore, we tested the dynamic range of the system by imaging one solution containing free fluorophore and one containing Ca^{++} bound fluorophores and determined the green to red pixel ratios for both solutions (Fig. 2).

2.6. Measurement of cell length

Cell contraction was evaluated at room temperature by measuring the length of the cells at rest and during contraction and then expressed as a percentage of the resting cell length. Stimulation of the cells and video recording were performed as described in section 2.4. The recorded videos were analysed frame by frame and the cell length measured using the NIS - Elements AR 3.10 software.

2.7. Data Analysis

Statistical analyses for ROS and $[Ca^{++}]_i$ were calculated using the bootstrap package in R [21]. A bootstrap function was defined to produce replicates and the statistical comparisons between data groups were expressed as Boot Mean (Mean of the differences) and 95% Confidence Intervals. Each plotted line in the subsequent figures represents the mean of the differences and 95% Confidence Intervals between two groups. When there are no differences, between the two groups, the data point will fall on the zero line. Data points deviating from the zero line indicate that there are differences between the "test" group and the other group (represented by the zero axis line). Deviation above the line indicates higher levels and below the line indicates lower levels in the "test" group. Cell length data expressed as Mean ± S.E.M. The differences between means were considered significant at $p<0.05$.

3. Results

3.1. Comparison between cardiomyocytes isolated from female and male rats

3.1.1. Measurement of intracellular ROS production

Figure 3 shows that the normalised fluorescence intensity of CM-H_2DCFDA for control is significantly lower in female cardiomyocytes than male (represented by the zero axis)

cardiomyocytes after 3 to 6 minutes stimulation in the presence of 30 μM H_2O_2. However, this difference decreases with time and the generation of ROS in the female group (as reported by CM-H_2DCFDA fluorescence) is similar to the male group after 12 minutes of stimulation. However, when exposed to a higher concentration of H_2O_2 (200 μM), the female group generates less ROS than the male group even after 12 minutes of stimulation, although the differences decreased slightly with stimulation time.

When the rats were fed methionine, the female cardiomyocytes showed a significantly lower production of ROS than male cardiomyocytes during oxidative stress generated by the presence of 30 μM or 200 μM H_2O_2 with no significant changes over the time of stimulation (Fig. 4).

3.1.2. Measurement of $[Ca^{++}]_i$

Figure 5 shows that $[Ca^{++}]_i$ is significantly increased in the control group of stimulated female cardiomyocytes when compared to male (represented by the zero axis) cardiomyocytes during oxidative stress induced by 30 μM or 200 μM H_2O_2.

When the rats were fed methionine, the female group showed similar levels of $[Ca^{++}]_i$ under oxidative stress in presence of 30 μM H_2O_2 compared to the male group at the beginning of the stimulation (Fig. 6). However, with increasing stimulation time, the female group showed a progressive increase in $[Ca^{++}]_i$ compared to the male cardiomyocytes, which became significantly different after 9 minutes of stimulation.

When the oxidative stress was induced by 200 μM H_2O_2, (Fig. 6) there was significantly higher $[Ca^{++}]_i$ in the female cardiomyocytes compared to the male group.

3.1.3 Measurement of cell shortening

While there was no significant differences between the different groups and treatments, oxidative stress resulted in decreased cardiomyocyte shortening for the female methionine group (Fig. 7, panel A) and male control group (Fig. 7, panel B). In addition, in male the methionine treatment also resulted in a decreased cardiomyocytes shortening but with no further significant decrease under oxidative stress (Fig. 7, panel B).

4. Discussion

In this study, we have presented new data showing that female cardiomyocytes produce significantly less ROS than male cells from both control and methionine fed groups under acute oxidative stress in both 30 and 200 μM H_2O_2 (Figs. 3 and 4). This could be due to the role of oestrogen in protecting cardiomyocytes in females, suggesting that females have a characteristic cardioprotective advantage. These findings are in agreement with studies that show the risk of cardiomyocyte apoptosis in normal and cardiac failure is higher in men than women [22-24]. It is also known that basal Hcy levels are higher in male than in female rats [25], which might explain their higher ROS production.

While lower $[Ca^{++}]_i$ has been reported in female compared to male cardiomyocytes under control conditions [26], during acute oxidative stress we observe a larger increase in $[Ca^{++}]_i$ in female compared to male in both control and methionine groups (Figs. 5 and 6). Oxidative stress has been shown to increase $[Ca^{++}]_i$ in correlation with the dose of H_2O_2 applied to the cardiomyocytes which is due to calcium influx through the sarcolemmal membrane (SLM), calcium release from the sarcoplasmic reticulum (SR) or other intracellular Ca^{++} store sites depending on the dose of H_2O_2 [26]. However, the latter study did not investigate the influence of gender on $[Ca^{++}]_i$ upon

incubation with H_2O_2 and the mechanisms involved in the difference reported in this study will need to be further investigated.

The contractility of the female cardiomyocytes in acute oxidative stress is not modified while there is a decreased contractility (although not significant) in male cardiomyocytes (Fig. 7, panels A and B). Also, the contractility in methionine fed female cardiomyocytes is identical to the control, while in male there is a significant decrease in contractility. Only when acute oxidative stress is induced in addition to the methionine feeding is contractility in female cardiomyocytes significantly reduced. Therefore, it is possible that in female rats the increased calcium levels observed upon oxidative stress may be a mechanism which helps maintain contractility of the female cardiomyocytes in the presence of ROS while these mechanisms (if present) are quickly overwhelmed in male cardiomyocytes. One possible mechanism to interpret the reduction in heart cell shortening in Hhcy is a defect in sensitivity of myofilaments to Ca^{++}. This is in agreement with Zivkovic *et al* [27] who found that isolated rat heart contractility is reduced by acute administration of homocysteine.

These results describe disturbances in cellular function in terms of ROS production, $[Ca^{++}]_i$ and cell contractility under oxidative stress and methionine fed conditions. In addition, the data provide new insights regarding sex differences. One limitation of this study results from the lack of perfusion during the imaging. This was necessary to avoid movement of the cells during the time of the experiments.

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Disclosures

The authors declare no conflicts of interest.

Geolocation Information

The work carried out in this manuscript was performed in Australia.

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Figure legends

Fig. 1: Shows 2 isolated cardiomyocytes that have been loaded with 10 μM of the fluorescent dye, CM-H_2DCFDA.

Fig. 2: Shows the relationship between green to red pixel ratio and % calcium bound.

Fig. 3: Mean differences in cardiomyocytes ROS production under stimulation in the female group compared to the male group as a function of stimulation time. Data shown are Bootstrap Mean of differences and 95% Confidence Intervals calculated using R (n=6-8) cells. Male groups for each treatment are represented by the zero axis. * $p < 0.05$ and ** $p < 0.01$ vs the male group at this time point.

Fig. 4: Mean differences in cardiomyocytes ROS production under stimulation in the female methionine group compared to the male group as a function of stimulation time. Data shown are Bootstrap Mean of differences and 95% Confidence Intervals calculated using R (n=6-8) cells. Male groups for each treatment are represented by the zero axis. * $p < 0.05$ and ** $p < 0.01$ vs the male group at this time point.

Fig. 5: Mean differences in intracellular Ca^{++} concentration under stimulation in the female group compared to the male group as a function of stimulation time. Data shown are Bootstrap Mean of differences and 95% Confidence Intervals calculated using R (n=6-8) cells. Male

groups for each treatment are represented by the zero axis. * $p < 0.05$ and ** $p < 0.01$ vs the male group at this time point.

Fig. 6: Mean differences of intracellular Ca^{++} concentration under stimulation in the female methionine group compared to the male as a function of stimulation time. Data shown are Bootstrap Mean of differences and 95% Confidence Intervals calculated using R (n=6-8) cells. Male groups for each treatment are represented by the zero axis. * $p < 0.05$ vs the male group at this time point.

Fig. 7: Cell contraction in rat heart cells isolated from control and methionine groups in response to stimulation at 0.2 Hz under normal and oxidative stress conditions with 200 µM H_2O_2. A. Female B. male. Cell contraction is expressed as a % of diastolic cell length. Data shown are the means ± SEM (n=6-8) cells from three hearts for each group.

Figure 1

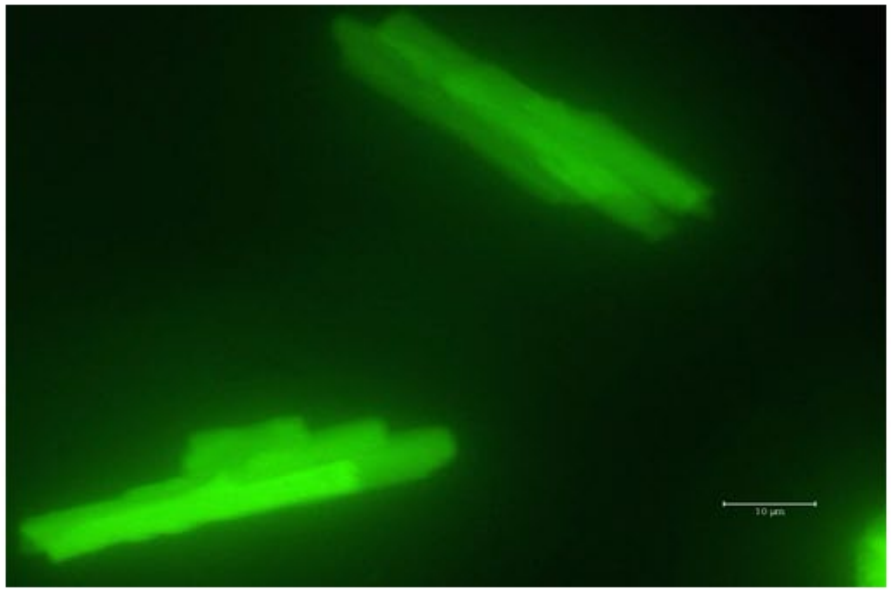
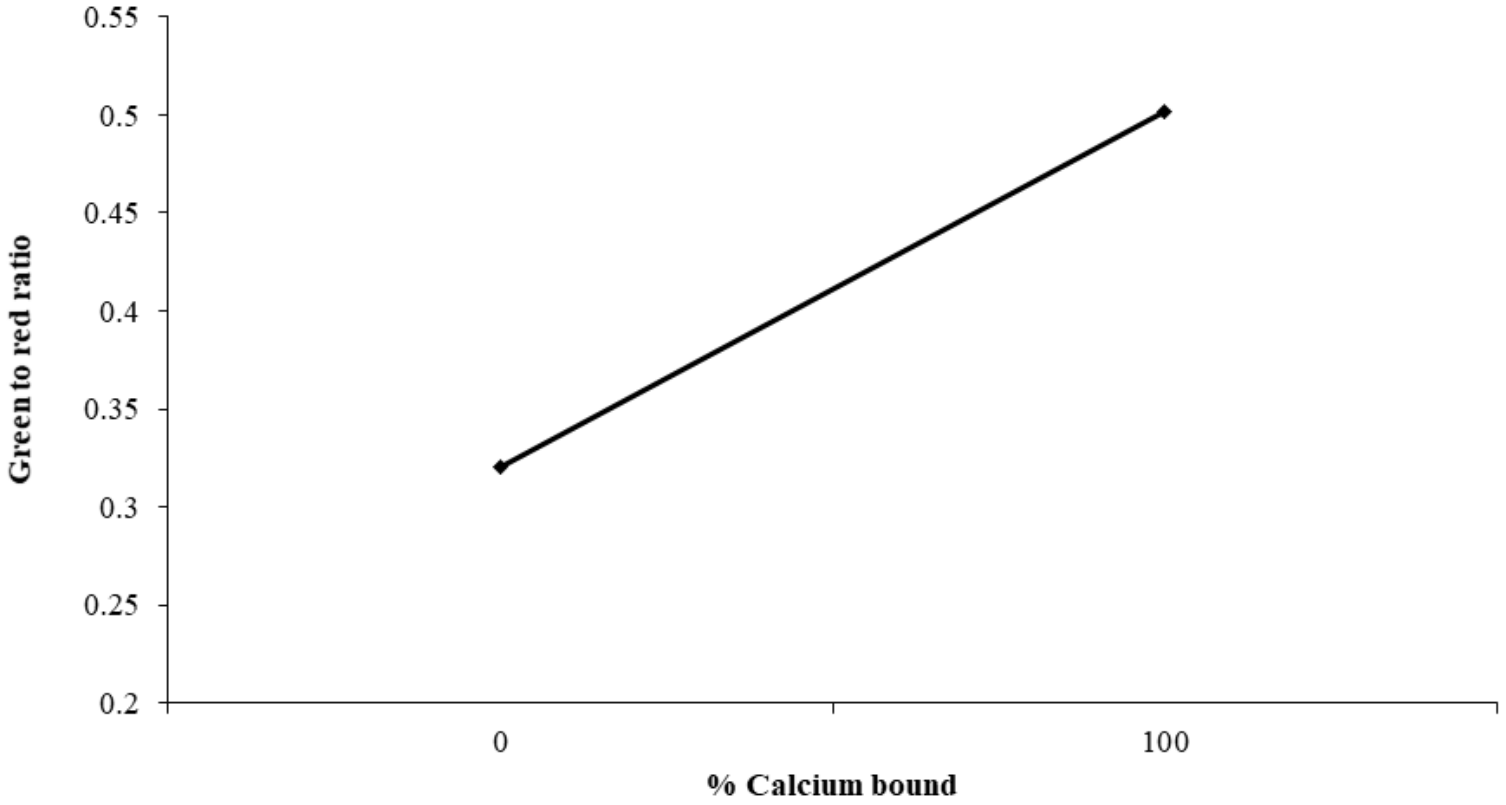


Figure 2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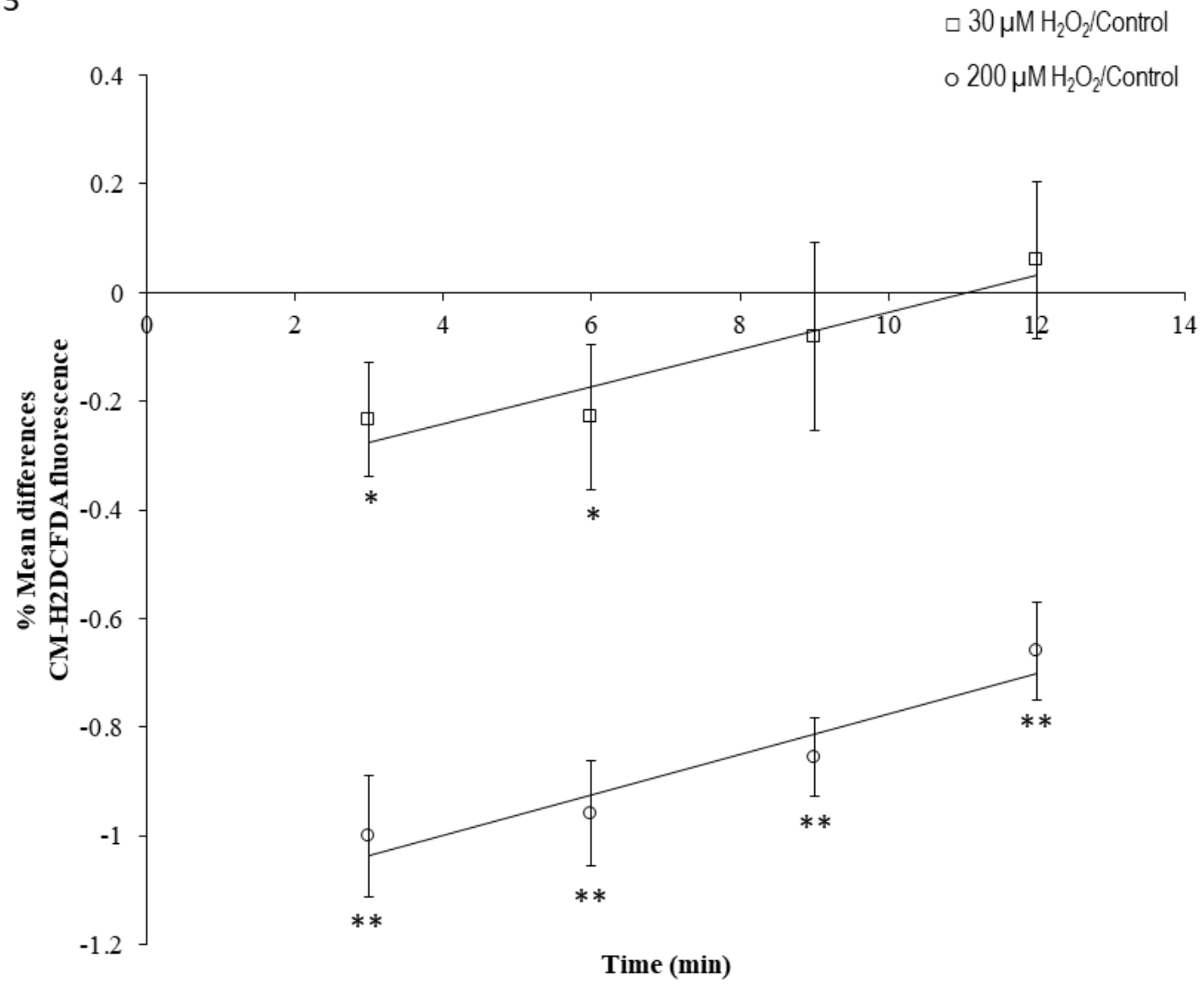
0.5
0.45
0.4
0.35
0.3
0.25
0.2
Green to red ratio
0
100
% Calcium bound

Figure 3


□ 30 µM H2O2/Control
○ 200 µM H2O2/Control
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0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
0
2
4
6
8
10
12
14
% Mean differences
CM-H2DCFDA fluorescence
Time (min)
*
*
**
**
**
**

Figure 4

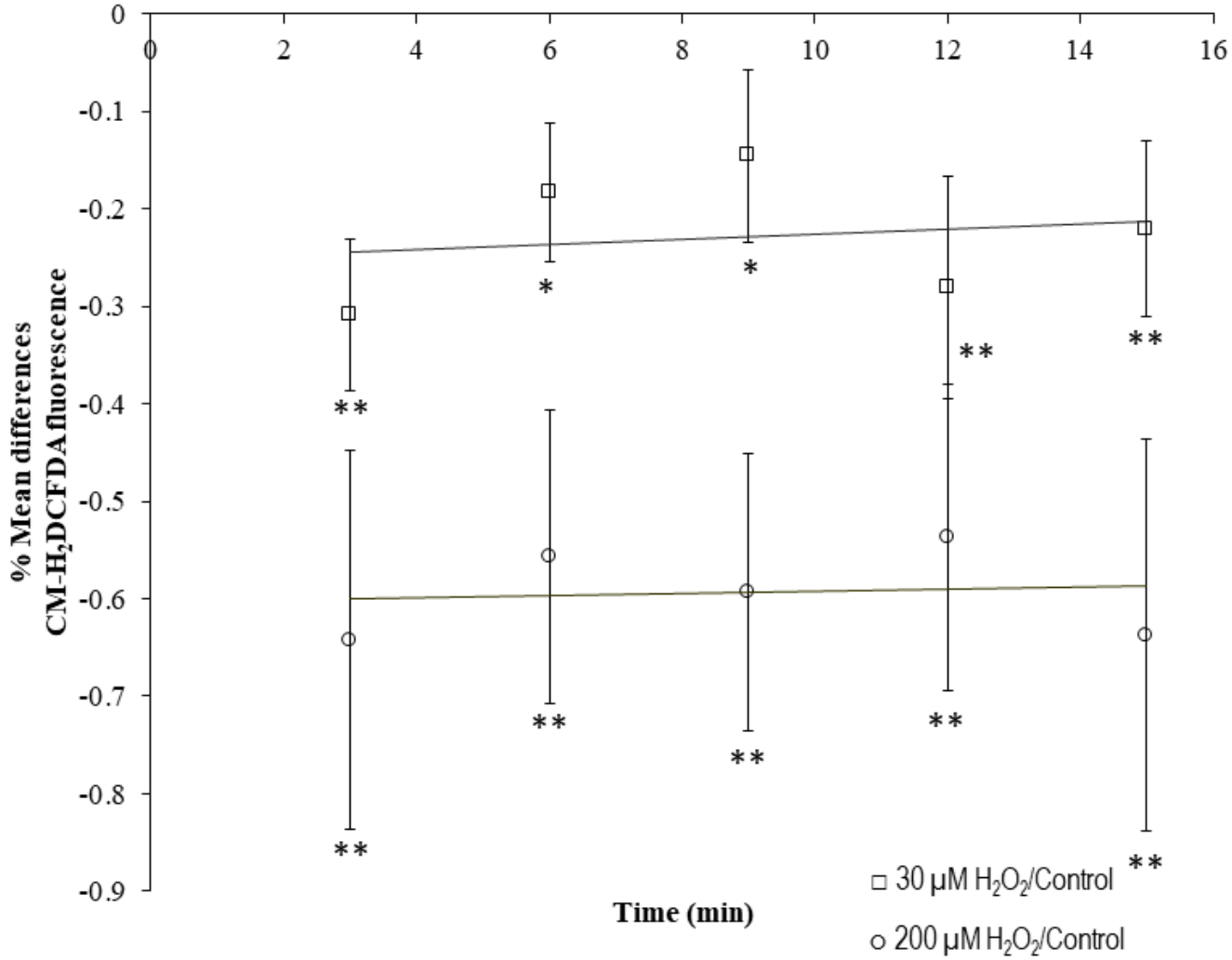
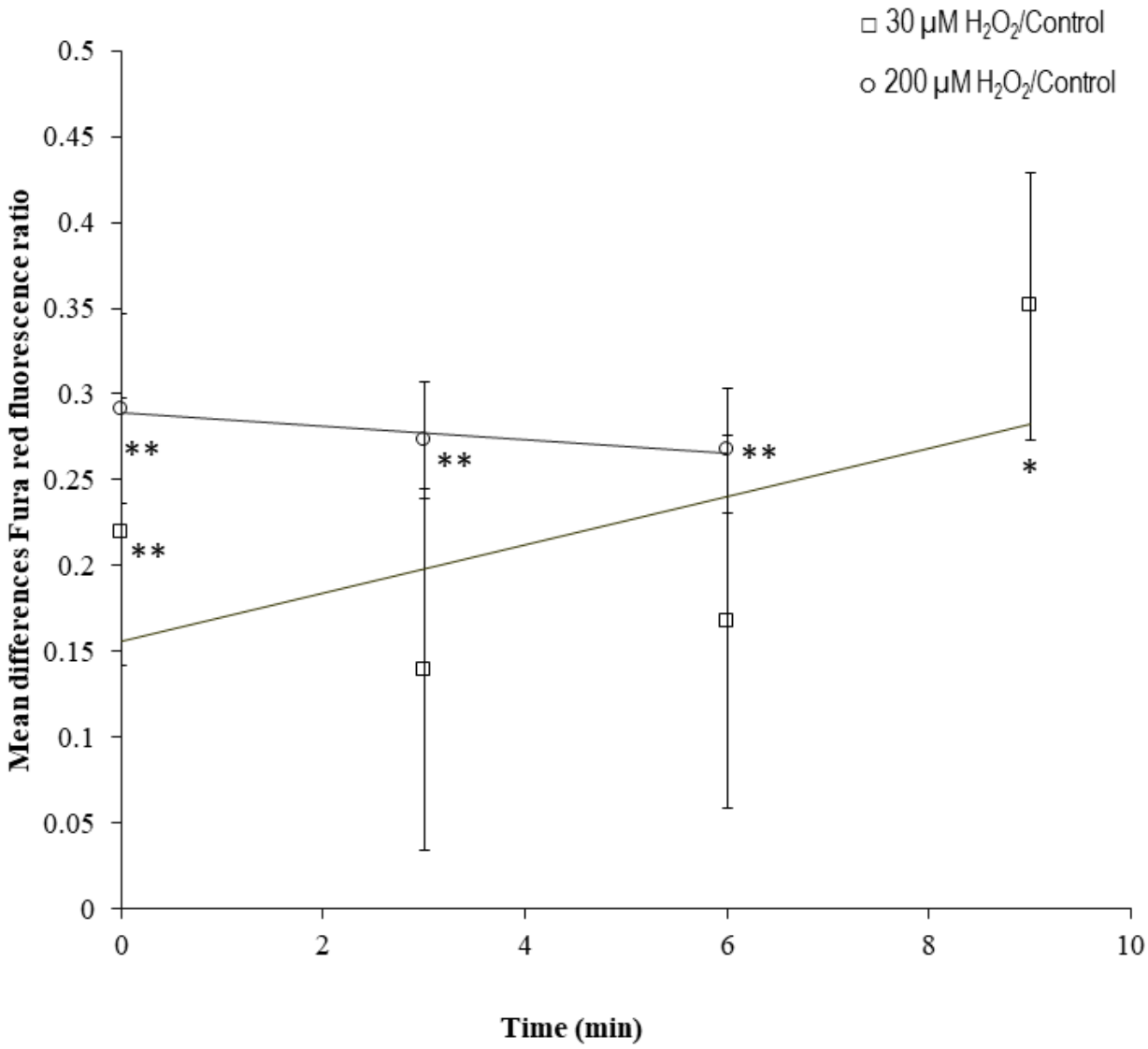

0
0
2
4
6
8
10
12
14
16
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
% Mean differences
CM-H2DCFDA fluorescence
Time (min)
□ 30 µM H2O2/Control
○ 200 µM H2O2/Control

Figure 5


□ 30 µM H_2O_2/Control
○ 200 µM H_2O_2/Control
Mean differences Fura red fluorescence ratio
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
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0
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2
4
6
8
10
Time (min)
**
**
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**
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Figure 6

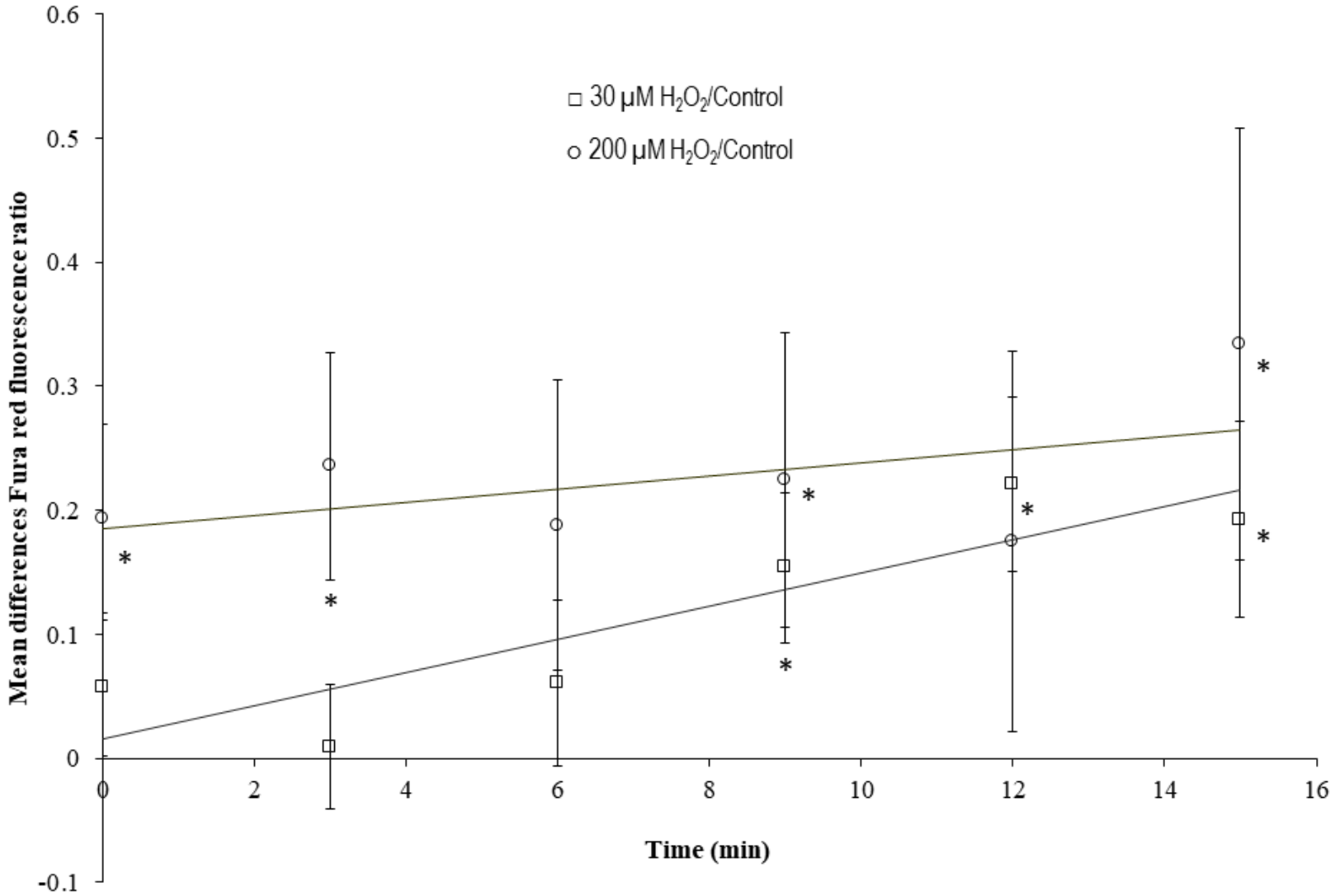
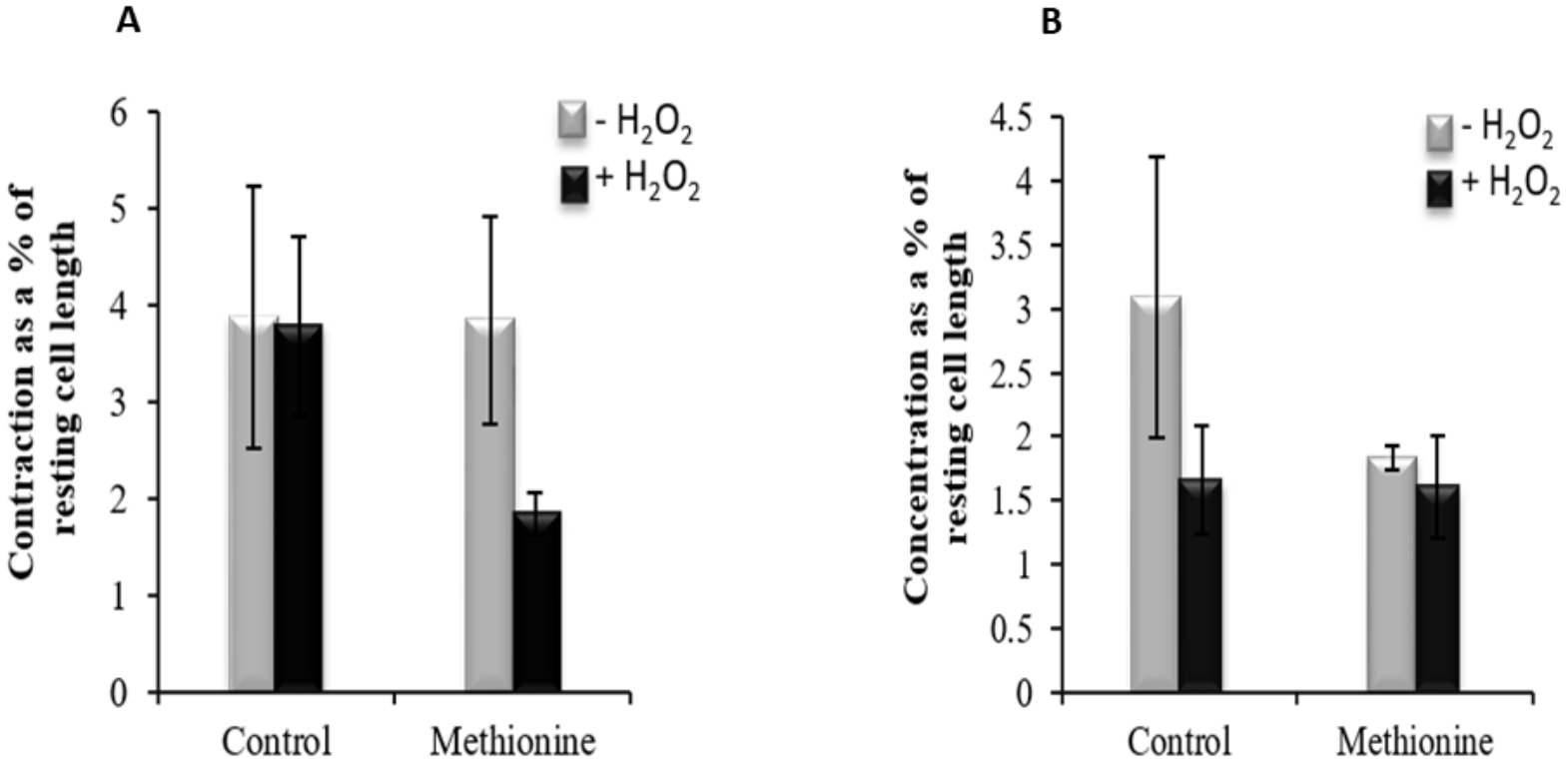

□ 30 µM H_2O_2/Control
○ 200 µM H_2O_2/Control
Mean differences Fura red fluorescence ratio
Time (min)
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
0
2
4
6
8
10
12
14
16

Figure 7


A
Contraction as a % of resting cell length
6
5
4
3
2
1
0
- H_2O_2
+ H_2O_2
Control
Methionine
B
Concentration as a % of resting cell length
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
- H_2O_2
+ H_2O_2
Control
Methionine