Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1780 (2008) 16–26
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbagen

Differential effect of creatine on oxidatively-injured mitochondrial and nuclear DNA

Chiara Guidi [a,1], Lucia Potenza [b,1], Piero Sestili [a,c,*,1], Chiara Martinelli [a], Michele Guescini [a], Laura Stocchi [a], Sabrina Zeppa [a], Emanuela Polidori [a], Giosuè Annibalini [a], Vilberto Stocchi [b]

[a] *Istituto di Ricerca sull'Attività Motoria, Università degli Studi di Urbino "Carlo Bo", Via I Maggetti 26, 61029 Urbino (PU), Italy*
[b] *Istituto di Chimica Biologica "Giorgio Fornaini", Università degli Studi di Urbino "Carlo Bo", Urbino, Italy*
[c] *Istituto di Farmacologia e Farmacognosia, Università degli Studi di Urbino "Carlo Bo", Urbino, Italy*

Received 14 March 2007; received in revised form 10 September 2007; accepted 18 September 2007
Available online 5 October 2007

Abstract

Creatine is a naturally occurring compound obtained in humans from endogenous production and consumption through the diet. It is used as an ergogenic aid to improve exercise performance and increase fat-free mass. Lately, creatine's positive therapeutic benefits in various oxidative stress-associated diseases have been reported in literature and, more recently, creatine has also been shown to exert direct antioxidant effects. Oxidatively-challenged DNA was analysed to show possible protective effects of creatine. Acellular and cellular studies were carried out. Acellular assays, performed using molecular approaches, showed that creatine protects circular and linear DNA from oxidative attacks. Nuclear and mitochondrial DNAs from oxidatively-injured human umbilical vein endothelial cells were analyzed. Creatine supplementation showed significant genoprotective activity on mitochondrial DNA. This evidence suggests that creatine may play an important role in mitochondrial genome stability in that it could normalize mitochondrial mutagenesis and its functional consequences. Thus, creatine supplementation could be used to prevent or ameliorate diseases related to mitochondrial DNA mutations, and possibly to delay aging.

Keywords: Creatine; Oxidative DNA damage; Mitochondrial DNA; Nuclear DNA; Antioxidant

1. Introduction

Creatine (Cr) is the most popular supplement proposed as an ergogenic aid. It is distributed throughout the body with 95% found in skeletal muscle and the remaining 5% in the brain, liver, kidney, and testes [1]. Cr is obtained through diet (~1 g/day for an omnivorous diet) and synthesized in the liver, kidney and pancreas (~1 g/day). The dietary intake and endogenous production of Cr match the spontaneous degradation of phosphoCr and Cr to creatinine at a rate of 2.6% and 1.1% per day, respectively. Once creatinine is formed, it enters the circulatory system by diffusion and is eliminated from the body through glomerular filtration. Intramuscular and cerebral stores of Cr, as well as its phosphorylated form, phosphoCr, increase with oral Cr-supplementation. The increase of these stores can offer therapeutic benefits by preventing ATP depletion, stimulating protein synthesis or reducing protein degradation, and stabilizing biological membrane [2].

Evidence from exercise literature has shown that athletes benefit from supplementation by increasing muscular force and power, reducing fatigue in repeated bout activities, and increasing muscle mass [3–7]. In the 1990s Cr supplementation became a popular ergogenic aid for many athletes to maintain a rapid rate of adenosine triphosphate (ATP) turnover during a brief period of high intensity activity [8–15].

In a different direction, Lawler et al. reported that Cr is capable of directly quenching aqueous radical and reactive

Abbreviations: CCC, Covalently closed circular; Cr, Creatine; DTT, DL-dithio-threitol; MFO, Mixed-function oxidase; mtDNA, Mitochondrial DNA; nDNA, Nuclear DNA; 8-OHdG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; PBS, Phosphate-buffered saline; ROS, Reactive oxygen species; PCR, Polymerase chain reaction; QPCR, Quantitative polymerase chain reaction

* Corresponding author. Istituto di Farmacologia e Farmacognosia, Università degli Studi di Urbino "Carlo Bo", Urbino, Italy. Tel.: +39 0722 303414; fax: +39 0722 303401.
E-mail address: piero.sestili@uniurb.it (P. Sestili).
[1] These authors (C.G., L.P., P.S.) contributed equally to the work described in this report.

0304-4165/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbagen.2007.09.018

species ions *in vitro* [16]. A more recent study from our laboratory showed that Cr exerts direct antioxidant activity *via* a scavenging mechanism in oxidatively-injured cultured mammalian cells [17]. Moreover, other authors have demonstrated that Cr supplementation not only improves exercise performance and increases fat-free mass, but is also beneficial in the prevention or treatment of some oxidative stress associated diseases [18–21]. In these diseases mitochondrial DNA (mtDNA) represents an important target for oxidative damage. Indeed, mtDNA mutations have recently been reported as being an etiological factor in oxidative stress-related disorders [22] including cardiovascular diseases and inherited [23] or acquired [24–26] neurodegenerative disorders, several types of tumors affecting the colon, bladder, lung, breast, kidney, head and neck [27–34] mitochondrial myopathies [35] and the normal aging process [36].

The human mitochondrial genome, completely sequenced in 1981 [37], is a 16,569-bp closed circular, duplex molecule present in a high copy number per cell, widely varying among cell types. It encodes 13 polypeptides, 22 transfer RNAs and 2 ribosomal RNAs, all essential for electron transport and ATP generation [38]. mtDNA has been observed to be more susceptible to damage than nuclear DNA because of several possible factors such as exposure to high levels of reactive oxygen species (ROS) produced during oxidative phosphorylation [39], lack of protective histones, and limited DNA repair pathways [40] having a robust base excision repair (BER) system but not nucleotide excision repair (NER) [41]. Oxidative damage to mtDNA may lead to loss of membrane potential, reduced ATP synthesis and cell death [42]. Mitochondria, being mediators of cell life and death, should therefore represent a potential target for new therapeutic approaches. Indeed, strategies are being developed for the targeted delivery of antioxidants or other cytoprotective agents to mitochondria. Cr might be a possible candidate as a mitochondrially-targeted antioxidant, in that it is actively taken up by mitochondria through specific transporters [43], unlike conventional antioxidants which have limited efficacy due to the difficulty of accumulating within these organelles. However, it is not known whether Cr protects from functionally relevant oxidative-induced mutations of mtDNA.

The aim of the present study is to evaluate possible protective effects of Cr on oxidatively-injured nuclear DNA (nDNA) and mtDNA in an attempt to better understand Cr physiology and to envisage its possible use in the prevention or amelioration of a wide range of oxidative stress-related human diseases where oxidative mtDNA damage plays an etiological role.

2. Materials and methods

2.1. DNA and chemicals

The plasmid pGEM-T (3000 bp) was purchased from Promega. All reagent grade chemicals were obtained from Sigma-Aldrich Inc. All primers were obtained from Sigma-Genosys Inc. and DL-dithio-threitol (DTT) from Clontech. H_2O_2, Fe^{2+}, Fe^{3+} and Cr were freshly prepared for each experiment.

2.2. Treatment of DNA with hydroxyl radicals and Cr

Different DNA sources (pGEM-T, PCR product, genomic DNA) were mixed with freshly prepared DTT (10 mM) and $FeCl_3$ (3 μM) (thiol/Fe^{3+}/O_2 mixed-function oxidase, MFO system) or H_2O_2 (2 mM) and Fe_2SO_4 (3 μM) (Fe^{2+}/hydrogen peroxide, H_2O_2 system) in the presence or absence of different Cr concentrations (from 1 mM to 10 mM), in a total volume of 20 μl of 40 mM HEPES (pH 7). Reaction mixtures were incubated for 100 min at 37 °C. DNA samples were applied to 0.8% agarose/Tris Borate EDTA (TBE) gel, stained with

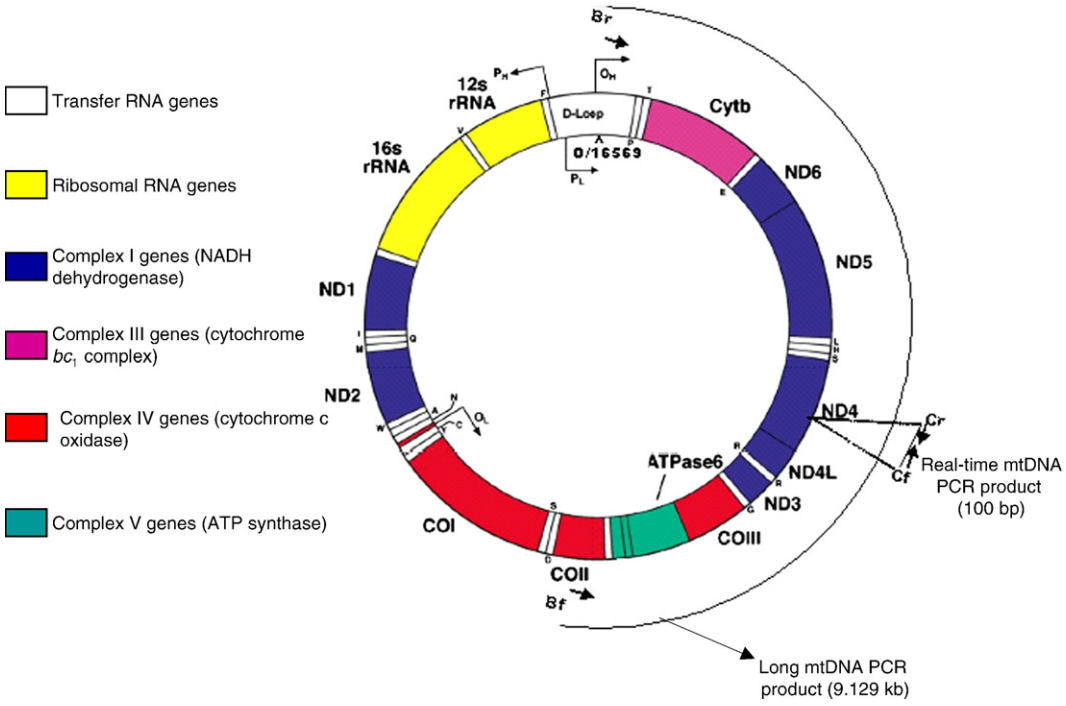

Fig. 1. Schematic representation of human mitochondrial genome and regions amplified by PCR. The four primers used for QPCR and Real-time PCR are indicated by arrows.

ethidium bromide (0.3 μg/ml) and visualized under UV light. Quantification was made by densitometric analysis using Quantity One Software 4.01 (Bio-Rad).

2.3. Isolation of total DNA

High molecular weight DNA was isolated with the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. Total cellular DNA concentration was determined at 260 nm using spectrophotometer (Beckman DU- 640) and nanodrop spectrophotometer (ND-1000 Nanodrop Technologies).

2.4. Cell culture and treatment conditions

HUVEC (human umbilical vein endothelial cells) were cultured at 37 °C in an atmosphere of 95% air and 5% CO_2 in M199 medium containing antibiotics, 1.4 mM glutamine, 10% fetal bovine serum and 50 μg/ml endothelial cell growth factor. HUVEC were seeded at an appropriate density 30–36 h before treatments. At the oxidative challenge stage, the cell number was between 3.5 and 4.5×10^5 cells/well. Cr (10 mM), Trolox (100 μM) or *o*-phenanthroline (10 μM) were added to complete culture medium and were given to cells 24 h, 10′ and 1 h prior to the challenge with H_2O_2, respectively. Trolox and *o*-phenanthroline were also added at the same concentrations to Saline A during treatment with H_2O_2. Oxidative challenge consisted in 30 min incubation of antioxidant-free or antioxidant-supplemented cells with 200 μM H_2O_2 at 37 °C in 2 ml of Saline A (0.145 M NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 5 mM glucose). After treatments, cells were washed with phosphate-buffered saline (PBS, 8 g/l NaCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, 0.2 g/l KCl), harvested by trypsination and processed for DNA damage or recultured in the original medium at different times for DNA repair and cytotoxicity studies.

2.5. Trypan blue exclusion assay

Monolayers were detached by trypsinization, an aliquot of cell suspension was diluted 1:1 with 0.4% trypan blue and cells were counted with a hemocytometer. Results are expressed as the number of viable (unstained) cells in treated and control samples.

2.6. Long PCR

Long PCR was performed in a final volume of 25 μl using a Mycycler machine (Bio-Rad). Specific primers were used to amplify a 9.129-kb fragment of the mitochondrial DNA (primers Bf–Br, long mtDNA PCR, Fig. 1) and a 7.3-kb fragment of the nuclear gene GBA (glucosidase, beta acid; primers Df–Dr, long nDNA PCR).

The reaction mixture contained 60 ng template total DNA, 2.5 μl buffer 1, 200 μM dNTPs, 0.5 μM of each primer and 1.5 U of Expand Long Template PCR system (Roche). The primer nucleotidic sequences and PCR parameters are reported in Tables 1 and 2.

Table 1
Primers employed

604 bp from D-Loop mitochondrial DNA (J01415):	
Af 35	5′ GGAGCTCTCCATGCATTTGG 3′
Ar 620	5′ GGGTGATGTGAGCCCGTCTA 3′
9.129 kb mitochondrial fragment (J01415):	
Bf 8080	5′ CCCCACATTAGGCTTAAAAACAGAT 3′
Br 620	5′ GGGTGATGTGAGCCCGTCTA 3′
100 bp mitochondrial fragment (J01415):	
Cf	5′CCATTCTCCTCCTATCCCTCAAC 3′
Cr	5′ CACAATCTGATGTTTTGGTTAAACTATATTT 3′
7.3 kb nuclear DNA of the GBA gene (NM_001005741):	
Df	5′ TTCTCCATGCAAATCTGTGT 3′
Dr	5′ GAACCAGATCCTATCTGTGC 3′
100 bp nuclear DNA of the GBA gene (NM_001005741):	
Ef	5′ AGCATCAGGGCGGAAGC 3′
Er	5′ TTTCTCCTTTAAGAGCTGCCATTT 3′

Table 2
Thermal cycling parameters

	Cycles	Temperature (°C)	Time
PCR 1	1×	95	5 min
		95	30 s
	30×	62	30 s
		72	45 s
	1×	72	7 min
	1×	4	hold
PCR 2	1×	95	2 min
	30×	95	30 s
		68	9 min
	1×	68	12 min
	1×	4	hold
PCR 3	1×	93	2 min
	10×	93	10 s
		60	30 s
		68	4 min 40 s
	20×	93	10 s
		60	30 s
		68	4 min 40 s + 20 s per cycle
	1×	68	7 min
	1×	4	hold
PCR 4	1×	95	10 min
	40×	95	1 min
		60	9 s
	1×	4	hold

2.7. Quantitative PCR (QPCR)

The amplification products obtained by long PCR were electrophoresed on 0.8% agarose/TBE gel, stained with ethidium bromide (0.3 μg/ml) and quantified by densitometric analyses of the intensity of bands using Quantity One Software 4.01 (Bio-Rad). Treated samples were then compared with controls and the relative amplification was calculated according to Santos et al. [44].

Results presented herein are the mean of two sets of PCR for each target of at least three different biological experiments.

2.8. PCR product purification

The Af–Ar mtDNA amplification products (Tables 1 and 2) were purified using the GenElute PCR Clean-up kit (Sigma-Aldrich Inc) according to the manufacturer's instructions.

2.9. Quantitative Real-Time PCR

Long mtDNA and nDNA PCR products, obtained from HUVEC DNA, were also quantified by Sybr Green Real-Time PCR, using primers Cf-Cr and Ef-Er, respectively, localized in the middle of the long PCR fragments (Table 1). After long amplification, samples were diluted 10^{-4}, while the corresponding genomic DNA samples were diluted 10^{-3}.

Quantitative Real-Time PCR was performed in a Bio-Rad iCycler iQ Multi-Color Real-Time PCR Detection System using 2× Quantitect SYBR Green PCR kit (Qiagen). The quantitative PCR reaction was performed at 95 °C for 10 min to activate HotStart DNA polymerase followed by 50 cycles of the two-step at 95 °C for 30 s and at 60 °C for 30 s. The specificity of the amplification products obtained was confirmed by examining thermal denaturation plots and by sample separation in a 3% DNA agarose gel.

Results were normalized by quantitating each sample for the amount of initial genomic DNA without previous long PCR amplification, in the same real-time PCR conditions.

Each sample was tested in triplicate, and the experimental groups (control DNA, H_2O_2-treated DNA with and without Cr time 0, after 2, 4, 24 and 48 h recovery) consisted of at least three independent experiments. The significance

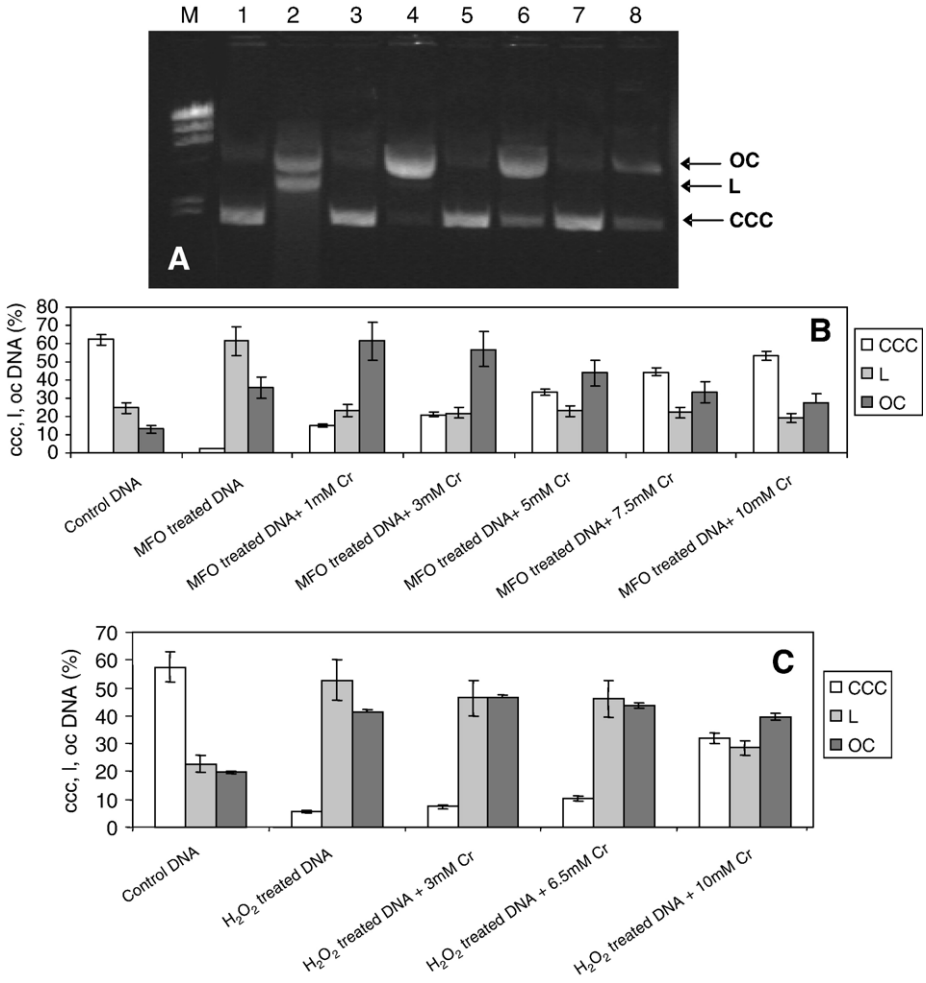

Fig. 2. Protective effect of Cr at different concentrations on plasmid DNA strand breaks induced by the MFO (A, B) and H_2O_2 systems (C). (A) Example of electrophoretic pattern obtained from MFO treatment. Lane M: DNA molecular weight λ/*Hin*dIII; lane 1: control DNA; lane 2: 100 min MFO-treated DNA; lane 3: 0 min MFO-treated DNA + 5 mM Cr; lane 4: 100 min MFO-treated DNA + 5 mM Cr; lane 5: 0 min MFO-treated DNA + 7.5 mM Cr; lane 6: 100 min MFO-treated DNA + 7.5 mM Cr; lane 7: 0 min MFO-treated DNA + 10 mM Cr; lane 8: 100 min MFO-treated DNA + 10 mM Cr. (B, C) Quantitation of Cr protection against DNA strand breaks induced by MFO (B) and Fe^{2+}/hydrogen peroxide (C) systems. Results are expressed as the percentage of the three forms on total plasmid DNA (CCC + OC + L). Values are means ± S.E.M. of results from three replicates.

of the difference in undamaged products among the groups has been evaluated by the Wilcoxon signed-rank test.

2.10. Determination of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-OHdG) by ELISA assay

DNA was extracted from HUVEC using the DNA Extractor WB Kit (Wako, Osaka, Japan) and suspended in 135 μl of 20 mM of sodium acetate (pH 4.8), digested to nucleotide with nuclease P1 (40 U/ml) at 37 °C for 1 h. Then, 15 μl of 1 M Tris–HCl (pH 7.4) was added to the samples and they were treated with alkaline phosphatase (25 U/ml) at 37 °C for 1 h. 8-OHdG levels in digested DNA were determined using 8-OHdG ELISA kit (Japan Institute for Control Aging, Japan), provided by LiStarFISH S.r.l. (Milano, Italy) [45], according to the manufacturer's instructions. The hydrolysates were filtered through Millipore Microcon YM-10 at 14.000 rpm for 10 min to remove enzymes and other macromolecules. The absorbance values for the test samples were read at 450 nm using the microplate reader Model 680 (Biorad) and the Microplate Manager Software Version 5.2.1 (Biorad).

2.11. Fast halo assay

The assay has been carried out as previously described [46]. Briefly, after the treatments, the cells were resuspended at 4.0×10^4/μl in ice-cold PBS containing 5 mM EDTA: this cell suspension was diluted with an equal volume of 2% low-melting agarose in PBS and immediately sandwiched between an agarose-coated slide and a coverslip. After complete gelling on ice, the coverslips were removed and the slides were immersed in NaOH 300 mM for 15 min at room temperature. Ethidium bromide (10 μg/ml) was directly added to NaOH during the last 5 min of incubation. The slides were then washed and destained for 5 min in distilled water. The ethidium bromide-labelled DNA was visualized using a Leica DMLB/DFC300F fluorescence microscope (Leica Microsystems, Wetzlar, Germany) and the resulting images were digitally recorded on a PC and processed with an image analysis software (Scion Image). The amount of fragmented DNA diffusing out of the nuclear cage, i.e. the extent of strand scission, was quantified by calculating the nuclear diffusion factor, which represents the ratio between the total area of the halo and nucleus and that of the nucleus. Data are expressed as relative nuclear diffusion factor, calculated by subtracting the nuclear diffusion factor of control cells from those of treated cells.

3. Results

3.1. Acellular experiments

We investigated the effects of Cr in acellular systems using different DNA sources, such as plasmid DNA and amplification

products obtained by polymerase chain reaction, which represent circular and linear DNAs, respectively. Fe^{2+}/hydrogen peroxide and thiol/Fe^{3+}/O_2 mixed-function oxidase were used as oxidative systems. In this latter system, autoxidation of thiols (DTT) in the presence of iron generates reactive oxygen species (ROS) such as superoxide anion, H_2O_2 and hydroxyl radical ($OH^{\cdot}$) [47]. In particular, hydroxyl radical generated in close proximity to nucleic acid molecules can add hydrogen atoms to DNA bases or abstract hydrogen atoms from the sugar moiety leading to modified bases, DNA strand breaks or abasic sites [48].

Aliquots of 600 ng pGEM-T plasmid were used in order to investigate and assess the induction of single and double strand breaks in the covalently closed circular (CCC) form. In DNA preparations from prokaryotic cells the plasmids show three topoforms, being the prevalent CCC, which may be converted in relaxed (open circular) or linear forms due to single or double strand breaks during experimental manipulations (Fig. 2). Control DNA, H_2O_2 and MFO treated samples in the absence or presence of Cr were assayed through 0.8% agarose gel electrophoresis to quantify the three plasmid topoforms. Electrophoresis analysis showed that both oxidation systems perturbed plasmid stability with a drastic reduction of the supercoiled form and an increase of circular and linear forms, indicating the development of single and double strand breaks, respectively. The addition of Cr resulted in a partial inhibition of the conversion of supercoiled to linear and open circular forms in a dose-dependent manner (Fig. 2).

In another in vitro assay a 600-bp region amplified by polymerase chain reaction (primers Af-Ar in Table 1 and PCR1 in Table 2) was treated as described for the plasmid DNA. The amplification product treated for 100 min with oxidant systems with or without Cr did not show any difference in the agarose-gel electrophoresis patterns. Nevertheless, after an overnight exposure Cr-free samples showed an almost complete DNA degradation while Cr-supplemented samples were partially protected (Fig. 3).

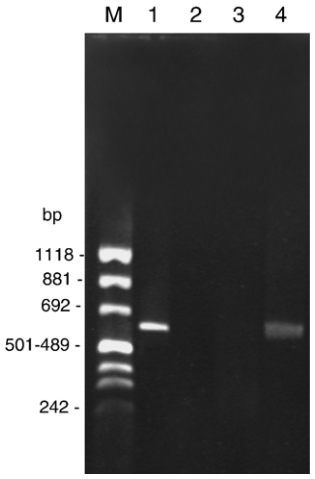

Fig. 3. Protective effect of Cr on a MFO-treated 600 bp mtDNA amplification product. Lane M: DNA molecular weight pUC Mix Marker 8; lane 1: control amplified product; lane 2: no template control; lane 3: MFO-treated PCR product; lane 4: MFO-treated PCR product in the presence of 10 mM Cr.

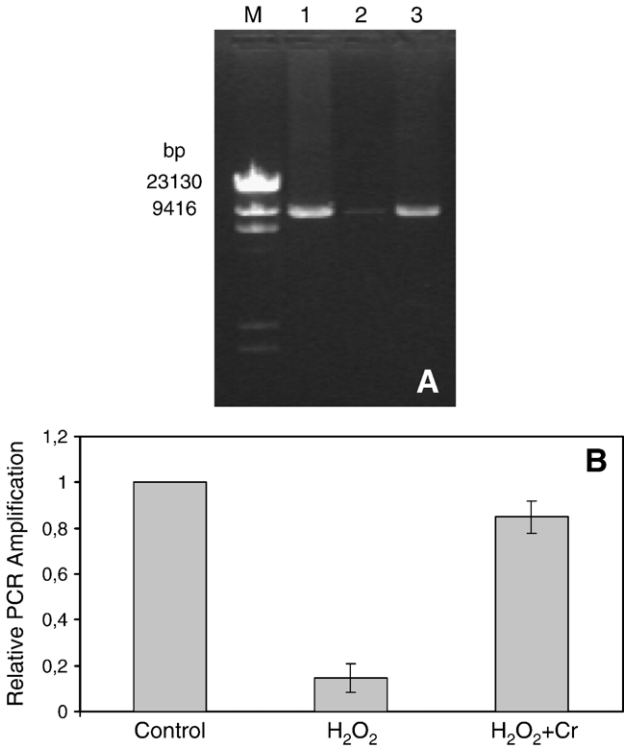

Fig. 4. Protective effect of Cr on mtDNA. (A) Electrophoretic profile of a 9-kb mtDNA region PCR. Lane M: DNA molecular weight λ/*Hin*dIII; lane 1: control amplified product; lane 2: H_2O_2-treated PCR product; lane 3: H_2O_2-treated PCR product in the presence of 10 mM Cr. (B) Quantitation by densitometric analysis of the PCR products obtained in panel A. Values are means ± S.E.M. from at least three independent experiments.

The results obtained on these different DNA sources show that DNA damage develops at different times suggesting that both damage and Cr protection could be related to the conformation of the DNA source under analysis.

In a further acellular experiment, 60 ng of total genomic DNA were treated with Fe^{2+}/H_2O_2 in the absence or presence of Cr and then used as a template for the amplification of a long mtDNA region with a proofreading DNA polymerase (Table 2, PCR 2) to evaluate the extent of DNA damage. This technique is based on the premise that DNA lesions, including oxidative damage such as strand breaks, abasic sites and some base modifications (for example, 8oxodA) can hamper the progression of the polymerase [49,50]. The reduction in the relative yield of the long PCR product reflects the presence of blocking DNA lesions [44,51] rather than the exhaustion of a critical reagent. Thus, amplification is inversely proportional to DNA damage: the more lesions on the DNA target, the less amplification. The amplification products obtained by long PCR were quantified by densitometric analysis, after gel electrophoresis migration, obtaining a quantitative PCR (QPCR). To obtain a real quantitative assay it is necessary to detect the cycle number at which amplification products are in the exponential phase of the long PCR: this condition ensures that other components of the reaction such as dNTPs, primers and Taq polymerase are not limiting. Hence, the cycle number selected assures that by amplifying a 50% control (containing half of the amount of the

non-damaged template, that is 30 ng), a ~50% reduction of the amplification signal is obtained, as compared to a 100% control (data not shown). The protective effect of Cr was also observed in this assay (Fig. 4A, B).

3.2. Cell studies

The genoprotective effect of Cr in DNA acellular systems led us to investigate whether similar effects could be observed in nDNA and mtDNA from H_2O_2-injured cells.

HUVEC cells, which had been previously shown to be sensitive to the antioxidant activity of Cr [17] were preincubated for 24 h with or without 10 mM Cr and then treated for 30 min with 200 μM H_2O_2. The number of viable cells was determined after 24, 48 and 72 h of growth in fresh culture medium using the trypan blue exclusion assay. Under these conditions Cr-supplementation afforded significant and durable cytoprotection in H_2O_2-treated cells (Fig. 5).

In order to quantify the formation of nDNA and mtDNA lesions, the efficiency of mtDNA damage removal as well as the effect of Cr-supplementation, DNA analyses were performed. DNA from *o*-phenanthroline or trolox supplemented cells exposed to H_2O_2 was also analyzed being *o*-phenanthroline a reference iron chelator and Trolox a reference radical scavenger.

The QPCR and the Real-Time PCR were the strategies selected to quantify the yield of the long mtDNA PCR products and nDNA PCR products from H_2O_2-treated, Cr-supplemented or unsupplemented HUVEC cells. One of the main advantages of the QPCR and Real-Time PCR assays is that they allow monitoring of the integrity of mtDNA directly from total cellular DNA avoiding processes which can increase base oxidation such as isolation of mitochondria or separate mtDNA purification steps.

QPCR showed that oxidative challenge induces more extensive damage in mtDNA (Primers B in Table 1 and PCR 2 in Table 2) than in nDNA (Primers D in Table 1 and PCR 3 in Table 2) and that Cr protective effect is statistically significant only on mtDNA (Fig. 6).

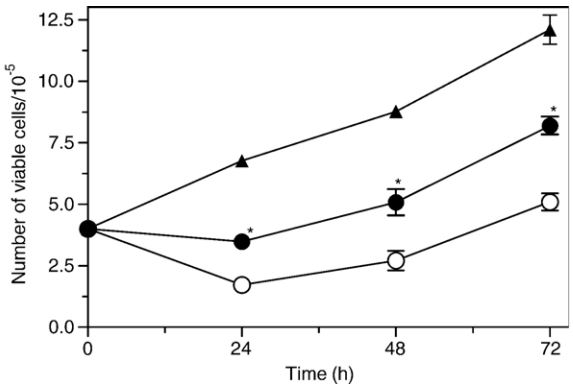

Fig. 5. Effect of Cr preloading on oxidant-induced cytotoxicity in HUVEC cells. Cells were pre-incubated for 24 h in the absence (open circles) or presence (closed circles) of 10 mM Cr, and then treated for 30 min in Saline A with 200 μM H_2O_2. Also shown is the growth curve of control cells (triangles). The number of viable cells was determined after 24, 48 and 72 h of growth in fresh culture medium using the Trypan blue exclusion assay (see Materials and methods). Results represent the means ± S.E.M. from at least 5 separate experiments. * $P<0.005$ (unpaired *t* test) compared to Cr unsupplemented cells.

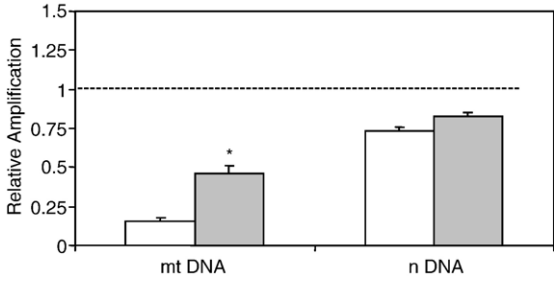

Fig. 6. QPCR on mt and nDNAs from Cr-supplemented (10 mM) and unsupplemented HUVEC. Cells were pre-incubated for 24 h in the absence (open bars) or presence (solid bars) of 10 mM Cr, treated for 30 min in Saline A with 200 μM H_2O_2 and immediately assayed for QPCR. The decrease in amplification was calculated comparing treated samples to undamaged control (dashed line). Data are expressed as the mean ± S.E. of at least three separate determinations in which two Long PCRs were performed per experiment. Student's *t* test was performed comparing controls to H_2O_2-treated groups or Cr untreated to treated groups. $P<0.05$ when controls and H_2O_2-treated groups were compared. *, $P<0.05$ comparing mtDNA from H_2O_2-treated cells to mtDNA from 10 mM Cr supplemented H_2O_2-treated cells.

Primer pairs located in the middle of mitochondrial and nuclear long PCR fragments were used in the Real-Time PCRs (Primers C and E in Table 1 and PCR 4 in Table 2). Standard curves have been established between DNA quantities used as templates in the long PCR and PCR amplicons detected by Real-Time PCR. Results were normalized by quantifying each sample for the amount of initial DNA without previous long PCR amplifications. This two-step assay was selected because a standard Real-Time PCR which uses total DNA, without previous long PCR amplification, produces overlapping curves either with or without induced DNA damages, since the amplification product is too short to show DNA polymerase proofreading blocking. These results were similar to those obtained with QPCR. Indeed, only with mitochondrial primers, significant shifts of the Real-Time PCR amplification curves to the right were obtained in H_2O_2-treated samples when they were compared with controls, reflecting the decrease in the long PCR and the presence of DNA lesions (Fig. 7A); notably, shifts to controls were obtained in Cr supplemented samples reflecting Cr protection and minor DNA damage on mitochondrial DNA with respect to H_2O_2-treated samples (Fig. 7A). Shifts to controls were not significant with nuclear primers suggesting that Cr is not protective on nuclear DNA.

The effect of Cr on mtDNA, as assayed immediately after oxidative challenge, was dose-dependent in the 3- to 10-mM range; concentrations lower than 3 mM were ineffective (not shown). Moreover, the protection afforded by 10 mM Cr, the dose used throughout this set of experiments by virtue of its higher activity, was significant from 0 up to 24 h repair periods, while no significant difference with Cr unsupplemented cells could be seen at 48 h (Fig. 7B). Importantly, that Cr effects are more pronounced at very early post-challenge times (compare the effect at 0 h with that at 24 h) might suggest that it prevents the induction of the lesions – i.e. a mechanism conceivable with a direct antioxidant activity – rather than acting downstream

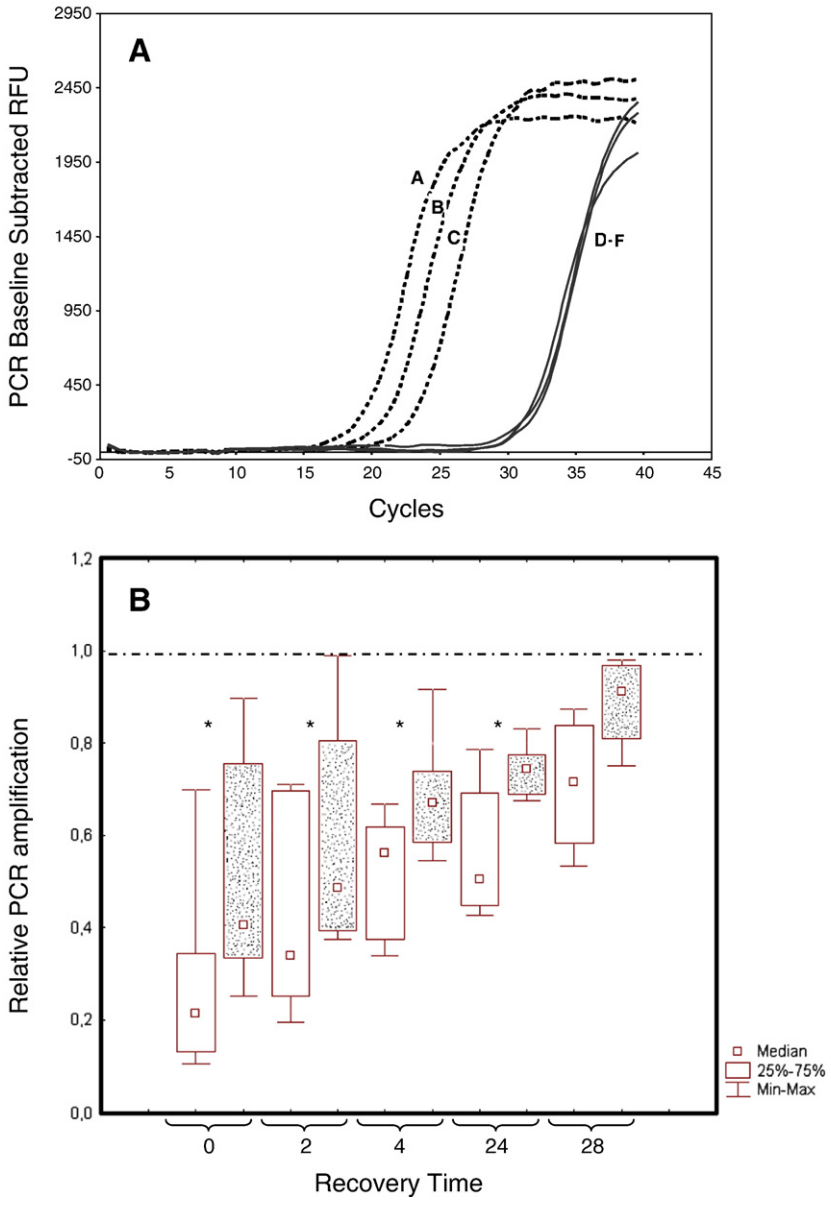

Fig. 7. Effect of Cr preloading on oxidant-induced HUVEC mtDNA damage. (A) Cr-supplemented (10 mM) or unsupplemented cells were treated with 200 μM H_2O_2 for 30 min and immediately assayed for mtDNA damage. Representative amplification curves of Real-time mtDNA PCR are shown: curves A–C were obtained with previous long PCR amplification of 9.129 kb mtDNA: curve A, control DNA; curve B, H_2O_2 + 10 mM Cr-treated DNA; curve C, H_2O_2-treated DNA; curves D–F were obtained without previous long PCR amplification. (B) HUVEC cells were pre-incubated for 24 h with 0 (open bar) or 10 mM (closed bar) Cr and then treated for 30 min in Saline A with 200 μM H_2O_2. The cells were harvested immediately (time 0) or allowed to grow in complete medium for the indicated times (Recovery time = hours). mtDNA damage was assessed by combining long PCR and Real-Time PCR. Results are expressed as relative PCR amplification; the decrease in amplification was calculated comparing treated samples to undamaged control (dashed line). The Box & Whisker graph was obtained from at least four independent experiments. * $P<0.05$, Wilcoxon signed-rank test.

their production. Finally, using the same approaches *o*-phenanthroline completely protects both n and mtDNA from oxidative damage while Trolox was ineffective (not shown).

Hydrogen peroxide is known to produce several types of lesions, including 8-OHdG. This specific lesion is not detected with full efficiency by QPCR in that it does not significantly stall the progression of DNA polymerase. In order to overcome this intrinsic limitation of QPCR we employed an ELISA method which uses a monoclonal antibody against 8-OHdG to detect the amount of modified bases [45]. As shown in Fig. 8, challenge with 200 μM H_2O_2 caused a 2.8 fold 8-OHdG increase in HUVEC nDNA (3.1 ± 0.45 ng/mg of DNA vs. 1.12 ± 0.15); notably and according to the above QPCR and Real-Time PCR results indicating that Cr was not protective on oxidatively-injured nDNA, Cr supplementation failed to decrease the level of 8-OHdG accumulation in nDNA from H_2O_2-treated HUVEC (Fig. 8).

Fast halo assay was performed in order to investigate the Cr effect on nDNA with an unrelated and independent technique sensitive to a broad range of DNA lesions [46] and results are

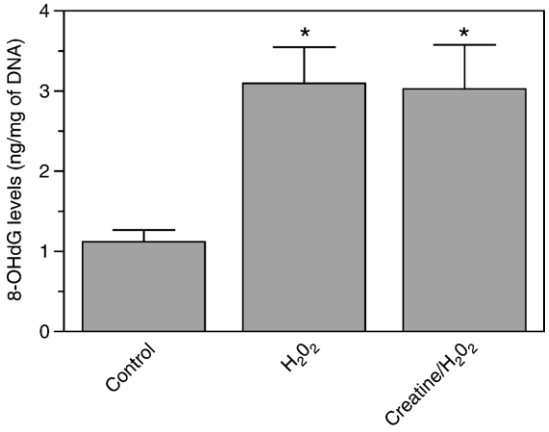

Fig. 8. Determination of 8-OHdG levels by ELISA. HUVEC were preincubated for 24 h with or without 10 mM Cr and then treated for 30 min with 200 mM H_2O_2. Results are expressed as ng of 8-OHdG/mg of DNA. Values represent the means±S.D. ($n=3$). Student's t -test was used to measure the significance of differences between groups. *$P<0.05$ control vs. H_2O_2-treated cells or control vs. H_2O_2-treated cells supplemented with 10 mM Cr. $P=0.887$ H_2O_2-treated cells compared to 10 mM Cr supplemented H_2O_2-treated cells.

shown in Fig. 9. As expected, H_2O_2-treated cells showed significant increase in halo size (see representative micrographs in Fig. 9), which reflects the degree of DNA single strand scission [46]. Pre-incubation of H_2O_2-treated cells with Cr did not show significant protective effect on the extent of nDNA breakage (Fig. 9), which is consistent with QPCR, Real-Time PCR and 8-OHdG data. Notably, according to previously published data [52,53], *o*-phenanthroline completely prevented H_2O_2-induced nDNA damage and, in sharp contrast, Trolox did not (Fig. 9). Finally, it is worth noting that, unlike mtDNA lesions, nDNA breaks induced by H_2O_2 in Cr-supplemented or Cr-unsupplemented cells, were rapidly repaired with identical kinetics ($t_{1/2}$ of approximately 12 min, not shown, see Ref.[54]).

4. Discussion

In this study we investigated the effect of Cr on DNA under oxidative-challenge conditions to understand whether this compound might be considered as a mitochondrially targeted antioxidant which could be helpful in the prevention of ROS-induced mtDNA oxidative damage. Acellular and cellular investigations were carried out.

Acellular assays were performed on circular and linear DNA and Cr protection from oxidative attack was shown. Results obtained from plasmid DNA are in agreement with other data on the assessment of DNA strand breaks obtained using either gel electrophoresis or other techniques that measured the average value of the extent of the lesions [55–60].

Cellular experiments also showed that Cr supplementation exerts genoprotective activity, although limited to mtDNA. The results obtained quantifying the oxidative damage of mtDNA and nDNA from HUVEC cells are consistent with previous researches performed on different human and rodent cell types, showing that mtDNA is more prone to oxidative damage than nuclear DNA [61–63]. In our investigation completion of mtDNA repair was not achieved even after a very long recovery period (48 h) whereas, according to previous observations [54], nDNA-damage was rapidly removed with a $t_{1/2}$ of ca. 12 min (not shown). The persistence of mtDNA lesions suggests that the selected oxidative treatment might have exceeded, at the mitochondrial level, the repair capacity. Indeed, in humans, numerous defence systems protect cellular macromolecules [64] from oxidative challenge, the oxidized DNA is continuously repaired and the oxidized bases excreted into the blood stream [65]. Nevertheless, other deleterious events, such as secondary ROS reactions, take place in the mitochondria and have the potential of overwhelming the mitochondrial repair capacity leading to a vicious cycle of damage which results in persistent mtDNA lesions.

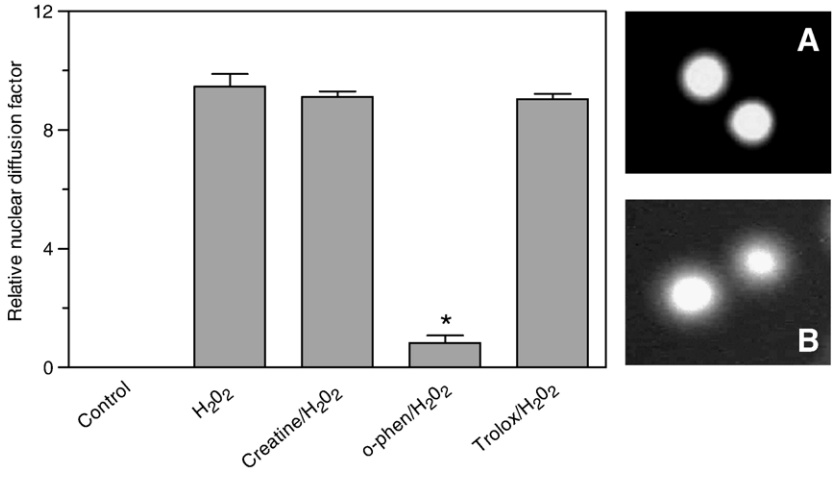

Fig. 9. Effect of Cr on oxidant-induced nDNA damage as assayed with the fast halo assay. HUVEC cells were pre-incubated for 24 h with or without 10 mM Cr, or for 20 min in the absence or presence of 10 μM *o*-phenanthroline or 1 mM Trolox, and then treated for an additional 30 min with 200 μM H_2O_2. nDNA damage was assayed immediately after treatments with the fast halo assay, and the results are expressed as relative nuclear diffusion factor (see Materials and methods). Data are the mean±S.E.M. from three separate experiments, each performed in duplicate. * $P<0.001$ (unpaired t test) as compared to H_2O_2-alone treated cells. Representative micrographs of HUVEC cells processed for DNA damage with the fast halo assay are also shown in panels A (control cells) and B (H_2O_2-treated cells).

Several studies have documented the powerful protective effects of Cr in oxidative stress associated diseases [18–21] and in neuropathologies, such as Huntington disease, Parkinson disease and amyotrophic lateral sclerosis [66–68]. The novelty of the present work is the finding that Cr protects oxidatively-injured DNA, as shown by both acellular experiments and cell based assays, respectively. In particular, our results show that Cr supplementation significantly protects only mtDNA. That nDNA is not sensitive to Cr-protection (Figs. 6–8) is not surprising. nDNA damage following oxidative stress is usually thought to be a function of site-specific DNA-associated Fe-based Fenton chemistry [69]. It is commonly assumed that iron is normally associated with nDNA and many reports indicate that only iron-chelators, unlike scavenging antioxidants such as Trolox [52,53] (Fig. 9), are capable of protecting cells against oxidative nuclear damage [53,70–72]. Importantly, Cr has been shown to exert its antioxidant, cytoprotective activity *via* a scavenging mechanism rather than through iron-chelation [17]: hence, the Cr lack of protective effects on oxidatively-injured nDNA, as assayed with QPCR, Real-Time PCR, 8-OHdG ELISA and fast halo assay, is not surprising. Conversely our data, as well as others [73–76], imply that mitochondria-accumulating scavenging compounds such as Cr are capable of preventing mtDNA oxidative damage. This differential activity, i.e. mtDNA protection in the absence of nDNA protection, might be due to the different conformation of the two types of DNA. Some indications of this can be observed in acellular experiments on circular and linear DNA (Figs. 2 and 3), which could resemble mitochondrial and nuclear DNA, respectively: plasmidic DNA showed oxidative damages just after 100 min treatment, while a PCR amplification product was damaged after a longer treatment time. Thus it is likely that circular DNA, because of its negative supercoiled structure, is more prone to damage as well as more accessible to Cr and sensitive to its protective effect, than linear DNA. Furthermore, in the specific case of Cr, it should be noted that the Cr-protective effect on mtDNA might be related to its mitochondrial localization. Indeed Cr is actively taken up by specific 55 and 70 kDa mt-Cr transporters [43] into mitochondria, where it is utilized for the energy transport between the site of ATP production and consumption by ATPases. Isolated, respiring mitochondria incubated in 15 mM Cr have been shown to accumulate 20 mM Cr [43]. Notably, under our supplementing conditions, HUVEC intracellular free-Cr level has been shown to be 48.5 nmol/mg of proteins [17], i.e. 8.82 mM (HUVEC volume = 5.5 μl/mg of proteins, [77]). As a corollary, the importance of the capacity of an antioxidant to accumulate within mitochondria in order to protect mtDNA from oxidative damage is indirectly emphasized by the observation that Trolox, which is not known to accumulate within these organelles, lacks any protective effect.

Finally, it is worth noting that our results implicitly raise the question of whether oxidative mtDNA and nDNA lesions represent lethal events. Indeed our data confirm the notion that H_2O_2-induced nDNA single strand breaks do not represent a cytotoxically relevant damage [69,78] since in Cr-supplemented H_2O_2-treated cells we observed reduced cytotoxicity in the absence of reduced nDNA breakage. In sharp contrast, we also showed that reduced mtDNA damage is paralleled by a decreased cytotoxic response in H_2O_2-injured Cr-supplemented cells. Thus, it could be inferred that, at least under mild stressing conditions, oxidative mtDNA damage, unlike nDNA damage, represents a cytotoxically relevant type of lesion. However, to answer this important question further studies will be necessary to understand whether this phenomenon is simply incidental or it reflects a causal relationship.

Our study is one of the few reporting the protective effect of Cr on mtDNA. Berneburg et al. [79] demonstrated that 1 mM Cr abolished the induction of mtDNA mutations generated in normal human fibroblast by repetitive UV-A irradiation. In our investigation we used a different cell line, different molecular approaches and a different cellular insult (acute oxidative stress) and we found that mtDNA is protected by Cr.

Data reported by Sestili et al. [17] indicate that Cr affords cytoprotection *via* direct antioxidant capacity with a radical scavenging mechanism: the results presented herein also point to the role of free-Cr as an antioxidant and suggest that the effect of Cr on oxidatively-injured mtDNA might represent an important mechanism contributing to its cytoprotective activity [17] in cells subjected to oxidative stress.

Other antioxidants including vitamins or cofactors such as Coenzyme Q10, ascorbic acid, vitamin E, riboflavin, thiamine, niacin, vitamin K (phylloquinone and menadione), and carnitine have already been used in the treatment of oxidative phosphorylation disorders to increase mitochondrial ATP production and slow or arrest the progression of clinical symptoms [68,80].

Results obtained from cellular experiments suggest that Cr supplementation may play an important role in mitochondrial genome stability in that it could normalize mitochondrial mutagenesis as well as functional consequences such as the decrease of oxygen consumption, mitochondrial membrane potential and ATP content and finally cell survival. Controlling cell life and death, the mitochondria have become the “new cellular brain” and hence represent a new and attractive therapeutic target for the wide range of pathologies where mitochondrial oxidative damage is known to play an etiological role.

On the basis of the results presented herein and because of its biochemical and nutritional features, Cr could be a promising antigenotoxic agent for the treatment of the above-mentioned diseases as well as for the delaying of aging. At this regard, it is worth noting that a very recent and independent study by Bendler et al. [81] has shown that long term Cr supplementation increases health and survival of mice: our results might contribute to the understanding of these important effects.

Further experiments on animal models, clinical and basic research will however be required in order to determine whether Cr antioxidant activity and mtDNA protection against oxidative damage contribute to the reported amelioration [66,67,81] of the symptoms of pathologies related to mtDNA mutations, and future research should also investigate whether long-term Cr supplementation is safe and may be used as a long-term supplement in the prevention of mtDNA damage.

References

[1] J.B. Walker, Creatine: biosynthesis, regulation, and function, Adv. Enzymol. Relat. Areas Mol. Biol. 50 (1979) 177–242.

[2] A.M. Persky, G.A. Brazeau, Clinical pharmacology of dietary supplement Cr monohydrate, Pharmacol. Rev. 53 (2001) 161–176.

[3] C.P. Earnest, P.G. Snell, R. Rodriguez, A.L. Almada, T.L. Mitchell, The effect of creatine monohydrate ingestion on anaerobic power indices, muscular strength and body composition, Acta Physiol. Scand. 153 (1995) 207–209.

[4] A. Casey, D. Constantin-Teodosiu, S. Howell, E. Hultman, P.L. Greenhaff, Creatine ingestion favorably affects performance and muscle metabolism during maximal exercise in humans, Am. J. Physiol. 271 (1996) E31–E37.

[5] K. Vandenberghe, M. Goris, P. Van Hecke, M. Van Leemputte, L. Vangerven, P. Hespel, Long-term creatine intake is beneficial to muscle performance during resistance training, J. Appl. Physiol. 83 (1997) 2055–2063.

[6] S.A. Smith, S.J. Montain, R.P. Matott, G.P. Zientara, F.A. Jolesz, R.A. Fielding, Creatine supplementation and age influence muscle metabolism during exercise, J. Appl. Physiol. 85 (1998) 1349–1356.

[7] J.S. Volek, N.D. Duncan, S.A. Mazzetti, R.S. Staron, M. Putukian, A.L. Gomez, D.R. Pearson, W.J. Fink, W.J. Kraemer, Performance and muscle fiber adaptations to creatine supplementation and heavy resistance training, Med. Sci. Sports Exerc. 31 (1999) 1147–1156.

[8] P.D. Balsom, K. Soderlund, B. Ekblom, Creatine in humans with special reference to creatine supplementation, Sports Med. 18 (1994) 268–280.

[9] I. Mujika, S. Padilla, Creatine supplementation as an ergogenic aid for sports performance in highly trained athletes: a critical review, Int. J. Sports Med. 18 (1997) 491–496.

[10] W.K.J. Volek, Cr supplementation:its effect on human muscular performance and body composition, J. Strength Cond. Res. 10 (1997) 200–210.

[11] M.S. Juhn, M. Tarnopolsky, Oral creatine supplementation and athletic performance: a critical review, Clin. J. Sport Med. 8 (1998) 286–297.

[12] T.W. Demant, E.C. Rhodes, Effects of creatine supplementation on exercise performance, Sports Med. 28 (1999) 49–60.

[13] A.S. Graham, R.C. Hatton, Creatine: a review of efficacy and safety, J. Am. Pharm. Assoc. (Wash.) 39 (1999) 803–810 (quiz 875–807).

[14] W.J. Kraemer, J.S. Volek, Creatine supplementation. Its role in human performance, Clin. Sports Med. 18 (1999) 651–666 (ix).

[15] G. Benzi, Is there a rationale for the use of creatine either as nutritional supplementation or drug administration in humans participating in a sport? Pharmacol. Res. 41 (2000) 255–264.

[16] J.M. Lawler, W.S. Barnes, G. Wu, W. Song, S. Demaree, Direct antioxidant properties of creatine, Biochem. Biophys. Res. Commun. 290 (2002) 47–52.

[17] P. Sestili, C. Martinelli, G. Bravi, G. Piccoli, R. Curci, M. Battistelli, E. Falcieri, D. Agostini, A.M. Gioacchini, V. Stocchi, Creatine supplementation affords cytoprotection in oxidatively injured cultured mammalian cells via direct antioxidant activity, Free Radic. Biol. Med. 40 (2006) 837–849.

[18] S. Kasparova, V. Brezova, M. Valko, J. Horecky, V. Mlynarik, T. Liptaj, O. Vancova, O. Ulicna, D. Dobrota, Study of the oxidative stress in a rat model of chronic brain hypoperfusion, Neurochem. Int. 46 (2005) 601–611.

[19] S. Kasparova, D. Dobrota, V. Mlynarik, T.N. Pham, T. Liptaj, J. Horecky, Z. Braunova, A. Gvozdjakova, A study of creatine kinase reaction in rat brain under chronic pathological conditions — chronic ischemia and ethanol intoxication, Brain Res. Bull. 53 (2000) 431–435.

[20] M. Wyss, A. Schulze, Health implications of creatine: can oral creatine supplementation protect against neurological and atherosclerotic disease? Neuroscience 112 (2002) 243–260.

[21] J.P. Pearlman, R.A. Fielding, Creatine monohydrate as a therapeutic aid in muscular dystrophy, Nutr. Rev. 64 (2006) 80–88.

[22] D.C. Wallace, Mitochondrial diseases in man and mouse, Science 283 (1999) 1482–1488.

[23] S. DiMauro, E.A. Schon, Mitochondrial respiratory-chain diseases, N. Engl. J. Med. 348 (2003) 2656–2668.

[24] I.J. Holt, D.H. Miller, A.E. Harding, Restriction endonuclease analysis of leukocyte mitochondrial DNA in Leber's optic atrophy, J. Neurol. Neurosurg. Psychiatry 51 (1988) 1075–1077.

[25] Z. Cao, J. Wanagat, S.H. McKiernan, J.M. Aiken, Mitochondrial DNA deletion mutations are concomitant with ragged red regions of individual, aged muscle fibers: analysis by laser-capture microdissection, Nucleic Acids Res. 29 (2001) 4502–4508.

[26] S. Wanrooij, P. Luoma, G. van Goethem, C. van Broeckhoven, A. Suomalainen, J.N. Spelbrink, Twinkle and POLG defects enhance age-dependent accumulation of mutations in the control region of mtDNA, Nucleic Acids Res. 32 (2004) 3053–3064.

[27] B.G. Heerdt, J. Chen, L.R. Stewart, L.H. Augenlicht, Polymorphisms, but lack of mutations or instability, in the promotor region of the mitochondrial genome in human colonic tumors, Cancer Res. 54 (1994) 3912–3915.

[28] L.J. Burgart, J. Zheng, Q. Shu, J.G. Strickler, D. Shibata, Somatic mitochondrial mutation in gastric cancer, Am. J. Pathol. 147 (1995) 1105–1111.

[29] W. Habano, S. Nakamura, T. Sugai, Microsatellite instability in the mitochondrial DNA of colorectal carcinomas: evidence for mismatch repair systems in mitochondrial genome, Oncogene 17 (1998) 1931–1937.

[30] W. Habano, T. Sugai, T. Yoshida, S. Nakamura, Mitochondrial gene mutation, but not large-scale deletion, is a feature of colorectal carcinomas with mitochondrial microsatellite instability, Int. J. Cancer 83 (1999) 625–629.

[31] W. Habano, T. Sugai, S.I. Nakamura, N. Uesugi, T. Yoshida, S. Sasou, Microsatellite instability and mutation of mitochondrial and nuclear DNA in gastric carcinoma, Gastroenterology 118 (2000) 835–841.

[32] K. Polyak, Y. Li, H. Zhu, C. Lengauer, J.K. Willson, S.D. Markowitz, M.A. Trush, K.W. Kinzler, B. Vogelstein, Somatic mutations of the mitochondrial genome in human colorectal tumours, Nat. Genet. 20 (1998) 291–293.

[33] M.S. Fliss, H. Usadel, O.L. Caballero, L. Wu, M.R. Buta, S.M. Eleff, J. Jen, D. Sidransky, Facile detection of mitochondrial DNA mutations in tumors and bodily fluids, Science 287 (2000) 2017–2019.

[34] P. Parrella, Y. Xiao, M. Fliss, M. Sanchez-Cespedes, P. Mazzarelli, M. Rinaldi, T. Nicol, E. Gabrielson, C. Cuomo, D. Cohen, S. Pandit, M. Spencer, C. Rabitti, V.M. Fazio, D. Sidransky, Detection of mitochondrial DNA mutations in primary breast cancer and fine-needle aspirates, Cancer Res. 61 (2001) 7623–7626.

[35] D.C. Wallace, A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine, Annu. Rev. Genet. 39 (2005) 359–407.

[36] G.A. Cortopassi, A neutral theory predicts multigenic aging and increased concentrations of deleterious mutations on the mitochondrial and Y chromosomes, Free Radic. Biol. Med. 33 (2002) 605–610.

[37] S. Anderson, A.T. Bankier, B.G. Barrell, M.H. de Bruijn, A.R. Coulson, J. Drouin, I.C. Eperon, D.P. Nierlich, B.A. Roe, F. Sanger, P.H. Schreier, A.J. Smith, R. Staden, I.G. Young, Sequence and organization of the human mitochondrial genome, Nature 290 (1981) 457–465.

[38] S. DiMauro, E.A. Schon, Mitochondrial DNA mutations in human disease, Am. J. Med. Genet. 106 (2001) 18–26.

[39] F.M. Yakes, B. Van Houten, Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 514–519.

[40] V.A. Bohr, Repair of oxidative DNA damage in nuclear and mitochondrial DNA, and some changes with aging in mammalian cells, Free Radic. Biol. Med. 32 (2002) 804–812.

[41] D.E. Sawyer, B. Van Houten, Repair of DNA damage in mitochondria, Mutat. Res. 434 (1999) 161–176.

[42] V.W.B. Van Houten, J.H. Santos, Role of mitochondrial DNA in toxic response to oxidative stress, DNA Rep. 5 (2006) 145–152.

[43] B. Walzel, O. Speer, E. Zanolla, O. Eriksson, P. Bernardi, T. Wallimann, Novel mitochondrial creatine transport activity. Implications for intracellular creatine compartments and bioenergetics, J. Biol. Chem. 277 (2002) 37503–37511.

[44] J.H. Santos, B.S. Mandavilli, B. Van Houten, Measuring oxidative mtDNA damage and repair using quantitative PCR, Methods Mol. Biol. 197 (2002) 159–176.

[45] Y. Ibuki, T. Warashina, T. Noro, R. Goto, Coexposure to benzo[α]pyrene plus ultraviolet A induces 8-oxo-7,8-dihydro-2′-deoxyguanosine formation in human skin fibroblasts: preventive effects of anti-oxidant agents, Environ. Toxicol. Pharmacol. 12 (2002) 37–42.

[46] P. Sestili, C. Martinelli, V. Stocchi, The fast halo assay: an improved method to quantify genomic DNA strand breakage at the single-cell level, Mutat. Res. 607 (2006) 205–214.
[47] K. Kim, I.H. Kim, K.Y. Lee, S.G. Rhee, E.R. Stadtman, The isolation and purification of a specific "protector" protein which inhibits enzyme inactivation by a thiol/Fe(III)/O2 mixed-function oxidation system, J. Biol. Chem. 263 (1988) 4704–4711.
[48] M.S. Cooke, M.D. Evans, M. Dizdaroglu, J. Lunec, Oxidative DNA damage: mechanisms, mutation, and disease, FASEB J. 17 (2003) 1195–1214.
[49] J.H. Santos, L. Hunakova, Y. Chen, C. Bortner, B. Van Houten, Cell sorting experiments link persistent mitochondrial DNA damage with loss of mitochondrial membrane potential and apoptotic cell death, J. Biol. Chem. 278 (2003) 1728–1734.
[50] J.A. Sikorsky, D.A. Primerano, T.W. Fenger, J. Denvir, Effect of DNA damage on PCR amplification efficiency with the relative threshold cycle method, Biochem. Biophys. Res. Commun. 323 (2004) 823–830.
[51] J. Cao, L. Jia, H.M. Zhou, Y. Liu, L.F. Zhong, Mitochondrial and nuclear DNA damage induced by curcumin in human hepatoma G2 cells, Toxicol. Sci. 91 (2006) 476–483.
[52] A. Guidarelli, P. Sestili, A. Cossarizza, C. Franceschi, F. Cattabeni, O. Cantoni, Evidence for dissimilar mechanisms of enhancement of inorganic and organic hydroperoxide cytotoxicity by L-histidine, J. Pharmacol. Exp. Ther. 275 (1995) 1575–1582.
[53] P. Sestili, A. Guidarelli, M. Dacha, O. Cantoni, Quercetin prevents DNA single strand breakage and cytotoxicity caused by tert-butylhydroperoxide: free radical scavenging versus iron chelating mechanism, Free Radic. Biol. Med. 25 (1998) 196–200.
[54] P. Sestili, R. Alfieri, D. Carnicelli, C. Martinelli, L. Barbieri, F. Stirpe, M. Bonelli, P.G. Petronini, M. Brigotti, Shiga toxin 1 and ricin inhibit the repair of H2O2-induced DNA single strand breaks in cultured mammalian cells, DNA Rep. 4 (2005) 271–277.
[55] M. Meriyani Odyuo, R.N. Sharan, Differential DNA strand breaking abilities of *OH and ROS generating radiomimetic chemicals and gamma-rays: study of plasmid DNA, pMTa4, in vitro, Free Radic. Res. 39 (2005) 499–505.
[56] W. Adam, J. Hartung, H. Okamoto, C.R. Saha-Moller, K. Spehar, N-hydroxy-4-(4-chlorophenyl)thiazole-2(3H)-thione as a photochemical hydroxyl-radical source: photochemistry and oxidative damage of DNA (strand breaks) and 2′-deoxyguanosine (8-oxodG formation), Photochem. Photobiol. 72 (2000) 619–624.
[57] B.F. Godley, F.A. Shamsi, F.Q. Liang, S.G. Jarrett, S. Davies, M. Boulton, Blue light induces mitochondrial DNA damage and free radical production in epithelial cells, J. Biol. Chem. 280 (2005) 21061–21066.
[58] G.M. Makrigiorgos, E. Bump, C. Huang, J. Baranowska-Kortylewicz, A.I. Kassis, A fluorimetric method for the detection of copper-mediated hydroxyl free radicals in the immediate proximity of DNA, Free Radic. Biol. Med. 18 (1995) 669–678.
[59] I. Banmeyer, C. Marchand, A. Clippe, B. Knoops, Human mitochondrial peroxiredoxin 5 protects from mitochondrial DNA damages induced by hydrogen peroxide, FEBS Lett. 579 (2005) 2327–2333.
[60] Y.Y.M. Su, G. Yang, Quantitative measurement of hydroxyl radical induced DNA double-strand breaks and the effect of N-acetyl-L-cysteine, FEBS Lett. 580 (2006) 4136–4142.
[61] S.W. Ballinger, B. Van Houten, G.F. Jin, C.A. Conklin, B.F. Godley, Hydrogen peroxide causes significant mitochondrial DNA damage in human RPE cells, Exp. Eye Res. 68 (1999) 765–772.
[62] G. Deng, J.H. Su, K.J. Ivins, B. Van Houten, C.W. Cotman, Bcl-2 facilitates recovery from DNA damage after oxidative stress, Exp. Neurol. 159 (1999) 309–318.
[63] B.S. Mandavilli, S.F. Ali, B. Van Houten, DNA damage in brain mitochondria caused by aging and MPTP treatment, Brain Res. 885 (2000) 45–52.
[64] C. Richter, J.W. Park, B.N. Ames, Normal oxidative damage to mitochondrial and nuclear DNA is extensive, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 6465–6467.
[65] K.B. Beckman, B.N. Ames, Oxidative decay of DNA, J. Biol. Chem. 272 (1997) 19633–19636.
[66] A. Bender, W. Koch, M. Elstner, Y. Schombacher, J. Bender, M. Moeschl, F. Gekeler, B. Muller-Myhsok, T. Gasser, K. Tatsch, T. Klopstock, Creatine supplementation in Parkinson disease: a placebo-controlled randomized pilot trial, Neurology 67 (2006) 1262–1264.
[67] A.C. Ellis, J. Rosenfeld, The role of creatine in the management of amyotrophic lateral sclerosis and other neurodegenerative disorders, CNS Drugs 18 (2004) 967–980.
[68] S.M. Hersch, S. Gevorkian, K. Marder, C. Moskowitz, A. Feigin, M. Cox, P. Como, C. Zimmerman, M. Lin, L. Zhang, A.M. Ulug, M.F. Beal, W. Matson, M. Bogdanov, E. Ebbel, A. Zaleta, Y. Kaneko, B. Jenkins, N. Hevelone, H. Zhang, H. Yu, D. Schoenfeld, R. Ferrante, H.D. Rosas, Creatine in Huntington disease is safe, tolerable, bioavailable in brain and reduces serum 8OH2′dG, Neurology 66 (2006) 250–252.
[69] O. Cantoni, P. Sestili, A. Guidarelli, L. Palomba, L. Brambilla, F. Cattabeni, Cytotoxic impact of DNA single vs. double strand breaks in oxidatively injured cells, Arch. Toxicol., Suppl. 18 (1996) 223–235.
[70] I. Latour, J.B. Demoulin, P. Buc-Calderon, Oxidative DNA damage by t-butyl hydroperoxide causes DNA single strand breaks which is not linked to cell lysis. A mechanistic study in freshly isolated rat hepatocytes, FEBS Lett. 373 (1995) 299–302.
[71] M. Melidou, K. Riganakos, D. Galaris, Protection against nuclear DNA damage offered by flavonoids in cells exposed to hydrogen peroxide: the role of iron chelation, Free Radic. Biol. Med. 39 (2005) 1591–1600.
[72] P. Sestili, G. Diamantini, A. Bedini, L. Cerioni, I. Tommasini, G. Tarzia, O. Cantoni, Plant-derived phenolic compounds prevent the DNA single-strand breakage and cytotoxicity induced by tert-butylhydroperoxide via an iron-chelating mechanism, Biochem. J. 364 (2002) 121–128.
[73] D.L. Hollins, H.B. Suliman, C.A. Piantadosi, M.S. Carraway, Glutathione regulates susceptibility to oxidant-induced mitochondrial DNA damage in human lymphocytes, Free Radic. Biol. Med. 40 (2006) 1220–1226.
[74] S.G. Jarrett, J. Cuenco, M. Boulton, Dietary antioxidants provide differential subcellular protection in epithelial cells, Redox Rep. 11 (2006) 144–152.
[75] J. Milano, B.J. Day, A catalytic antioxidant metalloporphyrin blocks hydrogen peroxide-induced mitochondrial DNA damage, Nucleic Acids Res. 28 (2000) 968–973.
[76] S.R. Pieczenik, J. Neustadt, Mitochondrial dysfunction and molecular pathways of disease, Exp. Mol. Pathol. (2007).
[77] U. Hillebrand, M. Hausberg, C. Stock, V. Shahin, D. Nikova, C. Riethmuller, K. Kliche, T. Ludwig, H. Schillers, S.W. Schneider, H. Oberleithner, 17beta-estradiol increases volume, apical surface and elasticity of human endothelium mediated by Na+/H+ exchange, Cardiovasc. Res. 69 (2006) 916–924.
[78] G.E. Iliakis, G.E. Pantelias, R. Okayasu, W.F. Blakely, Induction by H2O2 of DNA and interphase chromosome damage in plateau-phase Chinese hamster ovary cells, Radiat. Res. 131 (1992) 192–203.
[79] M. Berneburg, T. Gremmel, V. Kurten, P. Schroeder, I. Hertel, A. von Mikecz, S. Wild, M. Chen, L. Declercq, M. Matsui, T. Ruzicka, J. Krutmann, Creatine supplementation normalizes mutagenesis of mitochondrial DNA as well as functional consequences, J. Invest. Dermatol. 125 (2005) 213–220.
[80] S. DiMauro, M. Hirano, E.A. Schon, Approaches to the treatment of mitochondrial diseases, Muscle Nerve 34 (2006) 265–283.
[81] A. Bender, J. Beckers, I. Schneider, S.M. Holter, T. Haack, T. Ruthsatz, D.M. Vogt-Weisenhorn, L. Becker, J. Genius, D. Rujescu, M. Irmler, T. Mijalski, M. Mader, L. Quintanilla-Martinez, H. Fuchs, V. Gailus-Durner, M.H. de Angelis, W. Wurst, J. Schmidt, T. Klopstock, Creatine improves health and survival of mice, Neurobiol Aging (2007).