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Determination of Zn, Cu, Fe and Mn in Muscle Cells as Potential Markers of Oxidative Stress by Laser Ablation and Solution Based ICP-MS

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Abstract

Oxidative stress is imbalance between oxidant and antioxidant levels in living systems. Human cells are protected from reactive oxygen species (ROS) by endogenous enzymatic antioxidants, such as superoxide dismutase (SOD) and catalase. Most of these compounds require particular redox metals in their structures as cofactors to allow them to scavenge the free radicals, in particular Cu, Zn or Mn-with SOD and Fe with catalase. The aim of this study was to quantify these metals in human cells to evaluate their effectiveness as novel biomarkers for measuring oxidative stress. The metals (Zn, Cu, Fe, Mn) were measured *in vitro* in skeletal muscle cells (C_2C_{12}) which were incubated under hypoxia or hyperoxia conditions generated by varying oxygen levels from 1% - 60% for 24 and 48 hours. Two methods were used to perform the analyses. Solution-based inductively coupled plasma mass spectrometry (ICP-MS) was applied to quantify Zn, Cu, Fe and Mn in cell populations, and laser ablation (LA)-ICP-MS was employed to compare their relative levels in individual cells. The data acquired from both techniques were positively correlated confirming the validity of the two approaches. The results showed that the concentration of the measured elements increased dramatically in cells grown at 25% - 60% O_2, the most significant increase being in Cu at 60% O_2. None showed any increase at 5% - 15% O_2, indicating normoxia states. At 1% O_2, all elements, except Fe, showed a significant increase and the most remarkable growth was in Mn. Increasing the incubation time to 48 hours had differing effects on the elements. Zn and Cu concentrations were unaffected by increasing incubation time except at 60% O_2 where they showed further growth. In contrast, Mn concentration grew sharply for oxygen levels of 30% - 50% with no further effect at 1%, while Fe concentration decreased at 1% O_2 and grew steadily for oxygen levels of 5% - 60%. It can be concluded that all four elements were significantly affected by stress conditions applied to cells, but at different rates. Importantly, this paper describes a novel method for estimating oxidative stress in cells based on the determination of redox elements in single cells and cell populations using ICP-MS.

Keywords: biomarkers of oxidative stress, hypoxia, ICP-MS, laser ablation-ICP-MS, muscle cells, oxidative stress, single cell analysis

1. Introduction

Free radicals are chemically reactive species that possess one or more unpaired electrons. They are normally formed in living organisms and most of them are oxidants. They are scavenged by antioxidant compounds including vitamins and enzymes (Bloomer et al., 2006, Radovanovic et al., 2009). However, their generation is increased by certain conditions of physical and psychological stress, like exercise, leading to intracellular oxidative stress, an imbalance between oxidant and antioxidant levels in living systems (Bloomer & Smith, 2009, Bloomer, Ferebee, Fisher-Wellman, Quindry & Schilling, 2009). Human cells are protected from excess production of reactive oxygen species (ROS) by the body's antioxidant system. This defense system consists of endogenous and exogenous compounds. Superoxide dismutase and glutathione peroxidase are two examples of endogenous enzymatic antioxidants (Bloomer & Fisher-Wellman, 2008, Fisher-Wellman & Bloomer, 2009). Physiological function is positively or negatively affected by the important balance between ROS production and removal, which determines the intracellular redox state of living cells. Chronic exposure to excessive formation of ROS can potentially lead to a shift in the intracellular redox balance towards a more oxidative state, causing oxidative damage of biomolecules, inflammation and many chronic diseases (Bloomer & Fisher-Wellman, 2008, Cutler, Plummer, Chowdhury & Heward, 2005, Fisher-Wellman & Bloomer, 2009).

A variety of oxidative stressors can cause overproduction of free radicals, such as physical activities in which the consumption of molecular oxygen is increased (Bloomer & Fisher-Wellman, 2008, Fisher-Wellman & Bloomer, 2009). Moreover, many factors affect the formation of free radicals during physical activity such as intensity, duration, type of exercise and the amount of oxygen consumed. When the intensity and duration of physical activity increase, the oxidative damage to surrounding tissues is elevated as the defense compounds are no longer adequate (Fisher-Wellman & Bloomer, 2009).

ROS are generated from many sources during exercise such as the mitochondrial respiratory chain (Martinovic et al., 2009). It has been shown that during intense exercise oxygen consumption greatly increases, for example, the muscles' oxygen consumption is as much as 100-200 times greater than at rest. This means that the mitochondrial respiration in the active muscles is noticeably increased and as a consequence this rapid respiration can enhance the electron leakage from the electron transfer chain leading to an increase in ROS formation (Chevion et al., 2003, Nikolaidis et al., 2008, Sjodin, Westing & Apple, 1990).

In vitro, cells are commonly grown at 21% O_2 (normoxia conditions) which is the same concentration as in air we breathe. However, the levels of oxygen within our tissues are much lower than its concentration in the atmosphere (Bates, 2012). Oxygen pressure in human skeletal muscles is about 30-40 mmHg under normoxia (21% O_2) and this approximately equals 5% O_2 in the cell culture. Accordingly, human skeletal muscle cells should be grown at about 5% O_2 to achieve the physiological normoxic conditions and thus oxygen levels below 5% create hypoxic conditions (Deldicque & Francaux, 2013). In fact, it has been demonstrated that a low oxygen culturing environment (hypoxia) increases the proliferation of cells in culture and increases their lifespan by 25% compared to those grown under normal conditions. Further, it has been reported that cells in reduced oxygen environments grow faster and healthier with less DNA damage and less stress response (Bates, 2012). Interestingly, it has been shown that there are dramatic variations in gene expression profiles and phenotypic changes in cells cultured in these two conditions (normal and hypoxia) (Andrew, 2009). In vivo, the hypoxia state is originally generated by environmental conditions (high altitude or exercise) or pathological conditions (pulmonary disease or severe anemia) (Jamieson, Chance & Cadenas, 1986). It has been reported that hyperoxia (high oxygen availability) can also occur during severe-intensity exercise in humans and affect the muscle metabolic responses (Vanhatalo, Fulford, Dimenna & Jones, 2010).

Oxidative damage can be measured by biomarkers whose structures are harmfully affected by ROS and accordingly changed such as lipids, proteins and DNA (Mateos & Bravo, 2007, Powers & Jackson, 2008). Lipid peroxidation has been evaluated by measuring malondialdehyde (Fan, 2002, Mateos, Goya & Bravo, 2004, Rimbach et al., 1999, Wilson, Metz, Graver & Rao, 1997), and F_2-isoprostane (Dorjgochoo et al., 2012, Halliwell & Lee, 2010, Liu, Morrow & Yin, 2009, Milne et al., 2007, Nikolaidis, Kyparos & Vrabas, 2011). Determination of carbonyl groups is the method most widely used for measuring oxidative damage on proteins (Levine et al., 1990, Mateos & Bravo, 2007). The quantification of nucleotide 8-hydroxy-2-deoxyguanosine is the most frequently used method to measure DNA modification induced by free radicals (Crow et al., 2008, Henriksen, Hillestrom, Poulsen & Weimann 2009, Hu, Huang, Li & Chao, 2010, Mei, Yao, Wu & Xu, 2005). Additionally, the activity of enzymatic antioxidants, for example, superoxide dismutase (SOD) (Deitrich et al., 2010, Girotti et al., 2000, Ordóñez et al., 2011), and catalase (Firuzi et al., 2013, Trivić, Drid, Obadov & Ostojic, 2011) has been widely quantified in many investigations. This method is used to evaluate the antioxidant system at rest as well as after physical activity (Stanković & Radovanović, 2012). The concentration of these antioxidant compounds reflects their formation and consumption during oxidative stress (Girotti et al., 2000). Indeed, quantification of SOD can indicate the extent of free radical formation. There are numerous methods to measure this antioxidant enzyme (Girotti et al., 2000, Usui et al., 1991, Wang et al., 2015).

Unfortunately, none of the biomarkers mentioned above is sufficient to discover the potential role of oxidative stress in the pathogenic mechanisms of human diseases (Mateos & Bravo, 2007, Powers & Jackson, 2008). In addition, analytical methods applied for their measurement should be chemically robust, repeatable and with high sensitivity and low detection limit to compare between reference and changed values in living organisms. It would be highly beneficial to identify an ideal biomarker as well as an optimal method to assess oxidative stress (Nikolaidis, Kyparos & Vrabas, 2011). Trace biological elements like Zn, Cu, Mn and Fe can be used as oxidative stress markers due to their important role as cofactors of the antioxidant enzymes. Cu, Zn-superoxide dismutase and (Fe) catalase are two examples of the most important enzymatic antioxidants that protect human cells against intracellular oxidative stress. Their concentration depends on their formation and consumption during oxidative stress, resulting in an increase or decrease in their corresponding cofactor's level (Girotti et al., 2000). Several analytical techniques have been developed for the qualitative and quantitative study of metals in biological systems. ICP-MS is one of the most commonly used techniques because of its high sensitivity and low sample consumption (Usui et al., 1991).

2. Method

2.1 Instrumentation and Operating Parameters

For solution analyses, a Thermo Scientific ICAP-Qc quadrupole ICP mass spectrometer was used. A collision-reaction cell employing helium is fitted to reduce/remove interferences. A glass concentric nebulizer and a cyclonic spray chamber (Glass Expansion, Australia) were fitted with the instrument. A 0.25 mm I.D. probe (Elemental Scientific, USA) was used. For laser ablation analyses, a sector-field ICP-MS instrument (Element 2XR, Thermo Scientific, Germany) was coupled to a UP-213 laser ablation system (Electro Scientific Industries, UK). A tear-drop shaped cell, which has been described elsewhere (Horstwood, Foster, Parrish, Noble & Nowell, 2003), was fitted with the laser system. Helium was used for ablation at a flow rate of 0.55 L/min and introduced with argon through a Y shaped connector to the ICP-MS instrument. The operating parameters employed are shown in Table 1. Zn^{66}, Cu^{63}, Mn^{55} and Fe^{57} were measured in all cell samples which were incubated under stress conditions for 24/48 hours.

Table 1. Typical Operating Conditions for ICP-MS and LA-ICP-MS analyses

Parameter	ICP-MS	LA-ICP-MS
ICP radio frequency power, W	1550	1050
Cool gas flow rate, L/min	14	15.75
Auxiliary gas flow rate, L/min	0.8	0.9
Sample gas flow rate, L/min	1.1	0.985
Sample uptake time (s)	90	
Torch horizontal position	0.66	5
Torch vertical position	-0.11	3.4
Ablation gas flow, L/min		0.55 (He)
Laser spot size, μm		25
Repetition frequency, Hz		1 (single shot)
Laser output (power setting), %		50 for Zn, 60 for Cu, Fe and Mn

2.2 Cell Culture and Hypoxic/Hyperoxic Experiments

All cell culture work was carried out inside a Heraeus biological safety cabinet (Class II). Hypoxic and hyperoxic conditions were applied to cultured cells by employing a hypoxic incubator (Sanyo, O_2/CO_2 incubator, MCO-5M). A normal incubator (Thermo Scientific, Heracell 240i, CO_2 Incubator) was used to incubate all control cells. A similar temperature and level of CO_2 (37^0C and 5%) were maintained in both incubators. The cell line C_2C_{12} human skeletal muscle cells was used. Dulbecco's modified Eagle's medium (DMEM) with high glucose, 4.0mM L-glutamine and sodium pyruvate was used as a growth medium (Fisher Scientific, UK). It was supplemented with 20% fetal bovine serum (Dutscher Scientific, UK) and 1% penicillin-streptomycin solution (Fisher Scientific, UK).

In order to perform hypoxic/hyperoxic experiments, a known number of cells were added to two T-flasks containing a warm growth medium which were incubated in a normal incubator under normal conditions for 24 hours to allow the cells to attach to the surface of the flasks. The growth medium was changed on the following day before one of the two flasks was moved into the hypoxia incubator for 24 or 48 hours to become confluent at a certain concentration of oxygen as required. The other T-flask was returned to the normal incubator at 21% O_2 for 24 or 48 hours to be used as control samples. Twelve different concentrations of oxygen, from 1% to 60% at 5% intervals, were applied to the muscle cells by using the hypoxic incubator. All experiments were repeated twice (1A and 1B for 24 hours and 2A and 2B for 48 hours) by using new batches of cells.

2.3 Sample Preparation for Solution Based Work

All samples for solution based work were prepared by following a method used in other investigations to digest human cells (Managh et al., 2013). Briefly, after 24/48 hours, the cells, stressed and controls, were harvested by using trypsin and counted by a haemocytometer in order to prepare replicate samples for each oxygen level. One million cultured cells were added to 2 ml Eppendorf tubes and then centrifuged at 2000 rpm for 5 minutes at 4^0C. Then the growth medium was removed to obtain a dry pellet which was digested by concentrated nitric acid and hydrogen peroxide for 5 hours at 70^0C. The Eppendorf tubes were then placed into a Turbo Vap (Biotage, Sweden) to remove all remaining oxidizing agents by using a nitrogen gas stream at 60^0C for approximately half an hour. Finally, all the microtubes were stored in the fridge until they were analysed a few days later by ICP-MS. On the day of the analysis, 2 ml of 2% HNO_3

was added to each sample and mixed well. Two internal standards, rhodium and iridium, were added to each sample at a final concentration of 10 ppb. ICP-MS multi-element solution 2, SPEX CertiPrep (UK) was used to make all standard solutions.

2.4 Sample Preparation for Laser Ablation

Generally, after the cultured cells were counted they were split into two parts, one for solution work, as explained above, and the other part was used for laser work. 1x 10^5 each of the control cells and cells incubated under stress conditions were transferred to 15 ml metal free, screw capped centrifuge tubes (VWR International Ltd, UK), kept cool in an ice bath, before distributing the cells on a microscope slide using a Cytospin instrument (Hettich Universal 320R, DJB labcare, UK). Cell samples were transferred to angle Cytospin chambers of area 120 mm ²with filter cards (DJB labcare, UK) to mount the cells onto two types of slides (glass microscopic slides coated by nail polish and quartz slides, as described below). The CytoSpin was set at operating conditions of 2000 rpm, 5 minutes and 18^0 C to give an even distribution of cells across the slide. 5x10^4 of both kinds of cells (control and stressed) were mounted on both kinds of slides to give 2 equal samples for each type of cells.

2.5 Optimization of the Method Employed for Laser Analysis

The most challenging issue encountered for analysis by laser ablation-ICP-MS was the background of the four traces elements of interest in the microscopic slides (25.4 x 76.2 mm, 1mm-1.2mm thick, Scientific Glass Laboratories Ltd). It was found that their background was too high in the glass slides leading to interference with the signals obtained from the single cells. Therefore, the glass slides were coated with a monolayer of a transparent nail polish (Maybelline, Superstay, gel nail colour 59), which is Zn free, in order to cover the slides with a material that can absorb the laser radiation and prevent its absorption by slides. A mixture of 50:50 acetone and nail polish was applied to the slide by using the Cytospin to form a homogenous monolayer. This allowed higher laser fluence (laser output and spot size) to be applied to ablate the cells as given in Table 1. In addition to the coated glass slides, quartz slides (Agar Scientific Ltd, UK), which are relatively expensive but contamination free, were used for laser analyses under the same operating conditions.

3. Results and Discussion

3.1 Cell Population Analysis by ICP-MS

There was no significant difference between the results obtained from the two replicate experiments (1A & 1B) as confirmed by a paired t-test at the 5% significance level. The percentage change in the concentration of each element was calculated at each oxygen level by comparing between the stressed and control cell samples as shown in Figure 1 for Cu. Each stressed cell sample has its own matched control cell sample taken from the same batch, same passage number, at the same time, to enable valid comparison.

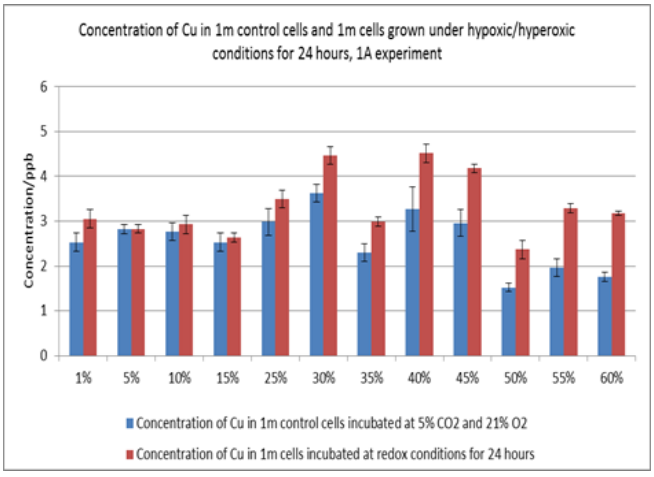

Figure 1. Concentration of Cu in 10^6 control cells and 10^6 cells grown under hypoxic/hyperoxic conditions for 24 hours, experiment (1A). Error bars show the standard deviation of 3 replicate measurements. X axis is oxygen levels

The concentrations of the four elements had increased dramatically in cells grown in hyperoxia conditions for 24 hours as shown for Zn in Figure 2. Figure 3 shows the pooled percentage of change in the four elements concentration after 24 hours incubation under hypoxia/hyperoxia conditions. It shows that all elements exhibited a gradual growth from 25% - 60% O_2 except Mn which started increasing from 30% with no change at 25% O_2. The most significant increase was in

Cu as it had gone up by 85% at 60% O_2 and then Mn by 43%. Zn and Fe showed lower increases at 60% O_2 of 27% and 26%, respectively. Importantly, none of the elements showed any increase at 5% O_2, indicating this to be a normoxia state for all of them. All elements, except Fe, showed a significant increase at 1% O_2 and the most remarkable growth was in Mn as it increased by 33%, then Cu by 21% with Zn showing the least increase of 17%.

Interestingly, Zn and Cu showed a small increase at 15% O_2 by 11% and 5%, respectively, whereas there was no noticeable change for Mn and Fe. None of the elements showed any significant change at 10% O_2 except Cu which had grown by 5% before going up sharply at 25% O_2. This change at 15% O_2 was unexpected as 15% is within the normoxia range 5% - 21% where the cells should not show any change. However, assays performed on cell populations may provide unexpected results which are difficult to interpret due to averaging of signals from many cells. In addition, it has been stated elsewhere that cells which are genetically identical may vary in their responses to stimuli (Di Carlo, Tse & Gossett, 2012).

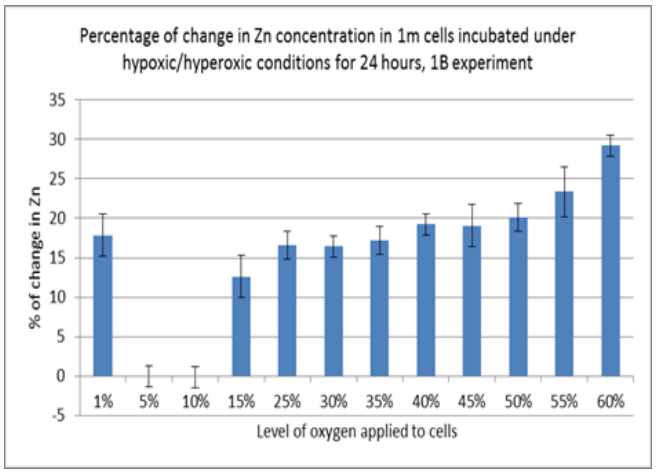

Figure 2. Percentage change in Zn concentration between stressed and control cells in 1m cells grown under oxidative stress for 24 hours, 1B experiment, error bars show the standard error (n = 5)

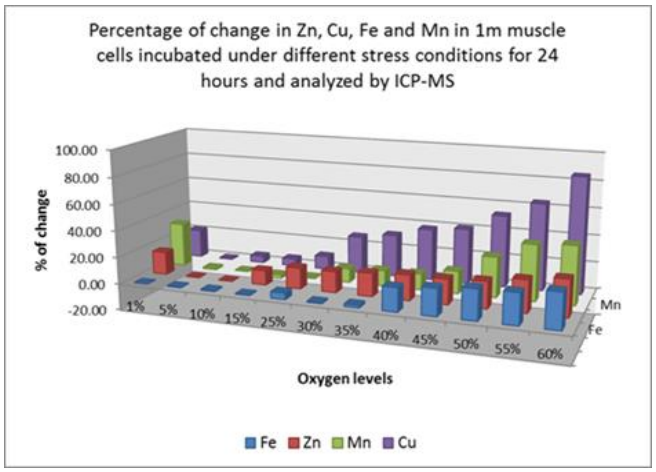

Figure 3. Pooled percentage change in the concentration of the four elements of interest in cells incubated under different levels of oxygen (hypoxia/hyperoxia conditions) for 24 hours (experiment 1A (n=3) and experiment 1B (n=5))

The cells proliferated slowly and weakly at high oxygen levels when they were incubated for 48 hours but no dead cells were found. The four elements responded differently to the 48 hours incubation, compared with 24 hours, under oxidative stress as shown in Figure 4. Zn concentration showed the same increases obtained after 24 hours incubation, except at 1% and 60% O_2 where it grew further to reach 22% and 39%, respectively, after 48 hours, increases of 5% and 12% over the 24 hour results. Similarly, the increase in the duration of cells' incubation had no further impact on Cu concentration at most levels of oxygen, with an average increase of 32% after 24 hours. However, the changes at 60% and 15% O_2 had further increased after 48 hours by 22% and 13%, respectively.

Surprisingly after 48 hours, Fe showed a decrease in its concentration by 30% at 1% O_2 after showing no change at this level after 24 hours. After 48 hours incubation, Fe concentration increased steadily at lower oxygen levels up to a 10% increase at 35% O_2, then more rapidly at higher oxygen levels, the overall increase leveling off at around 50% between

40% and 60% O_2. Thus, it is clear that Fe concentration was influenced by increasing the interval of incubation as it had changed over the four oxygen levels of (1% - 15%) whereas there was no noticeable change after 24 hours. As had been observed for Cu, Mn did not show any further change at 1% O_2 after the duration of incubation was increased to 48 hours. However, its concentration grew sharply by 35% after 48 hours over oxygen levels of 30%-50% and remained stable with no further effect at 55% and 60%.

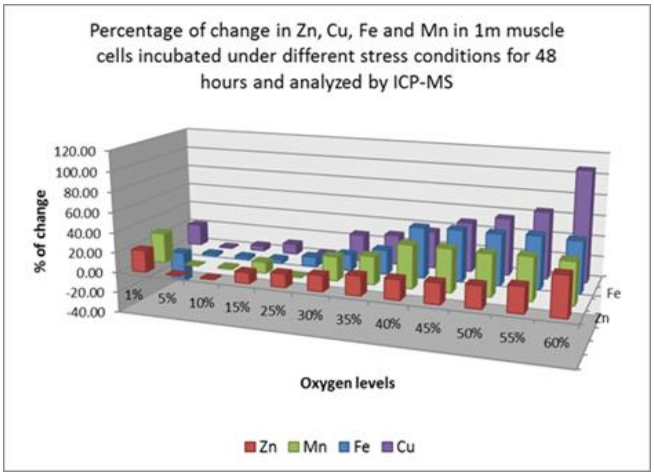

Figure 4. Pooled percentage change in the concentration of the four elements of interest in cells incubated under different levels of oxygen (hypoxia/hyperoxia conditions) for 48 hours (experiments 2A & 2B (n = 5 at 1%-25% O_2, n = 3 at 30%-35% O_2, n = 2 at 40%-60% O_2)

3.2 Single Cell Analysis by Laser Ablation-ICP-MS

All the elements except Mn were successfully detected in single cells incubated under hypoxia/hyperoxia conditions for 24 hours as shown in Figure 5 for Zn.

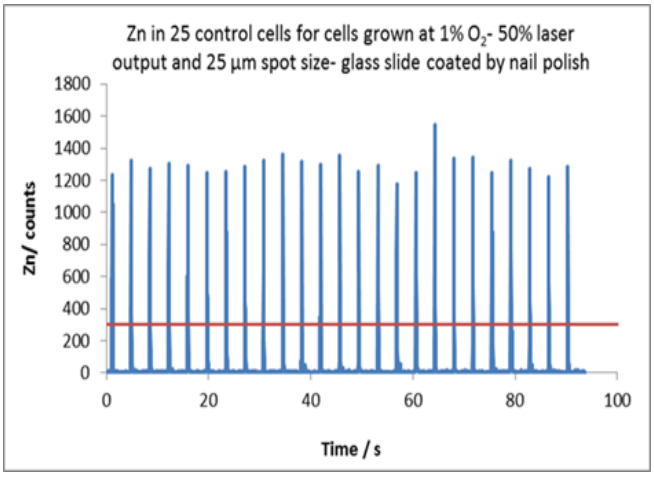

Figure 5. Signal intensities of Zn obtained when LA-ICP-MS was performed on 25 single control cells, the red line is the threshold at 300 counts

Zn, Cu and Fe exhibited a significant change in cells exposed to different stress environments as shown in Figure 6. Seven oxygen levels (1%, 5%, 10%, 30%, 40%, 50%, and 60%) were employed to generate stress conditions in cells. The two types of slides used produced the same results for all four elements at all oxygen levels as confirmed by a paired t-test. Importantly, there was a general variation between individual signals obtained from 100 cells in each sample especially with Fe where the variation was significant.

Zn, Cu and Fe showed a gradual growth in the cells incubated under oxygen levels of 30% - 60%. The most significant overall increase between 40% and 60% O_2 was in Cu by an average of 41% and then in Zn and Fe by 25% and 21%, respectively. Equally, Cu produced the highest increase at 1% O_2 by an average of 29% and then Zn by 20% whereas Fe showed a slight decrease of 5% which can possibly be ignored due to the significant variation between signals (RSD =

23%). Similarly to Fe at 1% O_2, Cu and Zn showed a very slight decrease of less than 10% at 5% and 10% O_2 which can be neglected if the variation among 100 single cells in each sample is taken into consideration, as the RSD for Cu and Zn at 5% O_2 was 6% and 8%, and that at 10% O_2 was 7% and 8%, respectively. Indeed, the average measurements can be ambiguous if the signals obtained from both kinds of cells, control and stressed, are variable over the same range of counts and accordingly the difference between them is not clearly seen. The same can be applied to Fe at these two levels of oxygen as it showed a change by an average of 2%. Since the variability of Fe signals is the most significant at all oxygen levels (RSD = 19%), so this slight change in Fe at 5% oxygen can also be ignored. Therefore, 5% and 10% oxygen are normoxia states for intracellular Zn, Cu and Fe and their intensities were not significantly changed in the cells exposed to these three oxygen levels.

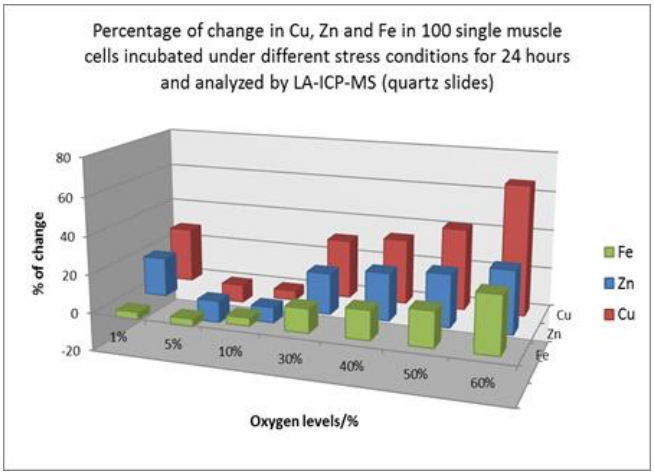

Figure 6. Percentages change in Cu, Zn and Fe in 100 single muscle cells incubated under different levels of oxygen (hypoxia/hyperoxia conditions) for 24 hours, mounted on quartz slides and analysed by LA-ICP-MS

4. Conclusion

In summary, this current study demonstrated that trace elemental analysis based on ICP-MS and LA-ICP-MS can be efficiently applied to measure intracellular oxidative stress in skeletal muscle cells exposed to hypoxia or hyperoxia conditions. Zn, Cu, Fe and Mn were successfully quantified as oxidative damage biomarkers in cell populations by utilizing ICP-MS as well as comparing their concentrations in individual cells by using LA-ICP-MS. The data acquired from both techniques are positively correlated. The results of this current study demonstrated that the intracellular concentrations of the four elements of interest were affected by changing oxygen levels as they all showed a dramatic increase at high oxygen levels (30% - 60%), albeit at different rates for each element. They also showed a change at 1% O_2 indicating hypoxia conditions but no significant change at 5%-15% O_2 indicating these levels to be normoxia environments for these elements. More importantly, the percentages of change in Zn, Cu and Fe recorded from both techniques were consistent and demonstrated a strong positive correlation coefficient (R); for Zn (0.98), Cu (0.95) and Fe (0.9). This finding shows the reliability of the method proposed, i.e. to use these vital cofactors for enzymatic antioxidant compounds, which protect human cells against oxidative damage, as oxidative stress biomarkers. The measurement of changes occurring in intracellular elements within skeletal muscle cells exposed to different stress conditions does not appear to have been previously reported. Therefore, the present work is novel and provides a potentially useful marker of oxidative stress conditions.

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References

Andrew, T. (2009). Advantages of Oxygen Control in CO2 Incubation for Research and Clinical Applications. https://doi.org/10.2144/000113035

Bates, M. K. (2012). *Culturing Cells Under Hypoxic Conditions for Biologically Relevant Results*. Retrieved June 02, 2016, from

https://www.americanlaboratory.com/913-Technical-Articles/123131-Culturing-Cells-Under-Hypoxic-Conditions-for-Biologically-Relevant-Results/

Bloomer, R. J., & Fisher-Wellman, K. H. (2008). Blood Oxidative Stress Biomarkers: Influence of Sex, Exercise Training Status, and Dietary Intake. *Gender Medicine*, *5*(3), 218-228. https://doi.org/10.1016/j.genm.2008.07.002

Bloomer, R. J., & Smith, W. A. (2009). Oxidative stress in response to aerobic and anaerobic power testing: influence of exercise training and carnitine supplementation. *Research in Sports Medicine*, *17*(1), 1-16. https://doi.org/10.1080/15438620802678289

Bloomer, R. J., Falvo, M. J., Fry, A. C., Schilling, B. K., Smith, W. A., & Moore, C. A. (2006). Oxidative stress response in trained men following repeated squats or sprints. *Medicine & Science in Sports & Exercise*, *38*(8), 1436-1442. https://doi.org/10.1249/01.mss.0000227408.91474.77

Bloomer, R. J., Ferebee, D. E., Fisher-Wellman, K. H., Quindry, J. C., & Schilling, B. K. (2009). Postprandial oxidative stress: influence of sex and exercise training status. *Medicine & Science in Sports & Exercise*, *41*(12), 2111-2119. https://doi.org/10.1249/mss.0b013e3181a9e832

Chevion, S., Moran, D. S., Heled, Y., Shani, Y., Regev, G., Abbou, B., … Epstein, Y. (2003). Plasma antioxidant status and cell injury after severe physical exercise. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(9), 5119-5123. https://doi.org/10.1073/pnas.0831097100

Crow, B., Bishop, M., Kovalcik, K., Norton, D., George, J., & Bralley, J. A. (2008). A simple and cost effective method for the quantification of 8-hydroxy-2'-deoxyguanosine from urine using liquid chromatography tandem mass spectrometry. *Biomedical Chromatography*, *22*(4), 394-401. https://doi.org/10.1002/bmc.946

Cutler, R. G., Plummer, J., Chowdhury, K., & Heward, C. (2005). Oxidative stress profiling: part II. Theory, technology, and practice. *Annals of the New York Academy of Sciences*, *1055*, 136-158. https://doi.org/10.1196/annals.1323.031

Deitrich, C. L., Braukmann, S., Raab, A., Munro, C., Pioselli, B., Krupp, E. M., … Feldmann, J. (2010). Absolute quantification of superoxide dismutase (SOD) using species-specific isotope dilution analysis. *Analytical and Bioanalytical Chemistry*, *397*(8), 3515-3124. https://doi.org/10.1007/s00216-010-3680-1

Deldicque, L., & Francaux, M. (2013). Acute vs. chronic hypoxia: What are the consequences for skeletal muscle mass? *Cellular and Molecular Exercise Physiology*, *2*(1). https://doi.org/10.7457/cmep.v2i1.e5

Di Carlo, D., Tse, H. T., & Gossett, D. R. (2012). Introduction: why analyze single cells? *Methods in Molecular Biology*, *853*, 1-10. https://doi.org/10.1007/978-1-61779-567-1_1

Dorjgochoo, T., Gao, Y. T., Chow, W. H., Shu, X. O., Yang, G., Cai, Q., … Dai, Q. (2012). Major metabolite of F-2-isoprostane in urine may be a more sensitive biomarker of oxidative stress than isoprostane itself. *American Journal of Clinical Nutrition*, *96*(2), 405-414. https://doi.org/10.3945/ajcn.112.034918

Fan, X. (2002). Measurement of malonaldehyde in apple juice using gc-ms and a comparison to the thiobarbituric acid assay. *Food Chemistry*, *77*(3), 353-359. https://doi.org/10.1016/s0308-8146(02)00110-3

Firuzi, O., Shakibazad, N., Amoozgar, H., Borzoee, M., Abtahi, S., Ajami, G., … Miri, R. (2013). Effects of Omega-3 Polyunsaturated Fatty Acids on Heart Function and Oxidative Stress Biomarkers in Pediatric Patients with Dilated Cardiomyopathy. *International Cardiovascular Research Journal*, *7*(1), 8-14.

Fisher-Wellman, K., & Bloomer, R. J. (2009). Acute exercise and oxidative stress: a 30 year history. *Dynamic Medicine*, *8*, 1. https://doi.org/10.1186/1476-5918-8-1

Girotti, S., Fini, F., Ferri, E., Budini, R., Piazzi, S., & Cantagalli, D. (2000). Determination of superoxide dismutase in erythrocytes by a chemiluminescent assay. *Talanta*, *51*(4), 685-692. https://doi.org/10.1016/s0039-9140(99)00332-x

Halliwell, B., & Lee, C. Y. (2010). Using isoprostanes as biomarkers of oxidative stress: some rarely considered issues. *Antioxidants & Redox Signaling*, *13*(2), 145-156. https://doi.org/10.1089/ars.2009.2934

Henriksen, T., Hillestrom, P. R., Poulsen, H. E., & Weimann, A. (2009). Automated method for the direct analysis of 8-oxo-guanosine and 8-oxo-2 '-deoxyguanosine in human urine using ultraperformance liquid chromatography and tandem mass spectrometry. *Free Radical Biology and Medicine*, *47*(5), 629-635. https://doi.org/10.1016/j.freeradbiomed.2009.06.002

Horstwood, M. S. A., Foster, G. L., Parrish, R. R., Noble, S. R., & Nowell, G. M. (2003). Common-Pb corrected in situ U–Pb accessory mineral geochronology by LA-MC-ICP-MS. *Journal of Analytical Atomic Spectrometry*, *18*(8), 837-846. https://doi.org/10.1039/b304365g

Hu, C. W., Huang, Y. J., Li, Y. J., & Chao, M. R. (2010). Correlation between concentrations of 8-oxo-7,8-dihydro-2'-deoxyguanosine in urine, plasma and saliva measured by on-line solid-phase extraction LC-MS/MS. *International Journal of Clinical Chemistry*, *411*(17-18), 1218-1222. https://doi.org/10.1016/j.cca.2010.04.029

Jamieson, D., Chance, B., Cadenas, E., & Boveris, A. (1986). The relation of free radical production to hyperoxia. *Annual Review of Physiology*, *48*(1), 703-719. https://doi.org/10.1146/annurev.ph.48.030186.003415

Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., … Stadtman, E. R. (1990). Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology*, *186*, 464-478. https://doi.org/10.1016/0076-6879(90)86141-H

Liu, W., Morrow, J. D., & Yin, H. (2009). Quantification of F-2-isoprostanes as a reliable index of oxidative stress in vivo using gas chromatography-mass spectrometry (GC-MS) method. *Free Radical Biology and Medicine*, *47*(8), 1101-1107. https://doi.org/10.1016/j.freeradbiomed.2009.07.028

Managh, A. J., Edwards, S. L., Bushell, A., Wood, K. J., Geissler, E. K., Hutchinson, J., … Sharp, B. L. (2013). Single Cell Tracking of Gadolinium Labeled CD4+ T Cells by Laser Ablation Inductively Coupled Plasma Mass Spectrometry. *Analytical Chemistry*, *85*(22), 10627-10634. https://doi.org/10.1021/ac4022715

Martinovic, J., Dopsaj, V., Dopsaj, M. J., Kotur-Stevuljevic, J., Vujovic, A., Stefanovic, A., & Nesic, G. (2009). Long-term effects of oxidative stress in volleyball players. *International Journal of Sports Medicine*, *30*(12), 851-856. https://doi.org/10.1055/s-0029-1238289

Mateos, R., & Bravo, L. (2007). Chromatographic and electrophoretic methods for the analysis of biomarkers of oxidative damage to macromolecules (DNA, lipids, and proteins). *Journal of Separation Science*, *30*(2), 175-191. https://doi.org/10.1002/jssc.200600314

Mateos, R., Goya, L., & Bravo, L. (2004). Determination of malondialdehyde by liquid chromatography as the 2,4-dinitrophenylhydrazone derivative: a marker for oxidative stress in cell cultures of human hepatoma HepG2. *Journal of Chromatography B*: *Analytical Technologies in the Biomedical and Life Sciences*, *805*(1), 33-39. https://doi.org/10.1016/j.jchromb.2004.02.004

Mei, S., Yao, Q., Wu, C., & Xu, G. (2005). Determination of urinary 8-hydroxy-2'-deoxyguanosine by two approaches-capillary electrophoresis and GC/MS: an assay for in vivo oxidative DNA damage in cancer patients. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *827*(1), 83-87. https://doi.org/10.1016/j.jchromb.2005.04.001

Milne, G. L., Yin, H., Brooks, J. D., Sanchez, S., Roberts, L. J., & Morrow, J. D. (2007). Quantification of F2-isoprostanes in biological fluids and tissues as a measure of oxidant stress. *Methods in Enzymology*, *433*, 113-126. https://doi.org/10.1016/s0076-6879(07)33006-1

Nikolaidis, M. G., Jamurtas, A. Z., Paschalis, V., Fatouros, I. G., Koutedakis, Y., & Kouretas, D. (2008). The Effect of Muscle-Damaging Exercise on Blood and Skeletal Muscle Oxidative Stress. *Sports Medicine*, *38*(7), 579-606. https://doi.org/10.2165/00007256-200838070-00005

Nikolaidis, M. G., Kyparos, A., & Vrabas, I. S. (2011). F-2-isoprostane formation, measurement and interpretation: The role of exercise. *Progress in Lipid Research*, *50*(1), 89-103. https://doi.org/10.1016/j.plipres.2010.10.002

Ordóñez, Y. N., Deitrich, C. L., Montes-Bayón, M., Blanco-González, E., Feldmann, J., & Sanz-Medel, A. (2011). Species specific isotope dilution versus internal standardization strategies for the determination of Cu, Zn-superoxide dismutase in red blood cells. *Journal of Analytical Atomic Spectrometry*, *26*(1), 150-155. https://doi.org/10.1039/c0ja00075b

Powers, S. K., & Jackson, M. J. (2008). Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiological Reviews*, *88*(4), 1243-1276. https://doi.org/10.1152/physrev.00031.2007

Radovanovic, D., Bratic, M., Nurkic, M., Cvetkovic, T., Ignjatovic, A., & Aleksandrovic, M. (2009). Oxidative stress biomarker response to concurrent strength and endurance training. *General physiology and biophysics*, *28*, 205-211.

Rimbach, G., Höhler, D., Fischer, A., Roy, S., Virgili, F., Pallauf, J., & Packer, L. (1999). Methods to assess free radicals and oxidative stress in biological systems. *Archiv für Tierernaehrung*, *52*(3), 203-222. https://doi.org/10.1080/17450399909386163

Sjodin, B., Hellsten, W. Y., & Apple, F. S. (1990). Biochemical Mechanisms for Oxygen Free Radical Formation During Exercise. *Sports Medicine*, *10*(4), 236-254. https://doi.org/10.2165/00007256-199010040-00003

Stanković, M., & Radovanović, D. (2012). Oxidative stress and physical activity. *SportLogia*, *8*(1), 1-11. https://doi.org/10.5550/sgia.120801.en.001s

Trivić, T., Drid, P., Obadov, S., & Ostojic, S. (2011). Effect of endurance training on oxidative stress in male wrestlers. *Journal of Martial Arts Anthropology*, *11*(2), 6-9.

Usui, A., Kato, K., Tsuboi, H., Sone, T., Sassa, H., & Abe, T. (1991). Concentration of Mn-superoxide dismutase in serum in acute myocardial infarction. *Clinical Chemistry*, *37*(3), 458-461.

Vanhatalo, A., Fulford, J., Dimenna, F. J., & Jones, A. M. (2010). Influence of hyperoxia on muscle metabolic responses and the power–duration relationship during severe-intensity exercise in humans: a 31P magnetic resonance spectroscopy study. *Experimental Physiology*, *95*(4), 528–540. https://doi.org/10.1113/expphysiol.2009.050500

Wang, L., Liu, S., Zheng, Z., Pi, Z., Song, F., & Liu, Z. (2015). Rapid assay for testing superoxide anion radical scavenging activities to natural pigments by ultra-high performance liquid chromatography-diode-array detection method. *Analytical Methods*, *7*(4), 1535-1542. https://doi.org/10.1039/c4ay02690j

Wilson, D. W., Metz, H. N., Graver, L. M., & Rao, P. S. (1997). Direct method of quantification of free malondialdehyde with high-performance capillary electrophoresis in biological samples. *Clinical Chemistry*, *43*(10), 1982-1984.