Research Article

Creatine supplementation prevents the inhibition of myogenic differentiation in oxidatively injured C2C12 murine myoblasts

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Creatine (Cr), one of the most popular nutritional supplements among athletes, has been recently shown to prevent the cytotoxicity caused by different oxidative stressors in various mammalian cell lines, including C2C12 myoblasts, *via* a direct antioxidant activity. Here, the effect of Cr on the differentiating capacity of C2C12 cells exposed to H_2O_2 has been investigated. Differentiation into myotubes was monitored using morphological, ultrastructural, and molecular techniques. Treatment with H_2O_2 (1 h) not only caused a significant (30%) loss of cell viability, but also abrogated the myogenic ability of surviving C2C12. Cr-supplementation (24 h prior to H_2O_2 treatment) was found to prevent these effects. Interestingly, H_2O_2-challenged cells preconditioned with the established antioxidants trolox or *N*-acetyl-cysteine, although cytoprotected, did not display the same differentiating ability characterizing oxidatively-injured, Cr-supplemented cells. Besides acting as an antioxidant, Cr increased the level of muscle regulatory factors and IGF1 (an effect partly refractory to oxidative stress), the cellular availability of phosphocreatine and seemed to exert some mitochondrially-targeted protective activity. It is concluded that Cr preserves the myogenic ability of oxidatively injured C2C12 *via* a pleiotropic mechanism involving not only its antioxidant capacity, but also the contribution to cell energy charge and effects at the transcriptional level which common *bona fide* antioxidants lack.

Keywords: Antioxidants / Creatine / Myogenesis / Nutritional supplements / Oxidative stress

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1 Introduction

The skeletal muscle tissue contains several cell types, including endothelial, mesenchymal-like, hematopoietic-like, and myogenic cells [1, 2]. The satellite cells, which represent the myogenic lineage, are quiescent and located beneath the basal lamina [3], they are involved in muscle regeneration upon mechanical, chemical, or degenerative lesion of the muscle fiber. In these situations, the satellite cells are activated, they start proliferating (at this stage satellite cells are renamed myoblasts), then participate to muscle regeneration through a differentiating process culminating in the fusion with remaining fibers or formation

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Abbreviations: Cr, creatine; **CrP,** phosphocreatine; **CRT,** creatine transporter; **DifD,** differentiation day; **DM,** differentiating medium; **GM,** growth medium; **GpX,** glutathione peroxidase; **MI,** myogenic index; **MRF,** muscle regulatory factor; **mtDNA,** mitochondrial DNA; **MTT,** 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; **NPSH,** nonprotein sulfydryls; **ROS,** reactive oxygen species; **SEM,** scanning electron microscopy; **TEM,** transmission electron microscopy

of new ones [4, 5]. Cultured myoblasts, which may differentiate into myotubes upon specific stimuli, represent an established model to mimic and study this process *in vitro* [6, 7].

The initiation of muscle differentiation is mostly governed by muscle regulatory factors (MRFs). The MRFs, which include Myo-D, Myf-5, MRF-4, and myogenin, are members of a family of basic helix-loop-helix proteins that act as transcription activators due to their inherent properties as DNA binding proteins [8]. Their binding to DNA initiates the transcription and regulates the expression level of some muscle-specific genes, such as myosin heavy and light chains, actin, or creatine (Cr) kinase [9]. The specific roles of the different MRFs in skeletal muscle differentiation have not yet been completely defined, essentially due to the existence of auto- and cross-regulatory loops between them [10, 11]. However, Myo-D and Myf-5 are early factors, mainly involved in myoblast formation and in satellite cell proliferation during regeneration, while myogenin and MRF-4 are late-acting factors, expressed during differentiation [12]. Also, other co-factors such as IGF-1 contribute to this process [7]. However, the full range of biochemical adaptations associated with myocyte formation is much more complex as compared to the above scenario [13], indeed Kislinger *et al*. [14] detected several hundreds of previously uncharacterized proteins expressed in a stage-specific manner during the differentiation of C2C12 murine myoblasts. It has recently been shown that MRFs and IGF1 are also involved in the hypertrophic response of skeletal muscle, particularly in regulating the net muscle protein synthesis and the addition of myonuclei to existing, terminally differentiated myofibers, by activation of satellite cells [4]. The roles of MRFs and IGF-1 in both muscle differentiation and hypertrophy [4, 8, 15 – 17] are depicted in Fig. 1.

Successful differentiation of satellite-derived myoblasts into functioning and integrated myotubes is a fundamental prerequisite for muscle regeneration, a repair process which is of primary importance in maintaining muscle function [18].

Oxidative stress, under conditions where it overwhelms the cellular antioxidant capacity, has been shown to directly or indirectly affect the proliferation/differentiation balance of satellite-derived cells and myoblasts [19] and to promote the loss of mature myofibers [20]. Increased levels of reactive oxygen species (ROS) targeting muscle fibers may be generated at multiple sites either in normal or pathological conditions. As an example, healthy exercising muscle is exposed to high levels of ROS derived either by intrinsic (sustained oxygen demand and electron flow through mitochondrial chain) or extrinsic (rise in body temperature and reduced blood flow mimicking ischemia/reperfusion) factors. From a pathological point of view, inflammation arising from mechanical traumas plays a bifaceted role in muscle repair processes [21] through the combined actions of free radicals, growth factors, and chemokines. As a rule, inflammation should promote and coordinate a cascade of events leading to muscle repair, but its persistence, through the action of infiltrated neutrophils, may cause further injury by oxidatively damaging differentiating cells and myotubes [22]. Notably, oxidative stress is known to play a concausal and detrimental role in a variety of multifactorial muscular pathologies characterized by proliferation/differentiation imbalance such as Duchenne dystrophy [23], myotonic dystrophy [24], sarcopenia [25], and cachexia [20].

Cr monohydrate is a popular nutritional supplement in the sports industry, reputed to maintain high-energy phosphates during exercise; although normal levels of Cr can be obtained through an omnivorous diet and/or by endogenous synthesis in the liver, kidney, and pancreas, dietary consumption of supplements containing Cr is on the rise. The wide use of Cr as an ergogenic aid among athletes finds its rationale in that 90% of total Cr in the body is stored in skeletal muscles [26] where its functional role, along with ATP, is the fundamental provision of energy. Cr is taken up from the blood by a specific Na^+ and Cl^- dependent creatine transporter (CRT). This process is controlled by many factors including substrate concentration, transmembrane Na^+ gradients and various hormones; CRT regulation, however, is poorly understood. Cr uptake modulation could involve changes in CRT gene expression and transporter intra-cellular localization, its post-translational modifications (phosphorylation, glycosylation) and its interaction with regulatory proteins [27]. Two-thirds of intracellular Cr is converted into its phosphorylated form phosphocreatine (CrP) by Cr kinases [28]. The total amount of the Cr pool (Cr plus CrP) depends on the rate of Cr uptake from blood and on the rate of non-enzymatic conversion to creatinine. As only about 2% of the Cr pool *per* day is converted into creatinine, the most important process that controls the Cr pool is the uptake from the extracellular fluid. Normally muscles maintain a high Cr concentration gradient which is 500 – 1000-fold higher inside the muscle than in the plasma [29] and this gradient is tightly regulated [26]. It is also well documented that high-dose-oral Cr supplementation further elevates intracellular Cr and CrP pools of muscle tissue, as well as serum Cr levels *in vivo* [30].

Interestingly, evidences accumulated over the past decade also indicate that Cr positively affects growth and differentiation of myogenic cells *via* non-ergogenic, poorly identified, mechanism(s) [6, 31 – 34]. Consistently, apart from its popularity as an ergogenic supplement among athletes, the use of oral Cr supplementation, is now being extending to the medical field to prevent and/or treat a number of muscular and cardiovascular diseases [35 – 46].

Recently, we and others have reported that Cr displays direct antioxidant activity either in acellular [47] or cellular systems [48], and has protective effects against oxidative stress in cultured mammalian cells, including undifferentiated C2C12 murine myoblasts [48, 49]. Notably, the antiox-

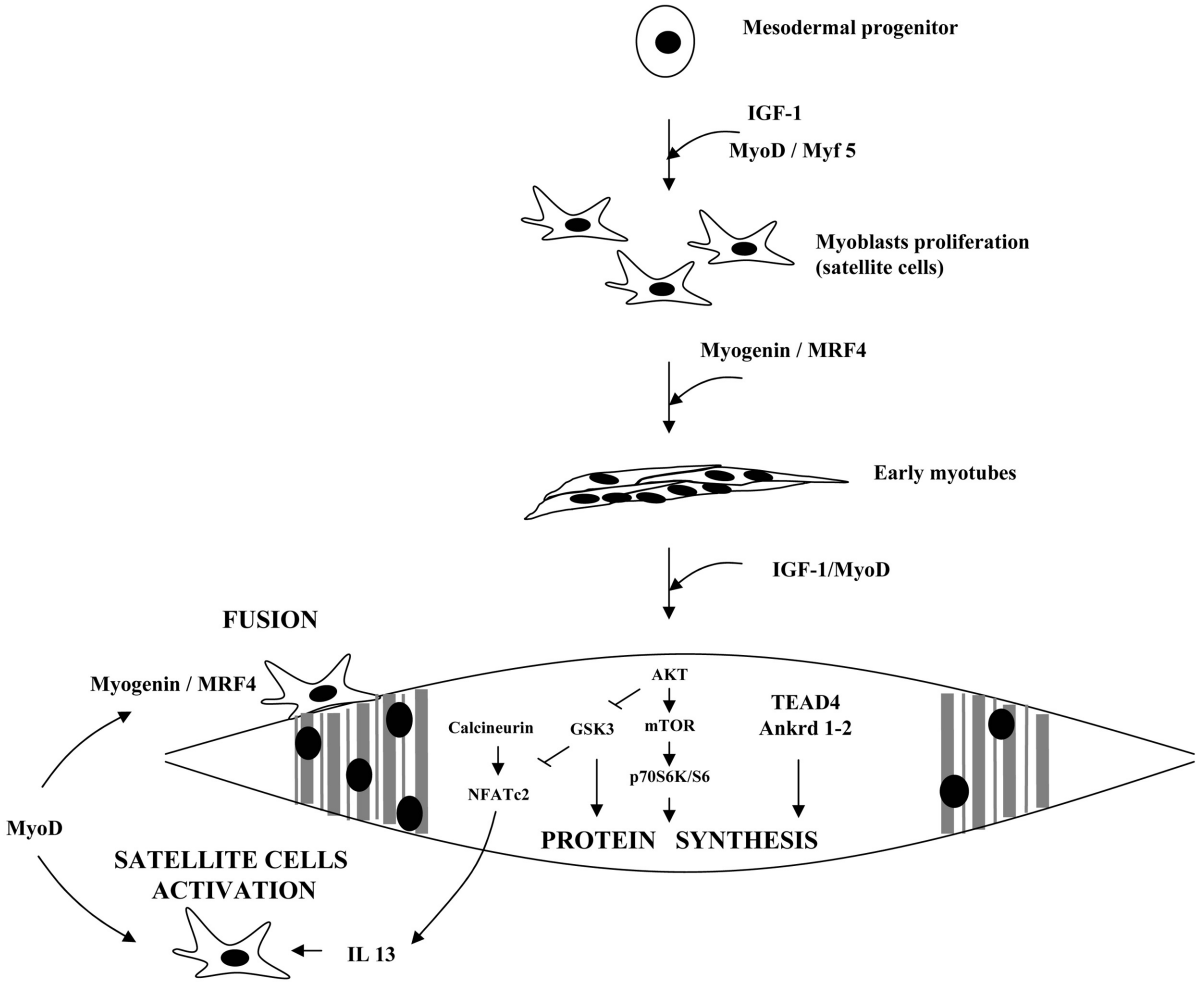

Figure 1. Mechanisms of MRFs and IGF-1-induced myotube differentiation and hypertrophy. Myf5 and MyoD are the first transcription factors activated during myogenic differentiation. Myf5 is involved in myoblast specification and maintenance, MyoD commits somatic cells to the myogenic lineage, whereas the "late" MRFs, Myogenin, and MRF4 promote terminal differentiation of myoblasts into multinucleated myotubes [8]. In addition MyoD transcriptionally promotes muscular hypertrophy by activating muscle-specific transcriptional modulators such as *TEAD4*, a member of the transcriptional enhancer factor family and the muscle ankyrin repeat proteins (*Ankrd1* and *Ankrd2*), involved in myofibril-based hypertrophic response signaling [15, 17]. IGF-1 induces an increase in the protein content of myotubes *via* phosphatidyl inositol-3-kinase and a serine/threonine kinase called Akt (*PI3K/Akt*). Besides Akt, IGF-1 acts on the *mTOR/p70S6K* and *GSK-3* pathways, both of which are involved in the control of protein translocation. IGF-1 also potentiates the recruitment of reserve cells for fusion *via* the secretion of IL-13 by myotubes under the control of NFATc2 [16].

idant capacity has been shown to be a function of the free-Cr intracellular pool and to be unrelated to CrP levels [48]. It has also been suggested that the Cr antioxidant capacity might contribute, *in vivo*, to the amelioration of the symptoms of the above muscular and cardiovascular pathologies. More recently, we have shown that Cr supplementation reduces the extent of oxidative mitochondrial DNA (mtDNA) damage [50] in HUVEC cells. Since the accumulation of free-radical dependent mtDNA damage over the lifetime is known to represent a major determinant of aging [51], we have hypothesized that Cr-supplementation might be a promising anti-aging strategy. Interestingly another group, using an independent and different experimental approach, has recently proposed Cr as an aging-delaying agent [52].

As it has been discussed above, oxidative stress impairs the ability of myoblasts to differentiate into mature myotubes, an event which is considered as pathologically relevant in specific human maladies and muscle aging [19, 20, 53]. Then it would be important to identify compounds and/or nutrients capable of counteracting the detrimental effect of oxidative stress in these specific situations. Cr, by virtue of its muscular positive tropism, anabolic effect, antioxidant activity, safety, low cost, and availability would be an attractive candidate to assist such pathophysiological events. In the present study, we have addressed the question

of whether Cr supplementation protects differentiating C2C12 myoblasts, a well established model of myogenic cultures, from oxidative injury and allows them to complete the differentiation into mature myotubes.

2 Materials and methods

2.1 Chemicals

Unless otherwise stated, reagent grade chemicals were obtained from Sigma – Aldrich (Milan, Italy). Cell culture media, sera, trypsin and antibiotics were from Cambrex Corporate (East Rutherford, NJ, USA).

2.2 Cell culture

C2C12 mouse myoblasts were routinely cultured in growth medium (GM) consisting of low-glucose DMEM supplemented with heat-inactivated 10% v/v fetal bovine serum, 2 mM glutamine, antibiotics (50 U/mL penicillin, 50 μg/mL streptomycin) and 25 mM Hepes, pH 7.5. The same medium composition was used to promote C2C12 cell differentiation, with the only exception that heat-inactivated fetal bovine serum was reduced to 1% v/v (differentiating medium (DM)). The cell monolayers were maintained in a humidified 5% CO_2 atmosphere at 37°C. Myogenic differentiation was achieved as previously described in [6]. Briefly, cells were plated at $10^4/cm^2$ in 100 mm plastic dishes and cultured in GM for 24 – 48 h, allowing them to reach ~80% confluency; to start differentiation GM was then replaced with DM. The cells were observed and processed for the experiments at critical time intervals, *i.e.*, at the undifferentiated stage and at selected differentiation days (DifDs), which are referred in the text as to "DifDn", where "n" is the time, in days, elapsed from the commencement of differentiation.

2.3 Treatment conditions

Cr-preloading of C2C12 was achieved by addition of 3 mM Cr to DM over the first 24 h of differentiation. At this time point (*i.e.*, at the end of DifD1), Cr-supplemented or -unsupplemented cultures were oxidatively challenged with H_2O_2 for 1 h in fresh, Cr-free DM. Cells were then extensively washed with PBS (8 g/L NaCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, 0.2 g/L KCl) and cultured in Cr- and oxidant-free DM for up to day 5. Trolox (0.1 mM) or *N*-acetylcysteine (3 mM) treatments were as follows: C2C12 were committed to differentiate in Cr-free DM and treated with H_2O_2 as described above, with the only exception that the two antioxidants were present during the oxidative challenge stage. Appropriate sham-treated samples were included in each experiment.

2.4 Determination of cell survival and viability

Cytotoxicity was determined using the trypan blue exclusion assay, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay or quantifying the protein content of the samples. For trypan blue: monolayers were detached by trypsinization, an aliquot of cell suspension was diluted 1:1 with 0.4% trypan blue and the cells were counted with a haemocytometer. Results are expressed as percent survival, that is the percent ratio between the number of viable (unstained) cells in treated *versus* control samples. MTT was added (50 μg/mL) to cell monolayers, the cells were then incubated for a further 1 h at 37°C, washed and dissolved in 1 mL of dimethyl sulfoxide. Formation of blue formazan was measured spectrophotometrically at 570 nm. Finally, the protein content of the cultures was determined using the method of Bradford [54], and taken as an index of cell viability.

2.5 HPLC determination of Cr, CrP, and ATP

The liquid chromatographic system from Beckman (Beckman Coulter S.p.a., Cassina dè Pecchi, Italy) consisted of a System Gold 126 solvent module, a Model 7725i sample injection valve and a System Gold 168 detector interfaced with an IBM 300PL computer. The separation of samples was performed using a 3 μm Supelcosil LC-18 DB column (15 cm × 4.6 mm id) protected by a 5 μm Supelcosil LC-18 DB guard column (2 cm × 4.6 mm id). Solvent A was 70 mM potassium dihydrogen phosphate, pH 6.0 containing 7 mM tetrabutylammonium hydrogen sulfate, and solvent B was 70 mM potassium dihydrogen phosphate, pH 6.0 containing 7 mM tetrabutylammonium hydrogen sulfate and 30% v/v CH_3OH. The injection volume used was 50 or 200 μL for Cr and CrP or for ATP analyses, respectively. The chromatographic separation was obtained by means of the following gradient of solvent B: 2.5 min at 0%, 0 – 20% in 2.5 min, 20 – 40% in 5 min, 40 – 100% in 8 min, and 7 min at 100. The gradient was then immediately returned to 0% and the initial conditions were restored in 2 min. The flow-rate was 2.5 min at 0.9 mL/min, then 1.2 mL/min and detection was performed at 218 nm. The samples were filtered before the analysis using a 0.22 μm filter (Millipore, Billerica, MA, USA).

2.6 Determination of catalase, glutathione peroxidase (GpX) and nonprotein sulfydryls (NPSH)

Catalase and GpX were determined spectrophotometrically in cell homogenates with the methods of Aebi [55] and Lawrence and Burk [56], respectively. NPSH were assayed spectrophotometrically in metaphosphoric acid extracts with the Elman's reagent as described in [57].

2.7 Assessment of the myogenic index (MI)

The MI, which represents the fraction of nuclei residing in myotubes containing three or more nuclei, was assessed examining pictures taken at the light microscope (see the next subheading) of cultures stained with May-Grunwald-Giemsa. Five fields/60 mm dish ($n = 3$) at a 100 × magnification were observed, and the total number of nuclei analyzed was 300 – 500/field.

2.8 Light, scanning (SEM) and transmission (TEM) electron microscopy

Light microscopy observations on control and treated monolayers were performed by means of a Nikon TE 2000-S reverted microscope (Nikon Instruments S.p.A Firenze, Italy), equipped with a DN 100 Nikon digital system, to monitor C2C12 response to the differentiation induction as well as to the different treatments.

The details were further investigated by SEM and TEM. For SEM analysis, the specimens were washed with PBS, immediately fixed *in situ* with 2.5% glutaraldehyde in PBS pH 7.4 for 45 min and post-fixed with 1% OsO_4 in the same buffer. After washings, they were alcohol dehydrated and critical point dried as previously described (40). Finally, the slides were mounted on conventional stubs, gold-sputtered and observed with a Philips 515 scanning electron microscope (FEI Company, The Netherlands)

For TEM analysis, C2C12 monolayers were fixed with 2.5% glutaraldehyde in PBS for 5 min, gently scraped and centrifuged. Pellets were fixed again in a new fixative solution for 30 min, OsO_4 post-fixed, alcohol-dehydrated and araldite embedded as reported in (7). Thin sections were collected on nickel 300 mesh grids and stained with uranyl acetate and lead citrate. The observations were carried out with a Philips CM10 transmission electron microscope (FEI Company) at 80 KV.

2.9 2-DE

Cells were detached, washed with PBS (pH 7.4) containing a protease inhibitors cocktail (Roche Diagnostics, Germany), and centrifuged. Pellets were then resuspended in 8 M urea, 4% 3-CHAPS, 65 mM DTE, 40 mM Tris base and sonicated for 5 s on ice. After centrifugation at 14 000 rpm, protein concentration was determined in the supernatants by the Bradford assay [54].

Forty-five microgram (analytical run) or 500 µg (semi-preparative run) of total protein were used for each electrophoretic run. Isoelectric focusing was carried out on Immobiline strips providing a non linear pH 3 – 10 gradient (GE Healthcare Italy, Milan, Italy) using an IPGphore system (GE Healthcare) and applying an increasing voltage from 200 V to 3500 during the first 3 h, then stabilized at 5000 V for 20 h. After isoelectric focusing, IPG strips were equilibrated by soaking in buffer containing 50 mM Tris-HCl pH 6.8, 6 M urea, 2% SDS, 30% glycerol and 2% DTE for 15 min, and then 50 mM Tris-HCl pH 6.8, 6 M urea, 2% SDS, 30% glycerol with 2.5% iodacetamide and trace amounts of bromophenol blue for further 15 min. The second dimension was carried out in a Laemmli system on 9 – 16% polyacrylamide linear gradient gels (18 cm × 20 cm × 1.5 mm) at 40 mA/gel constant current, until the dye front reached at bottom gel. Analytical gels were stained with silver nitrate [58]. Semi-preparative gels for mass spectrometric analysis were stained with Brilliant Blue G-Colloidal according to the manufacturer's procedure.

Gel images were acquired by Fluor-S MAX multi-imaging system (BioRad Laboratories Italy, Segrate, Italy) and the data were analyzed, including spots detection, quantification and normalization, using ImageMaster 2D Platinum version 5.0 software (GE Healthcare).

2.10 In-gel digestion

Protein spots excised from 2-D gels stained with Brilliant Blue G-Colloidal were rinsed with 100 mM ammonium bicarbonate and then dehydrated with ACN. The rinse/dehydration step was repeated two times. Prior to tryptic digestion proteins were reduced and alkylated by incubation with 10 mM DTT in 100 mM ammonium bicarbonate for 1 h at 60°C, followed by incubation with 55 mM iodacetamide in 100 mM ammonium bicarbonate for 45 min at room temperature. After dehydration of gel, using ACN, each protein was incubated overnight at 37°C with of trypsin in 50 mM ammonium bicarbonate. The digestion liquid was collected and the gel was added twice with 5% formic acid in ACN and stirred for 15 min. The two ACN extracts were combined with the digestion liquid and the solvent was completely removed by Speed-Vac.

2.11 MS

Protein identification by ESI-Q-TOF MS was performed sequentially by database search and then *de novo* sequencing using MS/MS spectra. Nanoscale capillary LC/MS/MS analysis of the digest proteins was performed using a CapLC capillary LC system (Micromass, Manchester, UK) coupled to a hybrid quadrupole orthogonal acceleration TOF tandem mass spectrometer (QTO Micro, Micromass). For protein identification, MS/MS spectra were searched by MASCOT (Matrix science, www.matrixscience.com, UK) using the NCBI nr database. Mass tolerance was set at 1.5 Da for the masses of peptide precursors and at 0.8 Da for the masses of fragment ions. Proteins containing at least one significant peptide were selected from data-base search results. For unmatched peptides, however, good quality MS/MS spectra were manually sequenced using *de novo* sequencing process (carried out by PepSeq of the Masslynx

4.0 software, Micromass), and the obtained sequence was subsequently used in Expasy TagIdent.

2.12 Electrophoresis and Western blotting

The C2C12 cells were homogenized in 200 µL of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.8, 250 mM NaCl, 1 µg/mL pepstatin, 10 µg/mL leupeptin, 2 mM sodium orthovanadate, 10 mM NaF, 5 mM EDTA, 40 µg/mL PMSF (PMSF), and 0.1% w/v Triton X-100) and sonicated for 20 s at 50 W. Samples were then centrifuged for 10 min at 14 000 × *g* to remove insoluble debris. Supernatants were mixed 1:1 v/v with sample buffer (0.5 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 4% of 2-mercaptoethanol, and 0.05% bromophenol blue) and 20 µg of sample proteins were loaded onto 10% SDS–polyacrylamide slab gels and subjected to electrophoresis [59]. The gels were electroblotted and stained with Coomassie [60]. Blots were probed with the specific primary antibodies: rabbit anti-myosin heavy chain (Santa Cruz Biotech, USA, 1:1000). Horseradish-peroxidase-conjugated goat anti-mouse and horseradish-peroxidase-conjugated goat anti-rabbit IgG (BioRad, Milan, Italy, 1:3000) were used as secondary antibodies. Immune complexes were visualized using an enhanced chemioluminescence Western blot analysis system (Amersham–Pharmacia, Milan, Italy), following the manufacturer's specifications. Blot images were digitized (Chemidoc, BioRad, Milan, Italy) and the OD of bands was quantified using the computerized imaging system (Quantity One, BioRad).

2.13 RNA extraction and real time quantitative PCR

Cells cultured and treated as described above (see "Treatment conditions" subheading) were analyzed for the expression of specific mRNAs at DifD0, 1, 2, and 3. At each differentiation time, plates ($n = 3$) were washed with PBS and total RNA was extracted from cell cultures using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was removed by DNase I digestion in column during the RNA extraction (Qiagen) and in order to eliminate any residual of genomic DNA, a further DNA digestion was performed using DNase I enzyme (Ambion, Austin, TX, USA). RNA was quantified spectrophotometrically (UV = 260 nm). One microgram of total RNA was reverse transcribed using Omniscript RT (Qiagen) and random hexamers (Promega Corporation, Madison, WI, USA) in a final volume of 20 µL as described in manufacturer's protocol.

The real time PCR approach was used to quantify the expression levels of the MRFs: oligonucleotide; primers for the MyoD, Myf5, myogenin, and Mrf4 transcription factors were designed for *Mus musculus* using Primer Express version 1.0 (Perkin Elmer Italia, Monza, Italy) from the GenBank database. The IGF-1 primers were designed by Louis *et al*. [7] and recognized all the IGF-1 isoforms; furthermore specific primers for the CRT gene were also selected on different exons in order to amplify a 117 nt cDNA region.

GADPH and the ribosomal protein S16 were used as housekeeping genes: in particular S16 has been chosen among several possible housekeeping genes since, it is used as control gene in differentiating myoblasts [61] and preliminary experiments showed that S16 mRNA was stable during the differentiation process and in all treatment conditions. All the primers are reported in Table 1.

Measurements were made by an iCycler machine (Bio-Rad) and QuantiTect SYBR green PCR kit (Qiagen). The RT-PCR was carried out in 96 well optical plate (BioRad). For each well, the 25 µL reaction contained: 12.5 µL of 2 × QuantiTect SYBR green PCR master mix, 0.3 µM each forward and reverse primer, 8 µL of RNase-free H_2O and 2 µL cDNA template. The cycling conditions were: 95°C for 15 min followed by 40 cycles of 94°C for 30 s and 60°C for 30 s. Primers efficiency was checked and real time PCR products were confirmed by melting curves, gel electrophoresis, and sequencing. Threshold cycle was defined as the fractional cycle number at which the fluorescence signal passed ten times value of ground fluorescence SD. The amount of the target transcripts was related to that of the reference S16 gene as described by Pfaffl *et al*. [62].

2.14 Statistical analysis

Unless noted otherwise, the results are expressed as mean values ± SEM for the indicated number of measurements. The significance of differences between the mean values recorded for different experimental conditions was calculated by student's *t*-test and *p*-values are indicated where appropriate in the figures and their legends.

With respect to the determination of myosin heavy chain, IGF-1 and MRFs levels (Figs. 8 and 9), the effects of all treatments were tested using a two tailed ANOVA analysis. Bonferroni-test was used as post-hoc. The significance threshold was set to 0.05.

3 Results

3.1 Effect of Cr pre-loading on the survival of oxidatively injured-, differentiating-C2C12 myoblasts

We had previously reported that exposure of growing, undifferentiated C2C12 myoblasts to a bolus of hydrogen peroxide resulted in necrotic cell death [48]. Here, to test the ability of differentiating C2C12 myoblasts to continue and/or complete the myogenic task under similar stressing conditions, we have exposed differentiating C2C12 to a brief (1 h) challenge with the oxidative stressor H_2O_2. The

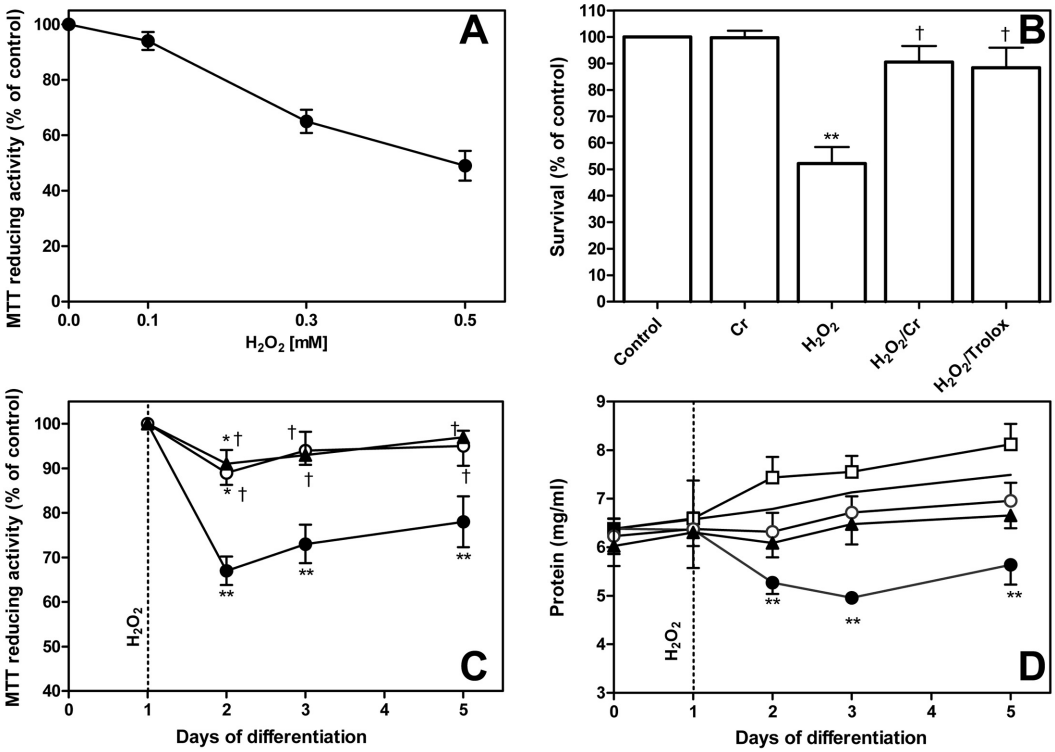

Figure 2. Effect of Cr on the viability of differentiating C2C12 cells exposed to hydrogen peroxide. (A) Cells were maintained for 24 h in DM medium; and then treated for 1 h with increasing concentrations of H_2O_2. Cytotoxicity was determined with the MTT assay 24 h post-challenge culture in H_2O_2-free DM. (B) Cells were maintained for 24 h in DM containing 0 or 3 mM Cr; the medium was changed with fresh Cr-free DM and cultures were then treated for 1 h with 0.3 mM H_2O_2. Cytotoxicity was determined with the trypan blue exclusion assay 24 h post-challenge. Also shown is the effect of 0.1 mM trolox on H_2O_2 cytotoxicity. (C, D) Cells were treated as detailed in panel B, cultured for up to DifD5 in fresh Cr and H_2O_2-free DM, and checked at the indicated time points by means of the MTT assay (C) or of determination of the protein content (D). In both panels C and D, solid circles refer to H_2O_2-treated, Cr-free cells; open circles to H_2O_2-treated, Cr-supplemented cells; triangles to the effect of 0.1 mM trolox on H_2O_2 cytotoxicity; the vertical dotted lines indicate the H_2O_2 challenge stage. In panel D only, solid line refers to Cr-free controls and squares to Cr- supplemented, unintoxicated cells. Data are the means ± SEM of five independent determinations. * $p < 0.05$ and ** $p < 0.001$ as compared to untreated, unsupplemented controls, † < 0.01 as compared to Cr- or trolox-free H_2O_2-treated cells (Student's *t*-test).

Table 1. Primers used for RTQ-PCR

Primer name	Sequence (5′ – 3′)	Accession number
S16 Fw	TGA AGG GTG GTG GAC ATG TG	NM_013647
S16 Rv	AAT AAG CTA CCA GGG CCT TTG A	
Myo-D Fw	TTC TTC ACC ACA CCT CTG ACA	NM_010860
Myo-D Rv	GCC GTG AGA GTC GTC TTA TCT	
Myogenin Fw	GCA CTG GAG TTC GGT CCC AA	NM_031189
Myogenin Rv	TTG TGG GCG TCT GTA GGG TC	
MRF-4 Fw	GTG GCC AAGTGT TTC GGA TC	NM_008657
MRF-4 Rv	AAA GGC GCT GAA GAC TGC TG	
IGF-I Fw	GCT ATG GCT CCA GCA TTC G	NM_010512
IGF-I Rv	TCC GGA AGC AAC ACT CAT CC	
CRT Fw	GCT TCC CCT ACC TGT GCT ACA	NM_133987
CRT Rv	TGA ACT GGC CCA ATG AGA TT	

oxidant was given during the early stage of the myogenic process, *i.e.*, 24 h (DifD1, see Section 2) after serum reduction; the viability of the cultures was checked 24 h (DifD2) following H_2O_2 treatment. As expected, the MTT reducing ability (a well established marker of cell viability) of the treated cultures decreased as a function of the oxidant concentration (Fig. 2A). Cell death was mainly necrotic (see the below "*Morphological analysis*" subheading). The dose of 0.3 mM H_2O_2, which causes a mild cytotoxic response was selected for the next experiments, where cell viability

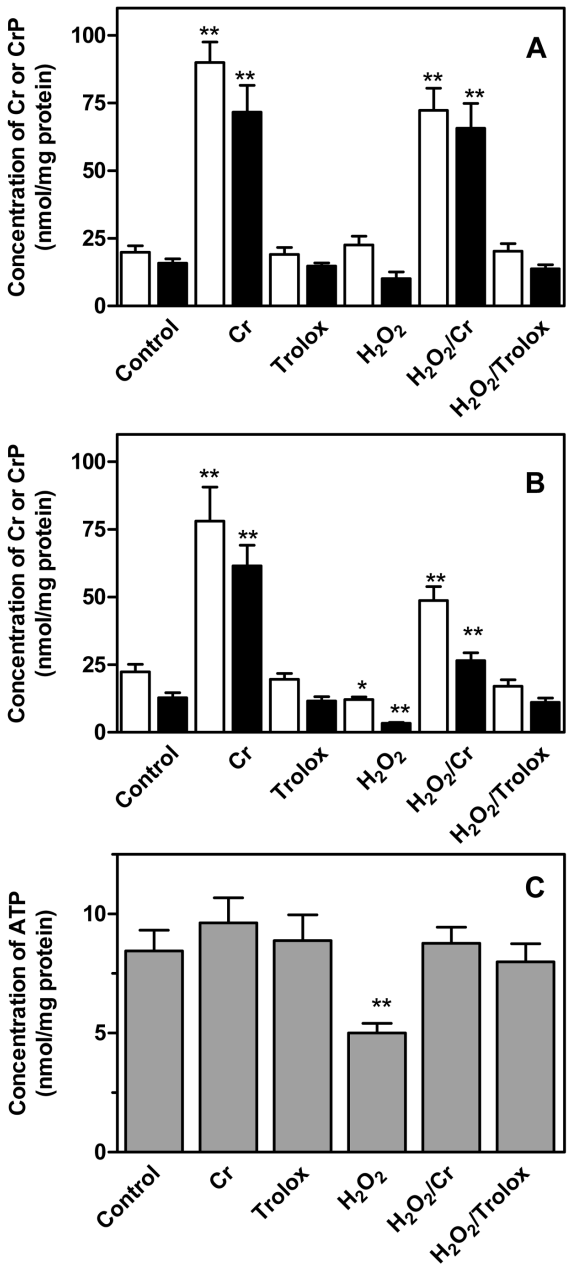

Figure 3. Intracellular levels of Cr, CrP, and ATP in differentiating C2C12 cells: the effect of Cr preloading and of oxidative challenge. Cr- or trolox-supplemented cells (see the "Treatment conditions" subheading in Section 2) were treated with 0.3 mM H_2O_2 and then assayed for Cr (white bars) and CrP (black bars) intracellular levels at 3 (A) and 48 h (B) post-challenge. Also shown (C) the levels of ATP at 48 h post-challenge. Data are the means ± SEM of four independent determinations. * $p < 0.05$ and ** $p < 0.001$ as compared to untreated, unsupplemented controls (Student's *t*-test).

was determined daily and up to DifD5. The MTT and protein assays were selected to determine cultures' survival up to DifD5 since, as differentiation continues, it is difficult and confusing to count cells which are progressively fusing together. However, the results obtained with the more straightforward trypan blue exclusion assay at DifD2 (*i.e.*, 24 h post-treatment, a time point where the fusion process is barely detectable and does not interfere with the experimental outcomes) are shown in Fig. 2B, and confirm that the oxidative challenge resulted in a significant reduction of viable cells. Interestingly, Cr or trolox (0.1 mM) prevented the effect caused by H_2O_2. The MTT assay, conducted up to DifD5, also showed that the maximal cell suffering observed at 24 h post-challenge was followed by a partial recovery in the course of the next days (Fig. 2C). Such a finding suggests that the effects of H_2O_2 on surviving cells' viability are in part reversible, probably reflecting the progressive normalization of the cellular oxidative metabolism. Again, Cr pre-loading exerted a marked and durable protection: indeed, the viability of H_2O_2-treated, Cr-preloaded cells was not statistically different from that of control ones. Not surprisingly, the reference antioxidant trolox (Fig. 2C) also protected C2C12 from H_2O_2 cytotoxicity. As to Cr-supplementation *per se*, it did not display any significant effect on untreated C2C12 cell viability as assayed with the trypan blue (Fig. 2B) or MTT assays (not shown).

The protein content of differentiating cultures confirmed the cytotoxic and cytostatic action of the oxidant: indeed intoxicated cultures showed a constantly lower protein content as compared to that of control ones (Fig. 2D); Cr and trolox prevented this effect.

Importantly Cr cytoprotection, as assessed with the MTT assay or determining the protein content, was abolished by the Cr uptake inhibitor β-guanidinopropionic acid (2 mM, not shown): thus the observed effects appear to depend entirely on the intracellular Cr fraction.

As a final note, due to the increasing size of maturing myotubes, control cultures' protein content slightly increased over time. Interestingly, Cr-supplementation induced a further, although non-statistically significant, increase in the protein accumulation (Fig. 2D) that is likely to depend on its modest hypertrophic effect previously reported by Louis *et al*. [63].

Taken collectively these data suggest that Cr- or trolox-preloading afford significant cytoprotection to differentiating, oxidatively-injured myoblasts.

3.2 Intracellular levels of Cr/CrP and ATP

The levels of Cr and CrP have been evaluated at 1 (DifD1, Fig. 3A) and 48 h (DifD3, Fig. 3B) after H_2O_2- or sham-treatment of cultures. In accordance with previous observations [48, 64], at DifD1 a significant intracellular accumulation of either Cr or CrP (Fig. 3A) could be observed in Cr-loaded C2C12 (four- and six-fold increase over controls, respectively). As expected, the addition of 2 mM β-guanidinopropionic acid prevented the rise in both Cr and CrP intracellular levels (not shown). Interestingly, the Cr and CrP levels at DifD3 were still elevated (Fig. 3B). This is likely to depend on the balance between the capacity of

Table 2. Catalase activity, GpX activity and NPSH levels in Cr-supplemented or unsupplemented C2C12 cells

Treatment[a)]	Catalase[b)] (U/mg protein)	GpX[b, c)] (mU/mg protein)	NPSH[b)] (nmol/mg protein)
Unsupplemented	26.8 ± 2.6	750 ± 2.3	16.6 ± 0.24
Cr-supplemented	27.1 ± 2.9	694 ± 1.9	16.8 ± 0.66

a) Cells were grown up to ~80% confluence, cultured for further 24 h in DM with or without 3 mM Cr and then assayed for catalase, GpX, and NPSH.
b) Catalase and GpX activities were measured as detailed in Section 2. Each point is the mean ± SEM of four to five determinations.
c) Total (Se-dependent and Se-independent) GpX activity.

skeletal muscle cells to retain Cr and the slow rate of Cr conversion into creatinine [26].

As to the H_2O_2 treatment, although it caused a slight, non-significant reduction of Cr levels at DifD1 (Fig. 3A), it led to a dramatic decrease of both Cr and CrP at DifD3 (Fig. 3B), an effect which could be prevented by Cr-supplementation. Notably, Cr-supplemented, oxidatively-injured C2C12 had Cr and CrP levels even higher than those of either Cr-free, H_2O_2-treated cells or of controls at both DifD1 and 3. Finally, the 24 h Cr supplementation regimen did not affect the mRNA expression of the CRT (not shown), as compared to Cr-free cultures, a finding similar to that reported *in vivo* by Tarnopolsky *et al*. [65].

When considering ATP, none of the treatments caused any significant variation of its levels at DifD1, as compared to control (12.53 ± 0.71 nmol/mg of proteins). However, at DifD3 a net decrease was found in H_2O_2-treated cells, an effect indicative of cell suffering and which could be prevented by both Cr and trolox (Fig. 3C).

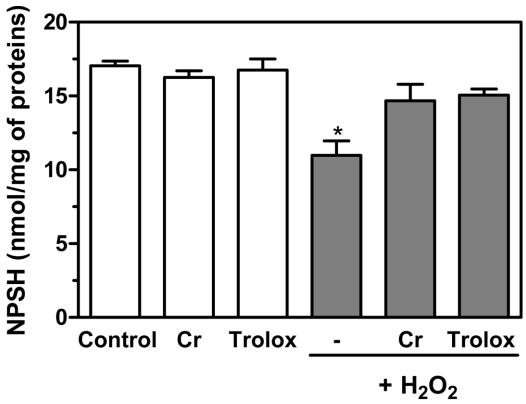

Figure 4. NPSH levels in oxidatively-injured differentiating C2C12: the effect of Cr or trolox supplementation. Cr- or trolox-supplemented cells were treated with 0.3 mM H_2O_2 and then assayed for NPSH levels immediately after oxidative challenge. Data are the means ± SEM of three separate determinations. *$p < 0.01$ as compared to control cells (Student's *t*-test).

3.3 Effect of Cr preloading on the intracellular levels of catalase, GpX, and NPSH

The activity of these two first-line antioxidant enzymes was tested 24 h following serum deprivation (DifD1) either in Cr-supplemented or -unsupplemented cells. NPSH (of which glutathione represents 90% or more [57]) levels were also determined in H_2O_2-treated cultures. Results shown in Table 2 indicate that Cr-loading had no effect on catalase and GpX activity or on the cellular NPSH content in untoxicated cells. However, despite the identical catalase- and GpX-dependent detoxifying capacity of Cr-loaded or -free cells, Cr supplementation partially prevented the fall of NPSH levels in cells treated with H_2O_2 (Fig. 4), as compared to Cr-unsupplemented cells. This effect has been reported previously by our group [48], and is likely to reflect the direct antioxidant capacity of the enriched intracellular free-Cr pool. Similarly and according to its antioxidant activity, 0.1 mM trolox (Fig. 4) also prevented the drop of NPSH caused by the oxidant.

3.4 Determination of the myogenic index

To quantify the fusion of myoblasts, the MI, defined as the number of nuclei residing in cells containing three or more nuclei divided by the total number of nuclei, was determined in May-Grunwald-Giemsa stained cells, with the results shown in Fig. 5. In agreement with recently published observations [7, 66] and as compared to controls, Cr *per se* significantly increased the MI of C2C12 at DifD5. Conversely and according to its toxicity, H_2O_2 treatment caused a dramatic drop of myoblasts' fusion. Again, Cr was protective against oxidative stress: Cr-supplemented, intoxicated cultures showed an MI similar to that of controls. Unexpectedly, trolox – whose effect on oxidatively-injured cells in viability experiments was comparable to that of Cr (see Figs. 2B–D) – with regard to the MI end point was still protective but to a significantly lower extent than Cr. Another established antioxidant, namely *N*-acetylcysteine, was tested under these conditions and behaved as trolox (not shown).

3.5 SEM and TEM analysis of the differentiating process

SEM and TEM ultrastructural analyses were performed on cultures at DifD3 and 5, with the micrographs described below and presented in Fig. 6.

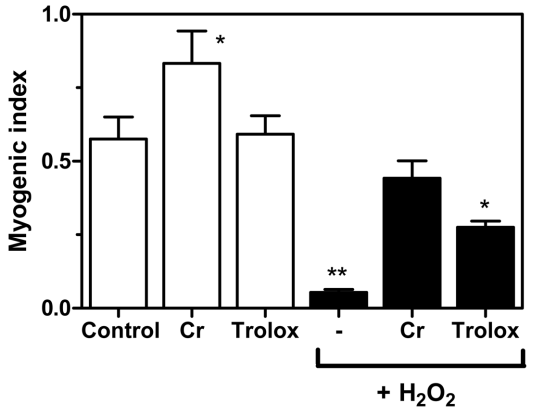

Figure 5. MI in oxidatively-injured differentiating C2C12: the effect of Cr or trolox supplementation. Cells were supplemented with Cr or trolox, treated with 0.3 mM H_2O_2 and allowed to differentiate in fresh DM. The MI (see Section 2) has been calculated analyzing micrographs of Giemsa stained cultures taken at DifD3. Data are the means ± SEM of three separate determinations. *$p < 0.01$ and **$p > 0.001$ as compared to control cells (Student's *t*-test).

At SEM analysis, early myotubes appeared at DifD3 and final cell maturation at DifD5, when an increased number of thicker and longer myotubes could be observed. Also, sporadic myotubes underwent spontaneous contractions at this stage (not shown), a feature characterizing the late differentiation stages of C2C12 cells [67]. The structure of differentiating myoblasts was further investigated by TEM, which permitted the identification of the early assembly of myofilaments, and of the significant increase of nuclear number.

The treatment of cells with Cr alone did not affect their morphology during differentiation. At SEM and TEM analysis and up to DifD5 their cell patterns were comparable to the control ones. However, a significant increase in myotube thickness, particularly at DifD5 appeared, as well as a higher number of mitochondria.

In contrast, H_2O_2 strongly affected C2C12 monolayer morphology. At DifD3 a general cell detachment appeared. Debris and rounding cells were observable by SEM on the underlying, discontinuous, monolayer. At TEM, the inner cell structure was deeply affected by oxidative challenge; degeneration and loss of mitochondria could be observed and diffuse cell hydration and vacuolization indicated the occurrence of necrosis.

At DifD5, when untreated monolayers are generally characterized by the presence of thick and long myotubes, deep changes appeared in intoxicated cells. A diffuse blebbing on myotube surface could be revealed by SEM; consistently a general cell necrosis was evident at TEM examination and surviving cells exhibited a deep alteration (swelling and disruption) of mitochondrial morphology and density (this effect is well documented by the inserts in the TEM micrographs of H_2O_2-treated cells at both DifD3 and 5).

Cr pre-loading significantly mitigated the effect of H_2O_2. Apart from the persistence of occasional debris, at both DifD3 and DifD5 the monolayers showed surface and inner cell features relatively comparable to the untreated control cell ones. In particular, a certain degree of differentiation occurred at DifD3 in Cr-supplemented, H_2O_2-treated cells. TEM analysis of the same samples revealed a relevant decrease in cytoplasmic vacuolization and a good mitochondrial morphology. At DifD5 a significant increase in myotube number could be observed at SEM, as well as the progressive disappearance of cell debris. TEM confirmed the formation of myotubes and the viability of mitochondria, and the good preservation of the rough endoplasmic reticulum, highly damaged after H_2O_2 treatment.

A comparison with the established antioxidant Trolox has also been made. According to survival data, addition of trolox attenuated the deleterious effects induced by H_2O_2. However, as compared to Cr-supplemented, oxidatively injured cells, fewer myotubes and more debris could be observed at DifD3. These neoformed myotubes, although showing a good preservation of organellar component, appeared generally thinner. At DifD5 myotube formation appeared still delayed. Again, the protective action of trolox seemed less evident than that of Cr.

3.6 Proteomic analysis of the differentiating process

The analysis of C2C12 proteomes, shown in Fig. 7, has been performed to compare the expression patterns of the cultures differentiating under our experimental conditions. The 2D maps of control C2C12 at DifD3 (Fig. 7C), as compared to that taken at the initial differentiation stage (DifD1) reflect the occurrence of marked alterations in protein expression. These changes are likely to parallel the ongoing differentiating process. In particular, a number of proteins were newly or more strongly expressed at DifD3 (compare Fig. 7C with Fig. 7A). Specifically, among these annexin 1 (spot 1), a calcium/phospholipid-binding protein promoting membrane fusion [68], gelsolin (spots 2 and 3), a calcium-regulated, actin-modulating protein [69] and ATP synthase D-chain (spot 4), an inner mitochondrial membrane protein whose expression increases over myogenesis and mitochondriogenesis [14], were identified with ESI-Q-TOF MS (spectra not shown). Cr-supplementation did not significantly modify this scenario (not shown). In contrast, the protein pattern of H_2O_2-injured, Cr-free cells at DifD3 (Fig. 7B) was remarkably different from those of unintoxicated controls at DifD1 and at DifD3 (Fig. 7C). Indeed many unidentified proteins indicated with the arrows in Fig. 7A reduced or even disappeared upon oxidative treatment. More interestingly, annexin 1, gelsolin, and ATP synthase D-chain were much less expressed. Notably, these alterations were apparently prevented by Cr supplementation. At DifD3 the protein pattern of H_2O_2-treated, Cr

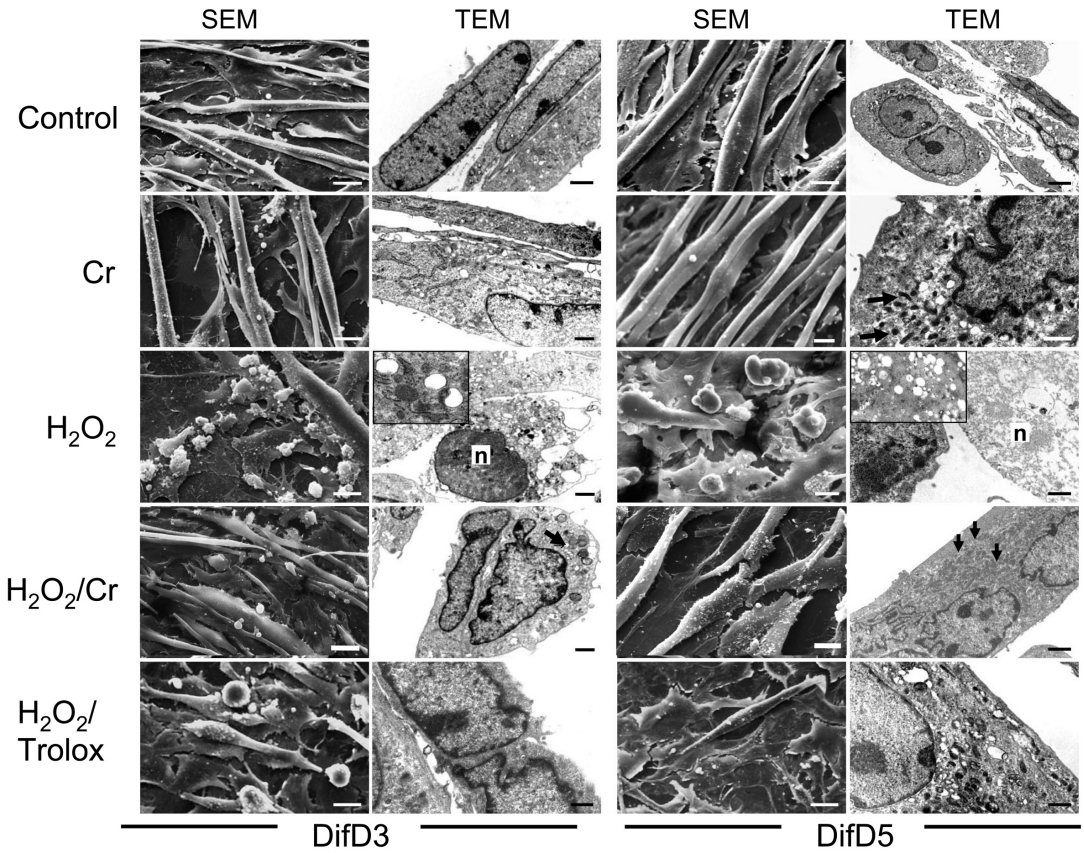

Figure 6. Differentiating C2C12 at SEM and TEM: the effect of oxidative challenge and of Cr supplementation. Micrographs were taken at DifD 3 and 5. Initial thin myotubes appeared in control cells at DifD3, while an increased number of thicker and longer myotubes were present at DifD5. Cr supplemented cells appeared comparable to the control ones, both at DifD3 and DifD5 with a higher number of mitochondria, particularly at DifD5 (→). H_2O_2 induced necrosis at DifD3 and DifD5 (n). The inserts in the TEM micrographs of H_2O_2-treated cells at DifD3 and 5 highlight the mitochondrial swelling and disruption caused by oxidative stress. Cr prevented the effect of H_2O_2: TEM showed a relevant decrease of cytoplasmic vacuolization and well preserved mitochondrial morphology (→). Bars: SEM 10 μm; TEM 1 μm.

preloaded cells (Fig. 7D), was virtually identical to that of untreated cells (Fig. 7C).

Thus, the oxidative challenge blocks the differentiative process, as suggested by the lack of a wide number of proteins highly expressed in differentiating cultures and Cr supplementation forestalls these alterations.

Western blot analysis of myosin heavy chain (MHC) – a reliable marker of myogenic maturation (Figs. 8A and B) further confirmed the latter inference. The MHC levels progressively increased over time up to DifD4 in control and Cr-supplemented cells; as expected, oxidative challenge resulted in a strong and irreversible depression of its synthesis in Cr-free cells, an effect which was significantly attenuated by Cr pre-loading (Figs. 8A and B).

3.7 mRNA expression of MRFs and of IGF-1

To assess whether the inhibitory effect of H_2O_2 on myogenesis was apparent at the mRNA level, differentiating C2C12 myoblast cultures were analyzed daily (from DifD0 through DifD3). In particular, the mRNA levels of MyoD, myogenin, MRF4, [12, 70], and IGF-1 [7, 71] (see also Fig. 2, which depicts the role of these factors in the myogenic process) were quantified. The real time PCR approach was used since, it allows to detect the low levels of mRNA transcripts found in H_2O_2-treated cultures (see below) and the results are shown in Fig. 9.

In control cultures, the selected gene transcripts increased after the induction of differentiation, although with different kinetics and to different extents. In most cases, Cr-preloading *per se* was capable of further boosting the induction of the same gene transcripts triggered by switching to DM, as compared to control cultures. Such effect was particularly evident in the case of IGF-1, MRF4 and myogenin at DifD2 and 3. As to MyoD transcript, a significant, durable but not progressive increase could be observed since DifD1. The "boosting" effect of Cr is similar to that reported by Louis *et al*. [7]: indeed these authors pointed out that the increased expression of IGF-1 transcript represents a key factor in the Cr-induced hypertrophy

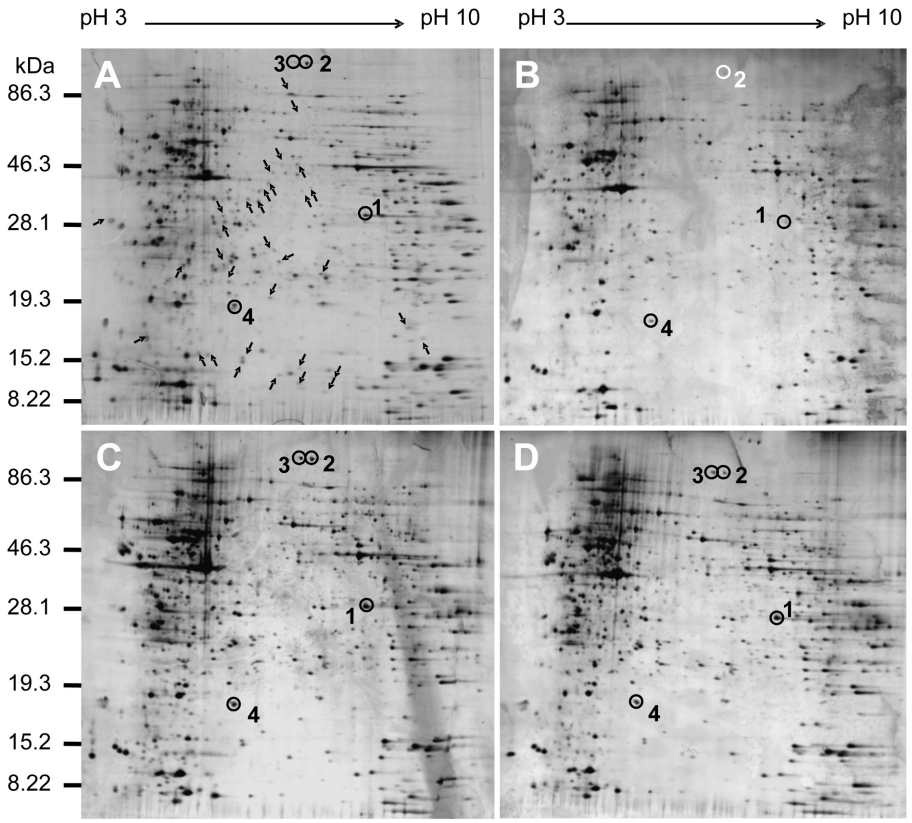

Figure 7. 2-DE electropherograms of differentiating C2C12: the effect of H_2O_2 treatment in Cr-free or Cr-supplemented cells. Cell extracts from confluent C2C12 cultures were subjected to 2-DE at 1 (A) or 3 days (*i.e.*, DifD3) after switching to DM (B, C, and D). A: control cells at DifD1; (B) cells exposed for 1 h to 0.3 mM H_2O_2 1 day after the beginning of differentiation and then cultured for further 2 days in fresh, H_2O_2-free DM. (C) Control cells at DifD3. (D) Cr-supplemented (3 mM Cr during the first day of differentiation) cells treated with H_2O_2 as in B. Spot 1, annexin 1; spot 2 and 3, gelsolin; spot 4, ATP synthase D chain. The arrows in A refer to the proteins that are less expressed or disappear following oxidative challenge.

of differentiating C2C12 cells; more recently the same group has also investigated the molecular bases for this effect [66].

In contrast, H_2O_2 challenge significantly reduced, as compared to untreated cells, the expression level of all the studied gene transcripts. Notably, among them, IGF-1 seems to be more profoundly and durably affected by oxidative challenge.

Interestingly Cr pre-loading prevented the reduced expression caused by H_2O_2. Indeed, at DifD3, Cr supplementation allowed H_2O_2-injured cells to express mRNA levels identical (MyoD and myogenin) to, or even higher (IGF-1 and MRF4) than those of control, Cr-free cells. The levels of these transcripts in H_2O_2-treated/Cr-loaded cells might represent the balance resulting from two distinct but concurring effects of Cr. On one hand Cr boosts transcripts expression, on the other, as an antioxidant, it prevents the inhibition of the same process caused by H_2O_2. Again, this finding correlates well with cytotoxicity, proteomic, and ultrastructural data.

The effects induced by trolox were far different than those elicited by Cr. Indeed, trolox lacked the capacity of stimulating the expression of IGF-1 (Fig. 10) as well as of the other MRFs (not shown). Furthermore, the protection against the inhibition of IGF-1 transcription caused by H_2O_2, was lower than that afforded by Cr, which allowed intoxicated cells to express this important gene transcript to a level even higher than that of untreated, Cr-free controls (compare Figs. 9 and 10).

4 Discussion

Cultured C2C12 murine myoblasts represent an established myogenic cell line: in the absence of mitogenic stimuli proliferating C2C12 withdraw from the cell cycle, elongate, adhere, and finally fuse together to form mature-like myotubes. In the present study, C2C12 cells completed this process in 5 – 6 days following the switching to DM, as assessed by means of biological, morphological, ultrastructural, and molecular approaches (see Figs 5 – 9).

The myogenic program is tightly coupled to a complex network of signal transduction pathways regulating the repression or expression of regulatory and muscle-specifying proteins [6, 14]. This program may be impaired by a number of external conditions, including oxidative stress [19, 72, 73], whose detrimental action is confirmed by the data presented herein.

4.1 Effect of H_2O_2 on C2C12 viability and differentiation

C2C12 cells exposed to a toxicologically relevant concentration (*i.e.*, resulting in a partial cell demise, see Figs. 2A

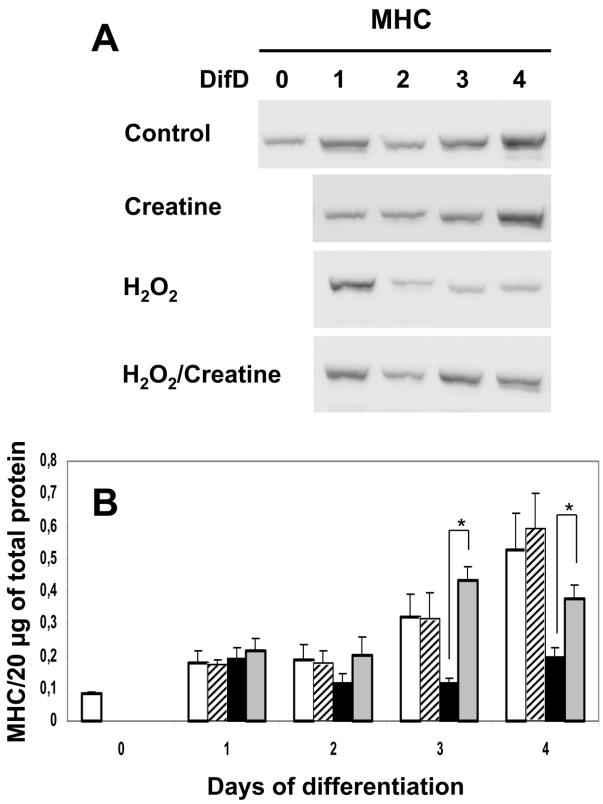

Figure 8. Myosin heavy chain profile in C2C12 cells as a function of the differentiation stage: the effect of H_2O_2 treatment in Cr-free or Cr-supplemented cells. (A) Representative immunoblots showing MHC levels in control, Cr-supplemented, and H_2O_2-challenged- Cr-free or Cr-supplemented cells. (B) Densitometric analyses of MHC blots (mean ± SEM from five independent experiments). Protein expression levels were reported as band optical densities (arbitrary units) normalized to total loaded proteins. Key: open bars, controls; striped bars, Cr-supplemented cells; black bars, 0.3 mM H_2O_2-treated cells; gray bars, Cr-supplemented, H_2O_2-treated cells. Statistical differences were assessed by ANOVA two tails analyses and Bonferroni post-hoc test. *$p < 0.01$. Ctrl *versus* Cr conditions and H_2O_2 versus Cr/H_2O_2 conditions.

and B) of H_2O_2 during the early stages of differentiation (24 h after serum reduction, DifD1), displayed a 30–40% reduction of their viability. More interestingly, the cells surviving the oxidative stressor, although exhibiting a partial and late recovery of protein synthesis and of MTT reducing ability (Figs. 2C and D), were unable to continue and execute the differentiative task (Figs. 5–9) within the considered time intervals. Indeed (i) cells surviving treatment did not show, up to DifD5, the typical morphological features of ongoing differentiation observed in control cultures, and their MI was very low; (ii) consistently, the overall 2D protein expression pattern was markedly different as compared to that of differentiating controls: in particular, specific proteins typically overexpressed during myotube formation such as gelsolin, annexin-1, ATP synthase D-chain, and MHC (this latter quantified by Western blotting) were significantly lower in H_2O_2-treated cells.

Thus, it can be concluded that a mildly toxic treatment with H_2O_2 blocks the myogenic process. Although the identification of the molecular targets responsible for the H_2O_2-dependent inhibition of myogenic differentiation was beyond the scope of the present research, we have identified some events that may account, at least in part, for the differentiative arrest. Firstly, the mRNA levels of selected regulatory or co-regulatory factors of myogenesis, namely MyoD, myogenin, and MRF4 were strongly and durably reduced in oxidatively-injured cells; transcription of IGF-1, which plays a major role in controlling muscle growth [7], was inhibited to an even greater extent. Secondly, the NPSH levels (of which GSH represents more than 90%) in intoxicated cells were markedly lower as compared to controls. Notably, Ardite *et al*. [74] have shown that the integrity of GSH pools is essential for myogenic differentiation of C2C12 cells. Thirdly, as assessed with TEM, H_2O_2-injured cells show signs of extensive mitochondrial degeneration and loss, a phenomenon which may be detrimental in a process typically requiring active mitochondriogenesis such as muscle differentiation [75, 76].

4.2 Cr *versus* trolox in preventing the oxidatively-induced differentiation arrest

The efficacy of two different approaches to shield cells from oxidative injury has been compared: the first, based on an orthodox approach, involved the use of the established radical scavenger trolox and the second that of Cr.

The concentrations of trolox and Cr used throughout this study were selected to obtain the same *bona fide* antioxidant capacity: indeed the two agents equally prevented the fall of NPSH caused by H_2O_2 (Fig. 4), which can be taken as an reliable marker of oxidative attack. Consistently, the capacity of Cr and trolox to prevent the reduction of cell viability (Figs. 2B–D) was similar. Nonetheless, when the ability of restoring the myogenic competence of oxidatively-stressed C2C12 cells was assayed using morphological, ultrastructural, and molecular approaches, the effect of Cr was clearly different. Indeed, Cr preloaded cells retained their ability to differentiate, while trolox or *N*-acetylcysteine-loaded C2C12 cells, although cytoprotected, did not display the same degree of differentiation (see Figs. 5–7, 9, and 10).

Thus, the above difference implies that Cr protection does not only depend on its antioxidant capacity, but also on other intrinsic properties. Cr is known to exert multiple effects on living cells, and in particular on muscle cells: *via* stimulation of MRFs and IGF-1 (see Fig. 9 and [7, 66]); it may promote hypertrophy on developing myotubes (Fig. 6 and [7]); it is highly taken up, concentrated and durably retained by muscle cells [26] and myogenic cultures (Fig. 3 and [64]); it is partly converted into CrP, which contributes

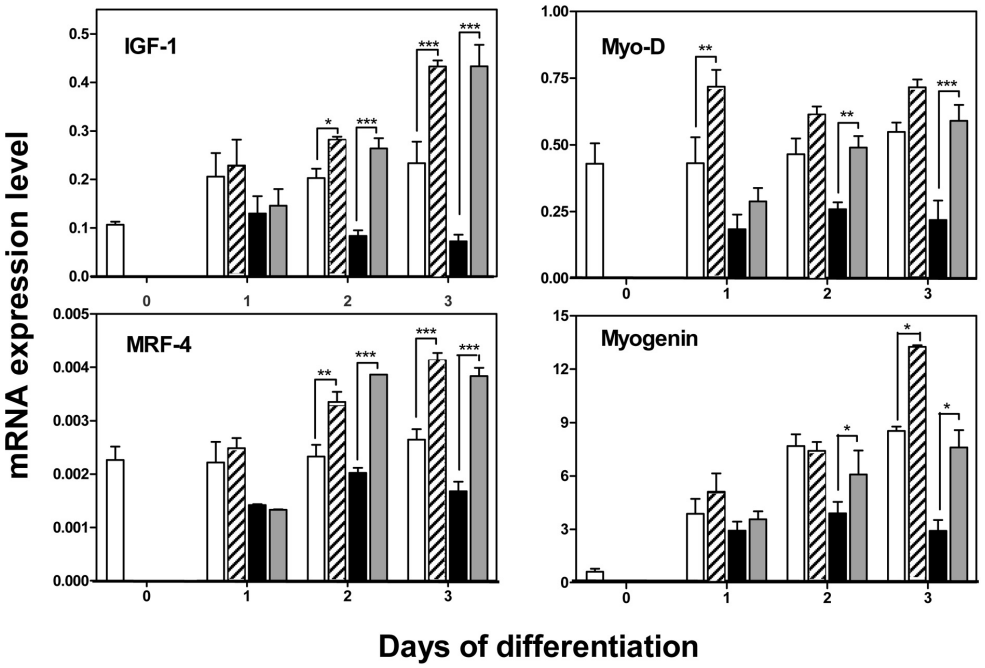

Figure 9. Expression of IGF-1, Myo-D, MRF-4, and myogenin mRNA in C2C12 at progressive differentiation stages: the effect of H_2O_2 treatment in Cr-free or Cr-supplemented cells. Cr-free or Cr supplemented cells were collected at DifD 0,1, 2, and 3 to determine the gene expression levels for *IGF-1*, *Myf-5*, *Myo-D*, *myogenin*, and *MRF-4* with RTQ-PCR. H_2O_2 was given 24 h after the beginning of differentiation (see the "Treatment conditions" subheading in Section 2 for details). At DifD1 the analyses were performed 1 h following oxidative stress. Key: open bars, controls; striped bars, Cr-supplemented cells; black bars, 0.3 mM H_2O_2-treated cells; gray bars, Cr-supplemented, H_2O_2-treated cells. Cr and H_2O_2 concentrations were 3 and 0.3 mM, respectively. Results are expressed as mean ± SEM from three independent experiments. Statistical differences were assessed by ANOVA two tails analyses and Bonferroni post-hoc test. * $p < 0.05$; ** $p < 0.01$ *** $p < 0.001$. Ctrl *versus* Cr conditions and H_2O_2 *versus* Cr/H_2O_2 conditions. The expression level of target genes were related to S16 mRNA gene level.

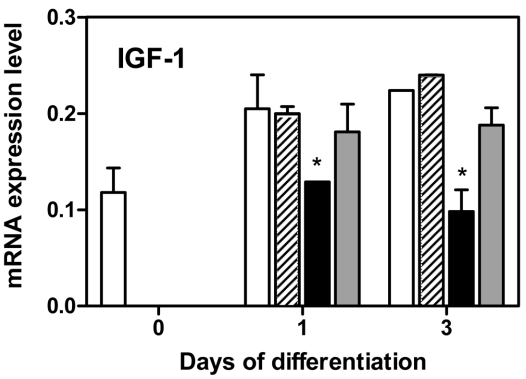

Figure 10. Expression of IGF-1 in C2C12 at progressive differentiation stages: the effect of H_2O_2 treatment in trolox-free or trolox-supplemented cells. Trolox-free or trolox supplemented cells were collected at DifD 0,1, and 3 to determine the gene expression levels for *IGF-1* with RTQ-PCR. Key: open bars, controls; striped bars, trolox-supplemented cells; black bars, H_2O_2-treated cells; gray bars, trolox-supplemented, H_2O_2-treated cells. Trolox and H_2O_2 concentrations were 0.1 and 0.3 mM, respectively. Results are expressed as mean ± SEM from three independent experiments. * $p < 0.01$ as compared to untreated control cells (Student's *t*-test). The expression level of target genes were related to S16 mRNA gene level.

to ameliorate cellular energy stores. Our data demonstrate that a 24 h Cr pre-loading results in higher, long lasting Cr and CrP intracellular levels; the increased availability of CrP ameliorates the energy status of cells undergoing oxidative stress, and renders them more resistant; the enriched free-Cr intracellular pool might also sustain the significant overexpression of relevant transcripts (MyoD, MRF4, myogenin, and IGF1). Interestingly, the positive effect of Cr "priming" on the expression level of these transcripts, although lower than in unintoxicated cells, could still be observed in H_2O_2-treated C2C12 (see Fig. 9 and related subchapters), and is likely to play a pivotal role in promoting myotube formation in intoxicated cultures. Importantly, trolox failed to stimulate the basal transcription level of IGF-1 (Fig. 10), as well as of the other MRFs studied (not shown).

Cr supplementation also prevented the detrimental effects of H_2O_2 on mitochondria, as suggested by TEM microscopy (Fig. 6): again, the extent of this effect seemed higher than that of trolox. Notably, we have recently shown that Cr, unlike trolox, mitigates H_2O_2-damage to mtDNA in cultured HUVEC endothelial cells [50]. This point has not been specifically addressed here, and will deserve extensive investigation in forthcoming studies. However – although

it is a tempting hypothesis – it is conceivable that, similarly to HUVEC, C2C12 mtDNA is sensitive to H_2O_2 challenge and that Cr pre-treatment reduces the extent of these lesions. Given the importance of mitochondrial activity and of mitochondriogenesis in muscle differentiation [76], it is plausible that the rescue of these organelles might concur to the overall protection of Cr. In our toxicity setting, for example, maintaining an adequate mitochondrial activity, along with the increased availability of CrP, might allow a better handling of intracellular calcium overflow in the course of oxidative stress. An indirect and incidental evidence of the Cr-mediated mitochondrial rescue consists in that Cr prevented the H_2O_2-induced inhibition of myogenin mRNA (Fig. 9), whose gene, incidentally, is a specific transcriptional target of mitochondrial activity [76]. Similarly, ATP synthase D-chain (spot 4 in Fig. 7), whose expression increases over mitochondriogenesis [14], was strongly reduced in Cr-free, H_2O_2 intoxicated cells, but normally expressed in Cr-supplemented, H_2O_2 challenged cells.

Thus the capacity of Cr to protect mitochondria in stressing conditions and its contribution to energy shuttling and buffering, might represent an additional mechanism concurring to maintain the differentiative ability of H_2O_2-injured myoblasts. It is also worth noting that the above effects were elicited by a concentration of Cr (3 mM) roughly comparable to the plasma levels attainable *in vivo* following high dose oral intakes [30].

The protective effect of Cr in this oxidative stress paradigm is likely to be the expression of its pleiotropic activity on multiple targets relevant to myogenesis, rather than the result of a single, highly specific mechanism. Indeed the enrichment of Cr intracellular pools (i) affords a significant sparing of intracellular thiols content, (ii) preserves the integrity of mitochondria, probably *via* its organelle-directed antioxidant activity [50] (iii) increases the expression of selected MRFs and of IGF-1 mRNAs (iv) augments the CrP stores. Only the first two of these effects are likely to be mediated by the antioxidant capacity of Cr; the other two effects are a peculiar of Cr and independent of its antioxidant capacity, as indirectly suggested by the fact that trolox increases neither MRFs and IGF-1 nor CrP pools. Indeed, the beneficial effects of Cr that are being observed at the therapeutic and preventive levels [31, 35–46] barely depend exclusively on the role of Cr in cellular energetics, but rather on multiple positive interactions with diverse cellular targets [7, 33, 47–50, 52, 64, 77–80]. The comprehension of Cr pleiotropism is an emerging issue in research literature, and the results in this direction might be important to rationally interpret the role and the therapeutic/preventive potential of Cr supplementation in specific situations. The present study is in line with this developing scientific concept.

Although the deleterious effect of ROS on muscle homeostasis are well documented [20–22], to the best of our knowledge there are few data in literature dealing specifically with the problem of oxidative damage on differentiating myoblasts [19, 25, 72–74, 81] and even fewer with its prevention [19, 74, 81].

A speculative implication of our data could also be that Cr might somehow promote differentiative signals (not necessarily through its antioxidant activity) alternative to the oxidation-sensitive ones more commonly prevailing under oxidizing conditions. At this regard, an attractive hypothesis might be that Cr somehow regulates the functional expression/balance of the ShcA proteins (SRC homology proteins), namely p66ShcA, p52ShcA, and p46ShcA [82]: these three isoforms are involved in the cell response to oxidative stress and also in skeletal muscle regeneration following ischemia [83]. In particular, p52ShcA promotes IGF-1-mediated hypertrophy while, in contrast, p66ShcA (whose signaling seems to be prevailing under oxidative conditions) inhibits IGF-1 effects leading to increased susceptibility to oxidative stress and poor survival of target cells [83]. The same authors also reported that p66ShcA/ko myoblasts – similarly to our Cr-supplemented C2C12 – are resistant to H_2O_2 differentiative arrest [83]. Thus Cr might somehow promote p52ShcA signaling and repressing at the same time that elicited by p66ShcA: this hypothesis should deserve consideration for future studies in this specific field.

4.3 Conclusions

Cr supplementation has the unique feature to protect myoblasts from cell death and to allow surviving cells to circumvent the differentiation arrest caused by oxidative challenge: in other words, Cr loading might increase the repair/regeneration balance of muscles subjected to stressing conditions. The implications of our findings are many-fold and raise clinical, nutritional and future research issues. As it has been discussed previously, muscle inflammation and a variety of muscular disorders are complicated by both persisting oxidative stress and impairment of the tissue repair capacity [25, 31, 36, 40, 43–45]. Our data might then concur to better understand the mechanisms whereby oral Cr supplementation is somehow beneficial in the above muscle and heart diseases as well as to lend support to its use in these situations.

In a further clinical perspective, the outcome of myoblast transplantation, a promising strategy for the therapy of muscular dystrophies and heart diseases, is affected by the poor survival of implanted myoblasts [53]. Since, it has been proposed that oxidative stress concurs to this problem, Cr pre-implant loading of myoblasts might result in a better survival and differentiative efficiency of implanted cells, and better clinical outcomes of this procedure.

From a nutritional point of view, our data might help to understand and reappraise the benefits arising from long-time Cr dietary supplementation, and support the advisability of elevating Cr levels not only in situations where meat

consumption is obligatorily reduced (hypercholesterolemia, cardiovascular diseases, aging, vegetarian diet), but also in specific groups (elderly, patients undergoing post-trauma rehabilitation, see also below) of omnivorous diet population. Indeed, plasma-Cr concentrations similar to those capable of eliciting the effects described herein (1 – 3 mM) can be achieved only through the adoption of specific oral supplementation regimens [30]. In this light, our findings strengthen the rationale of "long-term dietary Cr supplementations" to gain a longer and healthier lifetime, as suggested by Bender *et al*. in a recent report [52] showing that Cr improves health and survival of mice. The present study could also support the use of supplementary Cr intakes in post-trauma physical rehabilitation, *i.e*., a situation were better muscle regeneration is obviously advisable. Finally, the observed slight hypertrophic effects of Cr, along with its ability to favor myoblasts' differentiation and myotubes' formation in stressing conditions, could also raise the question of whether the mega-dose intakes of Cr consumed by professional athletes during their training programs can be considered as an illegal, doping procedure.

In closing, since Ducray *et al*. [78] have recently reported that Cr promotes differentiation of GABA-ergic neuronal precursors in cultured fetal rat spinal cord, the possibility that Cr is protective also in other oxidatively stressed, differentiating cell types would deserve consideration, and future studies in this direction should be encouraged.

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