TetraSOD® activates the antioxidant response pathway in human cells: An *in vitro* approach

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ABSTRACT

Combined action of both endogenous and exogenous factors stimulates the production of free radicals and reactive oxygen species (ROS) in cells. They are neutralized by an elaborate antioxidant defence system, superoxide dismutase (SOD) being the first line of enzymes involved in ROS scavenging. However, when ROS production cannot be counteracted by cellular antioxidant mechanisms oxidative stress happens inducing damages in proteins, lipids and DNA. It has been suggested that antioxidant supplementation may help to reduce oxidative stress. TetraSOD® is a unique marine healthy and functional ingredient that is produced under patent-protected technology, which exhibits a balanced nutritional composition and a significantly high SOD activity (>30,000 IU/g). In this work, we examined the antioxidant bioactivity of TetraSOD® in muscle cells (Normal Human Skeletal Muscle Myoblasts, HSMMs) using an *in vitro* approach. We detected an induction of primary antioxidant enzyme activities (SOD, GPx, and CAT) which was further related to gene expression up-regulation of a selected set of genes involved in response against oxidative stress, including *NRF2* and *HMOX1*. Overall, the results obtained in this study represent an important contribution to the understanding of the molecular basis underlying the cytoprotective effects induced by TetraSOD® consumption.

INTRODUCTION

Cells produce reactive oxygen species (ROS) as a consequence of the own metabolic processes, the superoxide anion being the precursor of all other ROS (Romao, 2015). The three main *in vivo* sources for superoxide anion are the mitochondrial respiratory chain complexes, the nicotinamide adenine dinucleotide phosphate-oxidase, and xanthine oxidases. ROS can provoke damages in proteins, lipids and DNA when antioxidant capabilities are overwhelmed by the burden of ROS, a state known as "oxidative stress" (Tebay et al., 2015). To detoxify ROS cells have developed an elaborate antioxidant defence system in which superoxide dismutase (SOD) is the first line of enzymes involved in scavenging of ROS. This enzyme reduces superoxide anion to hydrogen peroxide (H_2O_2), and then glutathione peroxidase (GPx) and catalase (CAT) convert H_2O_2 into H_2O and O_2 (Figure 1).

Occurrence of oxidative stress as a consequence of both endogenous metabolism and exogenous factors (diet, pollution, UV radiation, smoking, mental stress…) is considered to be involved in a number of diseases such as cancer, atherosclerosis, diabetes, chronic inflammation, and neurodegenerative and cognitive disorders (Fiedor and Burda, 2014). In this scenario, dietary factors seem to play a pivotal role in the regulation of the oxidant status as a diet low in antioxidants contributes to oxidative stress (Décordé et al., 2010). A wide range of studies have examined the beneficial effects of antioxidant supplementation to reduce oxidative stress; particularly, SOD is probably one of the antioxidant proteins most extensively addressed (Carillon et al., 2013a; Romao, 2015). In this regard, an increase in endogenous antioxidant enzymes (not only SOD but also GPx and CAT) has been observed after SOD supplementation both at protein and activity levels, supporting the hypothesis that exogenous SOD could induce endogenous antioxidant enzymes (Vouldoukis et al., 2004; Nelson et al., 2006; Carillon et al., 2013a; Carillon et al., 2013b; Carillon et al., 2014; Romao, 2015). However, the molecular mechanisms underlying such induction processes have not been elucidated yet.

TetraSOD® is a unique marine healthy and functional ingredient that is grown under patent-protected technology. In addition to its exhibited high SOD activity (>30,000 IU/g), it shows a balanced nutritional composition containing the three antioxidative enzymes (SOD, GPx, and CAT), essential fatty acids, vitamins, minerals, amino acids and pigments. In a previous work, we assessed *in vitro* the bioactivity of TetraSOD® in muscle (Normal human skeletal muscle myoblasts, HSMMs) cells (Evaluation of the potential bioactivity of TetraSOD® in muscle tissue using an *in vitro* approach; unpublished data). Transcriptional up-regulation of genes *FOXO3* and *SOD1* was observed, known to play a key role in antioxidant response. We have now determined the effect of TetraSOD® in the three main antioxidant enzyme activities (SOD, GPx and CAT) in HSMMs cells. Moreover, to gain additional insights on the TetraSOD® effect at molecular level, we have analyzed the expression profiles of a selected set of genes encoding for proteins involved in protection against oxidative stress: i) the three distinct identified isoforms of SOD in mammals. Two of the isoforms have Cu and Zn in the catalytic center and exhibit different localization, being either intracellular (Cu,Zn-SOD or SOD1) or extracellular (EC-SOD or SOD3). The last isoform has Mn as a cofactor (Mn-SOD or SOD2) and is localized to mitochondria (Zelko et al., 2002; Sheng et al., 2014); ii) glutathione peroxidases,

which couple oxidation of glutathione with detoxification of H_2O_2. The family comprised up to eight isoforms in mammals. Among them, both GPx1 and GPx4 are ubiquitous as have been identified in the cytosol, nucleus and mitochondria of the cells, and also exhibit a wide tissue distribution (Margis et al., 2008; Espinosa-Diez et al., 2015); iii) the human catalase, which is an heme-containing peroxidase that forms a tetramer composed of four subunits, each one divided in four domains, and plays a predominant role in controlling the concentration of H_2O_2 (Putnam et al., 2000; Goyal and Basak, 2010); iv) the heme oxygenase-1 (HMOX1) inducible phase II detoxifying enzyme, which is considered an important component involved in the adaptive and protective response to multiple oxidative insults (Solano and Arck, 2015; Liao et al., 2018); v) the nuclear factor erythroid 2-related factor 2 (NRF2), which is a transcription factor that under physiological conditions is sequestered by binding to Kelch-like ECH associated protein (KEAP1), thus inhibiting translocation of NRF2 into the nucleus. When KEAP1 changes its conformation due to interaction with different inducers, NRF2 is released and translocated to the nucleus, regulating the cytoprotective response to oxidative stress through transcriptional activation of phase II detoxifying and antioxidant enzymes (Dinkova-Kostova and Talalay, 2008; Tkachev et al., 2011). Main results of this research are presented hereinafter.

MATERIAL AND METHODS

Microalgae biomass (TetraSOD®)

TetraSOD® was produced by Fitoplancton Marino, S.L. using a patent protected technology. It corresponds to a freeze-dried powder of the microalgae species *Tetraselmis chuii* (belonging to Fitoplancton Marino Culture Collection), which is grown under outdoors conditions ensuring high SOD activity (>30,000 IU/g). Briefly, starter culture for the photobioreactors is grown in a thermoregulated room at 22 ºC with a 24 h light photoperiod provided by artificial daylight illumination (150 μEm^2s^{-1}). Cultures are up-scaled from an initial 50 mL flasks with f/2 culture medium in natural seawater at a salinity of 33 psu and air bubbled with an addition of 2% CO_2. Once the culture reaches the stationary phase it is used to inoculate 1 L culture flask. This process of scaling up in volume is repeated until 50 L culture is reached, which is then used to inoculate outdoor photobioreactors. These photobioreactors are automatically controlled with pH and temperature probes, and operate as a fed-batch mode to avoid nutrient limitation. Biomass is harvested when the culture reaches the mid-log phase and is further freeze-dried to render TetraSOD® final product.

Extract preparation and SOD activity determination in TetraSOD®

Extract was prepared from TetraSOD® using a proprietary methodology. A 10% dry weight solution in phosphate buffer (KH_2PO_4 200 mM pH=7.8) was disrupted using a high pressure homogenizer. Cell debris was removed by centrifugation at 20,000 g for 15 min at 4 ºC, and the supernatant was recovered and stored at -80 ºC. SOD activity was quantified using the SOD Assay Kit-WST (Dojindo) according to the Technical

Manual. The inhibition rate was determined by a kinetic method using a time range between 2-10 min, in which a very high linearity was observed (R^2>0.99).

Cell culture and incubation with TetraSOD® extract

Normal human skeletal muscle myoblasts (HSMMs, Clonetics™) were purchased from Lonza (Walkersville, USA) and cultured according to instructions provided by Lonza. They were grown in SkGM™-2 BulletKit™ medium in a humidified incubator at 37 °C with a 5% CO_2 atmosphere. Cells were subcultured when they were 50%-70% confluent and contained many mitotic figures throughout the flask.
Cells were incubated in 6-well plates for 24 h with TetraSOD® extract prepared at different dilutions (2 ml total volume per well) containing 30, 150 and 1500 IU per ml of culture media. As Control treatment, phosphate buffer was employed. Afterwards, cells were rinsed twice with PBS and directly processed for enzyme activities determination or RNA isolation as described below. The experiment was repeated twice, and in each one, treatments were performed in triplicate.

SOD, GPx and CAT activity determination in HSMMs cells

Cells were detached by gentle treatment with Trypsin/EDTA solution provided with the ReagentPack™ Subculture Reagents (Clonetics™). After cells were released, trypsin was neutralized with Trypsin Neutralizing Solution (Clonetics™). Thereafter, cells were washed twice in ice cold phosphate buffer by centrifugation at 220 g for 5 min at 4 ºC. Then cells were resuspended in phosphate buffer containing 1% peroxide-free Triton X-100. Complete lysis was achieved by softly pipetting up and down several times. Finally, cell lysate was centrifuged at 20,000 g for 10 min at 4 ºC, and the supernatant was recovered and immediately stored at -80 ºC until use.
SOD activity was quantified using the SOD Assay Kit-WST (Dojindo) according to the Technical Manual as previously indicated. GPx activity (nmol/min/ml) was quantified using the Glutathione peroxidase activity kit (Enzo) as described in the Manuel provided by the manufacturer. CAT activity (nmol/min/ml) was determined using the Catalase Assay Kit (Cayman Chemical) following the kit booklet. In all instances, activity values were transformed to percentages in relation to Control (without TetraSOD® incubation).

RNA isolation and gene expression analysis

Total RNA was isolated from HSMMs cells using the NucleoSpin® RNA Kit (Macherey-Nagel) in accordance with the manufacturer's protocols. A double on column DNase I treatment for 30 min was performed to avoid amplification of genomic DNA. Quality of RNA samples was first checked in agarose gels, and then A_{260}/A_{280} and A_{260}/A_{230} ratios (>2.1) were determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific). Total RNA (1 µg) from each sample was reverse-transcribed with the iScript™ cDNA Synthesis kit (Bio-Rad) following the manufacturer's protocol. Lack of genomic DNA contamination was confirmed by PCR amplification of RNA samples in the absence of cDNA synthesis.

Real-time analysis was carried out on a CFX96™ Real-Time System (Bio-Rad) using specific primers for *SOD1*, *SOD2*, *SOD3*, *GPx1*, *GPx4*, *CAT*, *HMOX1* and *NRF2* (Table 1). Real-time reactions were accomplished in a 10-µL volume containing cDNA generated from 10 ng of original RNA template, 300 nM each of specific forward and reverse primers, and 5 µl of iQ™ SYBR® Green Supermix (Bio-Rad). The amplification protocol included an initial 7 min denaturation and enzyme activation step at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Each PCR assay was done in duplicate. For normalization of cDNA loading, all samples were run in parallel with the reference gene beta actin (*ACTB*) as previously reported (Letsiou et al., 2017). Relative mRNA expression was determined using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Results were expressed as mean ± SD. Significant differences were determined with the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test. These analyses were performed using the Prism *v*6 software (GraphPad Software). Significance was accepted at $p<0.05$.

RESULTS

1. Antioxidant enzyme activities after TetraSOD® treatment

We have analyzed the effect of TetraSOD® on the three antioxidant enzyme activities SOD, GPx and CAT. In all instances, the lowest dose of TetraSOD® (30 IU) did no provoke a statistically significant change in activity when compared to the Control. However, in both 150 IU and 1500 IU treatments a significant increase was detected in the three activities. In SOD, the increase ranged around 34% in 150 IU and 37% in 1500 IU in relation to the Control. In GPx activity we observed the highest variation, ranging between 83% and 72% at 150 IU and 1500 IU, respectively. And in CAT, the increase in activity values ranged between 42% and 37% at 150 IU and 1500 IU, respectively (Figure 2).

2. TetraSOD® induces up-regulation of SOD1 and SOD2, but not of SOD3

Incubation of HSMMs with TetraSOD® extract up-regulated *SOD1* at the three doses employed (Figure 3), which is in agreement with previous results (Evaluation of the potential bioactivity of TetraSOD® in muscle tissue using an *in vitro* approach; unpublished data). With 30 IU per ml, a significant 4.49-fold increase in mRNAs with regard to the Control was observed. At 150 IU it increased to 4.32-fold, and it was 3.80-fold higher (although not significant with regard to the Control) at 1500 IU. No significant differences were found between any of the TetraSOD® treatments. Incubation of HSMMs cells with TetraSOD® also up-regulated *SOD2* at the three concentrations tested, with an increase in transcript amounts ranging between 2.02 at 1500 IU and 2.14 at 150 IU, with no statistically significant differences between treatments. In contrast to

SOD1 and *SOD2*, *SOD3* did not exhibit differences in mRNA abundance between any of the treatments and the Control.

3. GPx1 but not GPx4 expression is activated by TetraSOD®

TetraSOD® induced a significant up-regulation of *GPx1* at both 150 IU (2.14-fold higher than Control) and 1500 IU (2.23-fold increase). At 30 IU, a 1.85-fold increase was measured, although it was not statistically significant in relation to the Control. In contrast, no significant changes were detected in *GPx4* between any of the treatments and the Control (Figure 4).

4. TetraSOD® up-regulates CAT expression

Treatment of HSMMs cells with both 150 IU and 1500 IU of TetraSOD® provoked a significant increase in mRNA levels of *CAT* with regard to the Control (1.47-fold and 1.55-fold, respectively). A non-statistically significant 1.17-fold increase in transcripts was observed at 30 IU, which was significant when compared to 1500 IU treatment (Figure 5).

5. NRF2 and HMOX1 expression are induced by TetraSOD®

When HMMS cells were incubated with TetraSOD® at 30 IU, no significant changes in *NRF2* transcripts were detected (Figure 6). In contrast, a significant increase in mRNAs was measured both at 150 IU (2.05-fold) and 1500 IU (2.04-fold). Moreover, a significant up-regulation of *HMOX1* was observed after treatment with 30 and 150 IU, exhibiting 1.19- and 1.54-fold higher transcript amounts than the Control. However, no significant differences were observed at the highest TetraSOD® dose

DISCUSSION

Oxidative stress occurs when molecular defence systems are not able to counteract oxidation caused by both endogenous processes (such as mitochondrial metabolism) and/or exogenous factors including chemicals, pollution or diet. Hence, it reflects an imbalance of any biological system to detoxify ROS or repair resulting damages in cell components. In the present study, we have investigated the antioxidant effect of TetraSOD® using an *in vitro* approach in HSMMs cells, and further explore the underlying molecular mechanisms of antioxidant protection. In this regard, we have observed a significant induction in the activity of the antioxidant enzymes SOD, GPx and CAT after TetraSOD® treatment. In this regard, a literature searched revealed a wide range of compounds of different nature (peptides, chemical reagents, natural extracts…) able to induce activity of antioxidant enzymes in different cell systems. For instance, pretreatment of hippocampus cells with the cocaine- and amphetamine-regulated transcript (CART) peptide prevented the oxidative damage induced by amyloid-beta microinjection through a mechanism involving up-regulation of SOD activity (Jiao et al., 2018). An increase in not only SOD but also GPx activity has been observed in response

to oxidative damage induced by UV-A on normal human dermal fibroblasts after treatment with polypeptide from *Chlamys farreri* (Han et al., 2004). SOD activity was also up-regulated in human skin fibroblasts treated with the flavonoid baicalin in response to UV-A radiation (Zhou et al., 2012). Similar effects have been reported regarding SOD and CAT activity in human umbilical vein endothelial cells pretreated with pterostilbene (an homologous derivative of resveratrol) and further cultured under oxidative stress induced by uraemic serum (Chen et al., 2018). In addition, pretreatment of human HepG2 cells with the alkaloid epigoitrin increased SOD and GPx activity after exposure to H_2O_2 (Huo et al., 2017). All these responses found in cells have been associated to a general activation effect of antioxidant systems, in a complex scenario in which a battery of cytoprotective/defensive proteins including SOD, CAT and GPx are being up-regulated by the different compounds and treatments.

In addition to activities, the analysis of transcriptional response of genes encoding the antioxidant enzymes and the further regulation of polypeptide translation have been less addressed. Unravelling this link is a key factor in order to understand the molecular mechanisms of cellular response against oxidative stress For instance, a coordinated response in the induction of SOD, CAT and GPx activity, protein quantities and transcript amounts of *CAT*, *SOD1* and *GPx1* has been observed in HepG2 cells pretreated with the anthocyanin pelargonidin and then subjected to oxidative stress with citrinin (Babu et al., 2017). Similarly, human luteinized granulosa cells under oxidative stress induced by both H_2O_2 and dimethylfumarates exhibited a significant increase in *SOD1* and *CAT* mRNAs and their respective polypeptides (Akino et al., 2018). In this work, we have also found a parallel and positive response in both enzyme activities and transcripts for SOD (*SOD1* and *SOD2*), CAT and GPx (*GPx1*) encoding genes in HSMMs cells as a consequence of TetraSOD® treatment. Thus, present results unravel the molecular basis of the cytoprotective effect of TetraSOD® in relation to the primary antioxidant enzymes.

In an attempt to go deeper in the knowledge of basal transcriptional regulation of antioxidant enzyme-encoding genes, we then studied the response to TetraSOD® treatment of genes *NRF2* and *HMOX-1*. NRF2 is a transcription factor widely expressed in eukaryotic cells which is known to activate antioxidant defences. Actually, a number of reports have demonstrated that it is induced by antioxidant and chemopreventive compounds, which in turn regulates the expression of genes encoding both primary enzymes (that is, SOD, GPx, and CAT) and inducible phase II detoxifying enzymes such as HMOX1 (Krajka-Kuźniak et al., 2015; Fetoni et al., 2015; Tebay et al., 2015; Babu et al., 2017; Bahar et al., 2017; Hong et al., 2017; Liu et al., 2017; Teixeira et al., 2017; Chen et al., 2018; Wang et al., 2018). Our results have demonstrated up-regulation of both *NRF2* and *HMOX1* expression when HSMMs cells are incubated with TetraSOD®, which strongly suggests the participation of the NRF2/HMOX1 pathway in the increased expression of *SOD*, *GPx* and *CAT* genes, and hence in the protective antioxidant status induced by TetraSOD®. Additional regulation at translational level of primary antioxidant enzymes has not been addressed in this work, and hence cannot be ruled out in the absence of more data.

Cellular protection against oxidative damage involves two types of antioxidants: “direct” antioxidants, which can undergo redox reactions and scavenge ROS intermediates, and “indirect” antioxidants, which are inducers of cytoprotective proteins (Dinkova-Kostova

and Talalay, 2008). We have demonstrated that TetraSOD® exhibits a significantly high SOD activity, as well as GPx and CAT activities. The action of these enzymes might contribute to scavenging of extracellular ROS owing to their own antioxidant activities. But what is more important, TetraSOD® treatment induces a key set of genes belonging to the antioxidant machinery present in eukaryotic cells. *In vivo*, it has suggested that the antioxidant actions observed after consumption of a SOD-enriched product elaborated from melon might be triggered by a cascade of events possibly involving the NRF2 pathway (Carillon et al., 2013a; Carillon et al., 2013b; Carillon et al., 2014). Our present *in vitro* results with TetraSOD® fully agree with that hypothesis and represent an important contribution to the understanding of the more complex physiological protective and preventive effects derived from TetraSOD® consumption.

CONCLUSIONS

We can conclude from results presented in this work that:

1. TetraSOD® induces a coordinated activation of the primary antioxidant enzymes (SOD, GPx and CAT) in cells, with selected genes being additionally regulated at transcriptional level.
2. The NRF2/HMOX-1 antioxidant pathway is targeted as playing a pivotal role in the cellular response to TetraSOD® treatment.

FIGURES

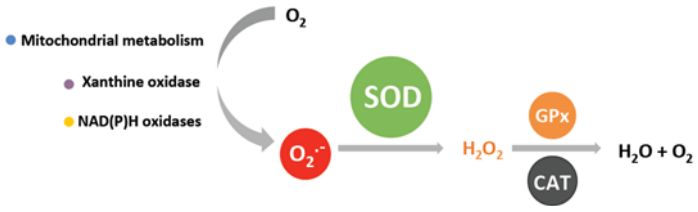

Figure 1. Action of SOD to control anion superoxide. Further reactions are needed to remove H_2O_2 derived from superoxide anion by catalase (CAT) and glutathione peroxidase (GPx) (TetraSOD® dossier, 2017).

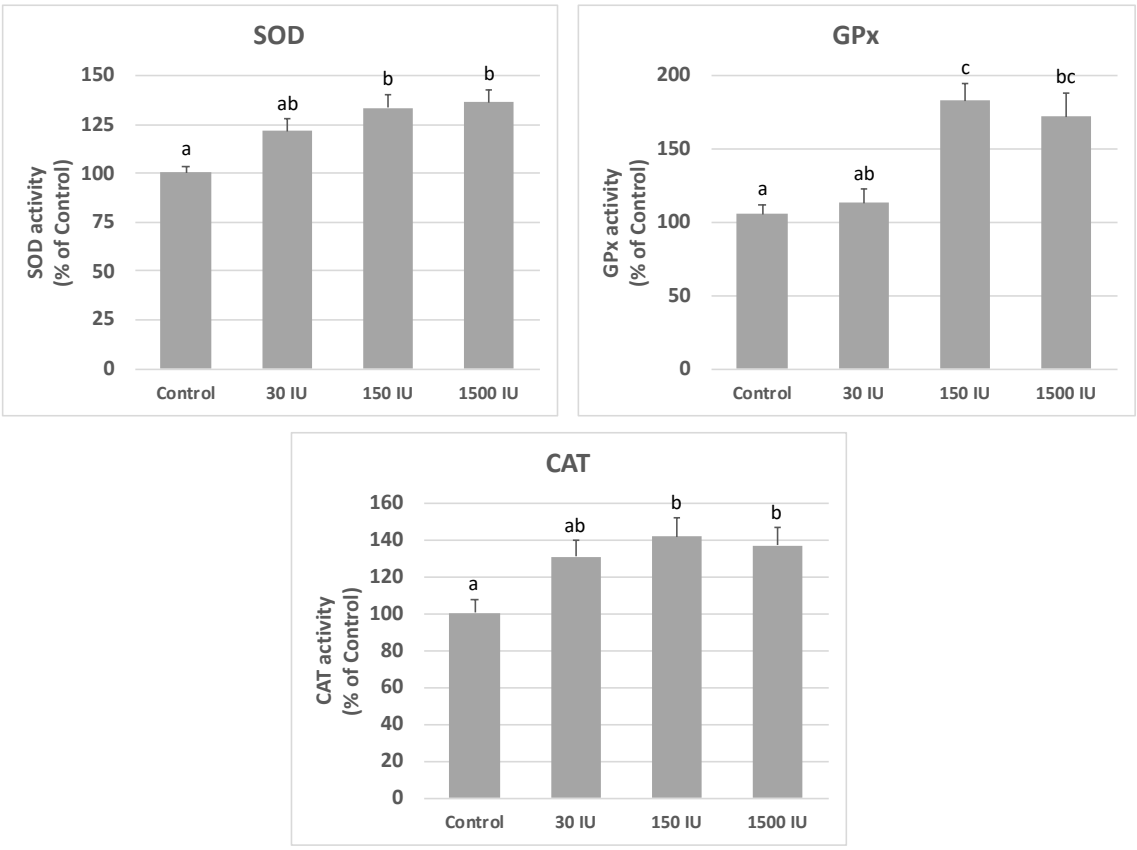

Figure 2. Relative enzyme activities after treatment of HSMMs cells with TetraSOD® extract at different doses of SOD (IU per ml of culture media) for 24 h. Values are expressed as percentages of activity with regard to the Control (100%). Data were expressed as the mean fold change (mean ± SD, n = 6; two assays in triplicate) from the calibrator group (Control). Different letters denote statistically significant differences among doses (p<0.05) as determined by the Kruskal-Wallis test (non-parametric one-way ANOVA) followed by Dunn's multiple comparisons test.

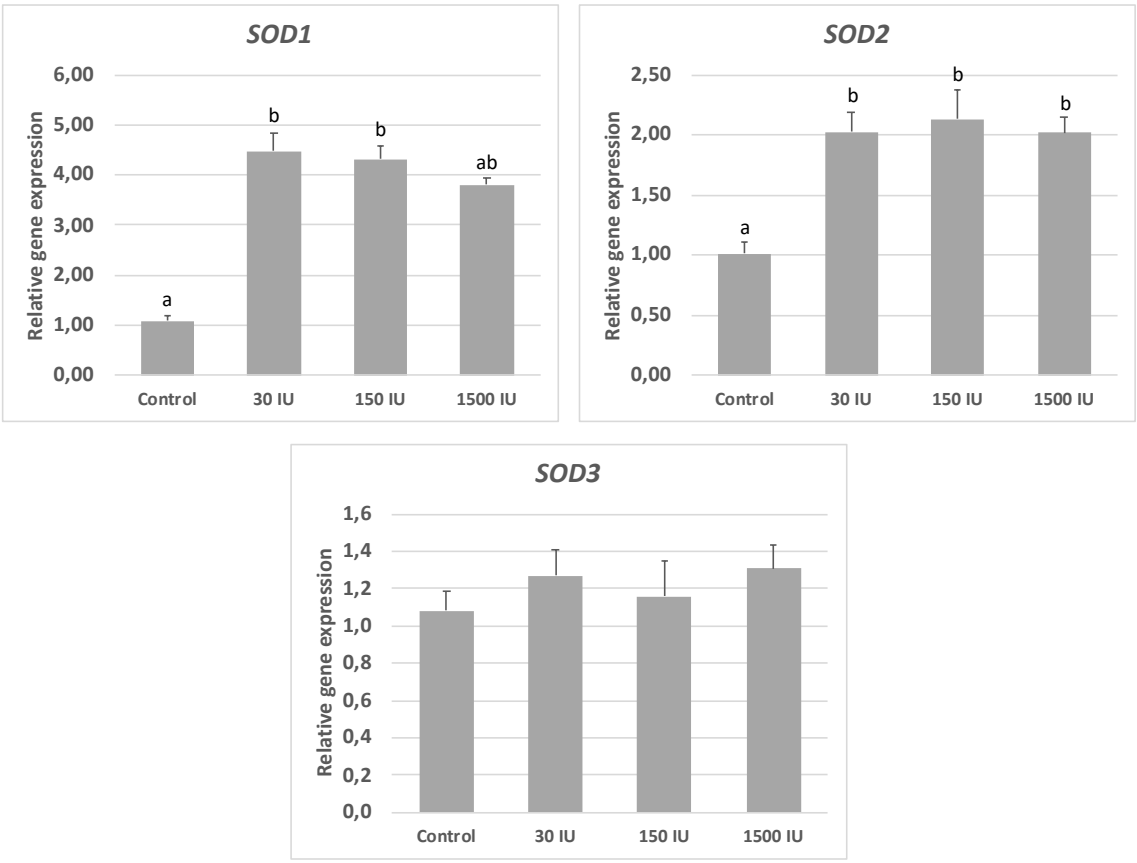

Figure 3. Relative gene expression levels of *SOD1*, *SOD2* and *SOD3* after treatment of HSMMs cells with TetraSOD® extract at different doses of SOD (IU per ml of culture media) for 24 h. Expression values were normalized to those of *ACTB*. Data were expressed as the mean fold change (mean ± SD, n = 6; two assays in triplicate) from the calibrator group (Control). Different letters denote statistically significant differences among doses (p<0.05) as determined by the Kruskal-Wallis test (non-parametric one-way ANOVA) followed by Dunn's multiple comparisons test.

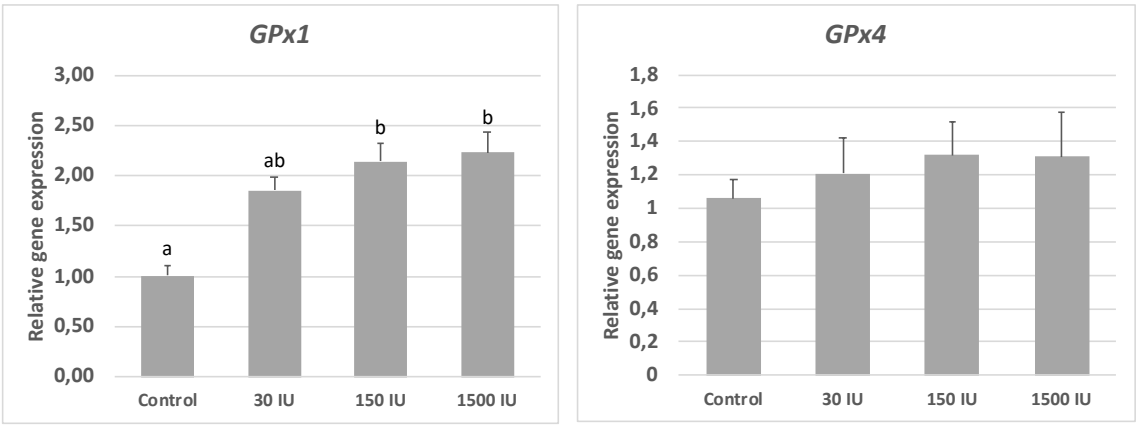

Figure 4. Relative gene expression levels of *GPx1* and *GPx4* after treatment of HSMMs cells with TetraSOD® extract at different doses of SOD (IU per ml of culture media) for 24 h. Expression values were normalized to those of *ACTB*. Data were expressed as the

mean fold change (mean ± SEM, n = 6; two assays in triplicate) from the calibrator group (Control). Different letters denote statistically significant differences among doses (p<0.05) as determined by the Kruskal-Wallis test (non-parametric one-way ANOVA) followed by Dunn's multiple comparisons test.

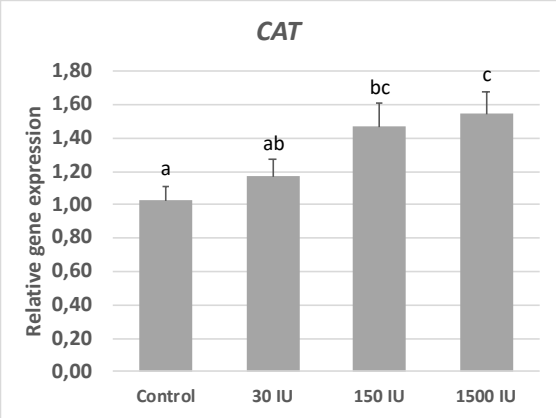

Figure 5. Relative gene expression levels of *CAT* after treatment of HSMMs cells with TetraSOD® extract at different doses of SOD (IU per ml of culture media) for 24 h. Expression values were normalized to those of *ACTB*. Data were expressed as the mean fold change (mean ± SEM, n = 6; two assays in triplicate) from the calibrator group (Control). Different letters denote statistically significant differences among doses (p<0.05) as determined by the Kruskal-Wallis test (non-parametric one-way ANOVA) followed by Dunn's multiple comparisons test.

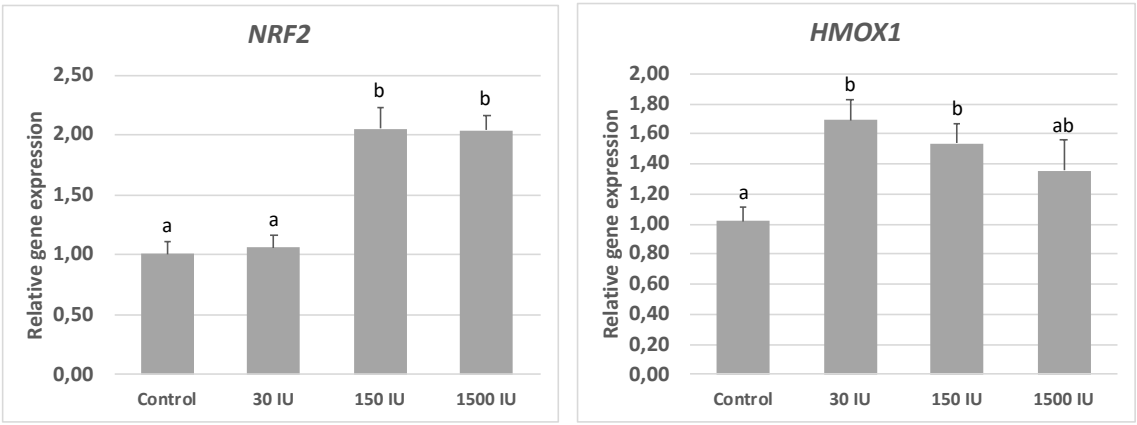

Figure 6. Relative gene expression levels of *NRF2* and *HMOX1* after treatment of HSMMs cells with TetraSOD® extract at different doses of SOD (IU per ml of culture media) for 24 h. Expression values were normalized to those of *ACTB*. Data were expressed as the mean fold change (mean ± SEM, n = 6; two assays in triplicate) from the calibrator group (Control). Different letters denote statistically significant differences among doses (p<0.05) as determined by the Kruskal-Wallis test (non-parametric one-way ANOVA) followed by Dunn's multiple comparisons test.

TABLES

Table 1. List of primers used in this study

Gene symbol	Gene name	Primer sequences	Amplicon length (bp)	Optimal Ta (ºC)	Reference
SOD1	Superoxide dismutase 1	F: 5'-GGATGAAGAGAGGCATGTTGGA-3' R: 5'-TAGACACATCGGCCACACCAT-3'	71	56.9	Letsiou et al., 2017
SOD2	Superoxide dismutase 2	F: 5'-ACAAGTTTAAGGAGAAGCTGACGG-3' R: 5'-CTCCCACACATCAATCCCCAG-3'	173	60.2	This work
SOD3	Superoxide dismutase 3	F: 5'-TGGATCCGAGACATGTACGCCAA-3' R: 5'-GGAAGAGGACGACGCCGGTCA-3'	154	66.5	This work
GPx1	Glutathione peroxidase 1	F: 5'-CGATGTTGCCTGGAACTTTGAG-3' R: 5'-ATGTCAATGGTCTGGAAGCGG-3'	90	60.5	Letsiou et al., 2017
GPx4	Glutathione peroxidase 4	F: 5'-CGCTGTGGAAGTGGATGAAGAT-3' R: 5'-AGCCGTTCTTGTCGATGAGGA-3'	99	59.5	Letsiou et al., 2017
CAT	Catalase	F: 5'-TGCTGAGAAGCCTAAGAATGCG-3' R: 5'-ACAGATTTGCCTTCTCCCTTGC-3'	80	57.9	Letsiou et al., 2017
HMOX1	Beta-ketoacyl ACP synthase	F: 5'-CGATGCACACCACATGACCGACC-3' R: 5'-CGCCTGCTTCATCGCCTTGACC-3'	122	60.0	This work
NRF2	Nuclear factor erythroid 2-related factor 2	F: 5'-CCAAAGAGCAGTTCAATGAAGC-3' R: 5'-GCAGCCACTTTATTCTTACCCC-3'	76	55.3	Letsiou et al., 2017

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