

Original article

Evaluation of anti-oxidant treatments in an *in vitro* model of alkaptonuric ochronosisDaniela Braconi¹, Marcella Laschi¹, Loredana Amato¹, Giulia Bernardini¹, Lia Millucci¹, Roberto Marcolongo², Giovanni Cavallo², Adriano Spreafico^{2,3} and Annalisa Santucci^{1,2}

Abstract

Objectives. Alkaptonuria (AKU) is a rare genetic disease associated with deficient homogentisate 1,2-dioxygenase activity in the liver. This leads to the accumulation of homogentisic acid (HGA) and its oxidized/polymerized products in connective tissues, which in turn become characterized by the presence of melanin-like pigments (ochronosis). Since at present, further studies are necessary to support the use of drugs for the treatment of AKU, we investigated the effects of various anti-oxidants in counteracting melanin-like pigmentation and oxidative stress related to HGA and its metabolites.

Methods. We set up an *in vitro* model using human serum treated with 0.33 mM HGA and tested the anti-oxidants ascorbic acid, *N*-acetylcysteine, phytic acid (PHY), taurine (TAU), ferulic acid (FER) and lipoic acid (LIP) for their ability to prevent or delay the production of melanin-like pigments, as well as to reduce oxidative post-translational modifications of proteins. Monitoring of intrinsic fluorescence of HGA-induced melanin-like pigments was used to evaluate the efficacy of compounds.

Results. Our model allowed us to prove efficacy especially for PHY, TAU, LIP and FER in counteracting the production of HGA-induced melanin-like pigments and protein oxidation induced by HGA and its metabolites.

Conclusions. Our model allows the opening of new anti-oxidant therapeutic strategies to treat alkaptonuric ochronosis.

Key words: Ascorbic acid, Ferulic acid, Homogentisic acid, Lipoic acid, *N*-acetylcysteine, Phytic acid, Protein carbonyls, Taurine, Protein thiols oxidation.

Introduction

Alkaptonuria (AKU) is a rare autosomal recessive inborn error of metabolism of the aromatic amino acids, phenylalanine and tyrosine, owing to the deficiency of homogentisate 1,2-dioxygenase (HGO; EC 1.13.11.5) activity in the liver [1]. This causes an inability of the intermediate homogentisic acid (HGA) to be converted into fumaric acid and acetoacetic acid [2, 3]. As a consequence, one of the first

symptoms of AKU is homogentisic aciduria, revealed by darkening of urine on standing or after alkalization. Tissue injury appears later in life with gradual bluish-black discolouration of connective tissues, mainly joints, cardiac valves, kidney and skin [1, 4, 5]. Such a pathological pigmentation is known as ochronosis [6]. In cartilage, ochronosis leads to tissue degeneration, chronic inflammation and ochronotic arthropathy [4, 5].

Despite an evident historical interest in AKU, the molecular characterization of the disease progressed very slowly. In fact, the *hgo* gene was still uncharacterized in the mid-1990s [7]. Also, the mechanisms by which the metabolic disturbance in AKU leads to ochronosis and arthropathy have not been completely clarified [8, 9]. Several theories have been proposed to explain why connective tissues affected by the deposition of ochronotic pigment become weak, brittle and prone to ruptures, leading to a rapid degeneration of joints [10]. Ochronotic

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Submitted 21 January 2010; revised version accepted 5 May 2010.

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pigment forms as patients grow older and kidney functions slow, allowing for the accumulation of HGA. HGA itself may act as a chemical irritant, causing inflammation and tissue degeneration, or may possibly physically bind to connective tissue and alter structures and interaction of macromolecules [11].

Other evidence suggests that it is rather a by-product of HGA oxidation that causes degeneration. As HGA accumulates, it is oxidized to benzoquinone acetate (BQA). This oxidation occurs spontaneously in urine under aerobic and alkaline conditions. BQA may polymerize to form ochronotic pigment *in vivo* and bind to collagen fibres, increasing intermolecular cross-linking with a process similar to that observed in ageing or tanning hides to make leather [12]. Hence, BQA, polymerized BQA or melanin-like pigments formed from BQA may also bind chemically to connective tissue macromolecules [11]. The oxidation of HGA into BQA induces the production of free radicals that are associated with tissue oxidative damage and are thought to cause degeneration by inciting inflammation [2]. The ochronotic pigment, soluble or bound to collagen irreversibly [13], can itself propagate oxidative stress by several electron transfer reactions [14].

One of the major obstacles to progress in developing therapeutic strategies for AKU and ochronosis has been the lack of *in vitro* and *in vivo* models to study ochronosis [15, 16]. In the present work, we introduce an *in vitro* model consisting of human serum treated with HGA to test the efficacy of some anti-oxidants in preventing or slowing down HGA-induced ochronotic pigmentation, as well as in reducing oxidative post-translational modifications of proteins. Interestingly, we found that HGA could not only enhance the production of ochronotic pigment in our serum-based model, but also significantly oxidize proteins either irreversibly (inducing protein carbonyls) or modulating the redox state of their thiol groups. The tested anti-oxidant compounds were able to significantly reduce the production of ochronotic pigment and protein oxidation, and may be the basis for establishing new therapies for AKU.

Materials and methods

Human samples and reagents

Human serum was obtained after informed consent was taken from a healthy donor. The study received approval from the local ethics committee (Comitato Etico Locale Per la Sperimentazione Clinica Dei Medicinali dell'Azienda Ospedaliera Universitaria Senese di Siena; EudraCT number 2009-012707-26). Unless otherwise indicated, all high-quality reagents and antibodies were from Sigma-Aldrich (Milan, Italy). The water used was Milli-Q (Millipore, Bedford, MA, USA).

Incubation of serum with HGA and anti-oxidants

Human serum was diluted 1:5 with sterile PBS, and 100 μ l aliquots were prepared in Eppendorf tubes and treated as indicated hereafter. In sterile PBS, 100 mM HGA stock

solution was prepared. 100 mM stock solutions of ascorbic acid (ASC), *N*-acetylcysteine (NAC) and taurine (TAU), a 15 mM stock solution of phytic acid (PHY) and a 20 mM stock solution of ferulic acid (FER) were prepared in sterile PBS. In sterile dimethyl sulphoxide (DMSO), 15 mM lipoic acid (LIP) was prepared. All the solutions were prepared freshly immediately before use and protected from sunlight.

HGA of 0.33 mM (final concentration) was added to human serum, using as a control human serum without any supplementation. Also, HGA was added together with 10 μ M anti-oxidants (final concentration) using as controls serum aliquots supplemented with each anti-oxidant added singularly. As vehicle controls for LIP, serum samples added with 6.6% (v/v) DMSO and 6.6% (v/v) DMSO + 0.33 mM HGA were prepared as well.

For co-treatment experiments, HGA and anti-oxidants were simultaneously added to serum and allowed to incubate at 37°C; for pre-treatment experiments, serum was pre-incubated for an hour at 37°C with anti-oxidants before adding 0.33 mM HGA. Incubation was allowed to proceed for 6 and 20 h, when samples were collected and processed for further analyses.

Analysis of HGA-induced melanin-like fluorescence

All samples were subjected to SDS-PAGE (4–12% polyacrylamide Criterion XT Precast Gel; Bio-Rad, Milan, Italy) following manufacturer's instructions. Before Coomassie staining [17], images of SDS-PAGE unstained gels were acquired at 530 nm with the Molecular Imager VersaDoc MP Imaging Systems 4000, Bio-Rad (filter applied: 530DF70; 2 min illumination UV trans, gain used: $\times 0.5$ and binning 2×2).

Analysis of protein carbonylation

Twenty micrograms of proteins were first incubated in the dark in 6% (w/v) SDS, 5% (v/v) trifluoroacetic acid and 5 mM 2,4-dinitrophenylhydrazine to derivatize protein carbonyls; then the buffer reaction was neutralized with 2 M Tris base containing 30% (v/v) glycerol and 2% (v/v) β -mercaptoethanol [18]. All samples were subjected to SDS-PAGE (4–12% polyacrylamide Criterion XT Precast Gel; Bio-Rad) and transferred onto nitro-cellulose (NC) sheets with the semi-dry Novablot transblot cell (Bio-Rad) applying 0.7 mA/cm² for a total time of 75 min.

For the immuno-revelation of protein carbonyls, NC sheets were incubated with rabbit anti-dinitrophenyl antibodies 1:10 000, followed by peroxidase-conjugated anti-rabbit antibodies 1:7000 and revelation was achieved through chemiluminescence (Immun-Star HRP Chemiluminescent Kit; Bio-Rad).

Analysis of BIAM-labelled protein thiols

Twenty micrograms of proteins were incubated for 15 min in the dark in 2-(*N*-morpholino) ethanesulfonic acid (MES)-Tris buffer (pH 6.5) containing 20 μ M biotinylated iodoacetamide (BIAM) to alkylate non-oxidized protein thiols [19]. The reaction was quenched by adding 20 mM β -mercapoethanol (final concentration) and

samples were subjected to SDS-PAGE (4–12% polyacrylamide Criterion XT Precast Gel; Bio-Rad) and transferred onto NC sheets with the semi-dry Novablot transblot cell (Bio-Rad) applying 0.7 mA/cm² for a total time of 75 min.

For the immuno-revelation of BIAM-alkylated protein thiols, NC sheets were incubated with peroxidase-conjugated streptavidin (1:100 000), and revelation was achieved through chemiluminescence (Immun-Star HRP Chemiluminescent Kit; Bio-Rad).

Image analysis

Images of gels and films were acquired (Image Scanner; Amersham Biosciences, Italy) and analysed with Image Master Platinum (Amersham Biosciences), choosing as the reference parameter the intensity of bands, which is automatically normalized by the software against the surrounding background.

Statistical analysis

All the experiments were carried out in triplicate. For western blot analysis, only representative films are shown. Analysis of variance was followed by Bonferroni-type multiple comparison to evaluate statistical differences. At least a $P \leq 0.05$ was considered significant.

Results

We evaluated HGA-induced melanin-like pigment production in human serum samples in the absence or in the presence of anti-oxidants such as ASC, NAC, ASC combined with NAC, PHY, TAU, FER and LIP. Our model was obtained according to Hegedus and Nayak [20] to reproduce in human serum the formation of HGA-induced pigment. Nonetheless, we lowered the concentration of HGA to be tested with respect to Hegedus and Nayak's work [20], in order to reproduce *in vitro* an HGA concentration within the range of AKU patients' level [16].

The anti-oxidants tested were added to human serum together with HGA (co-treatment) or an hour before the addition of HGA (pre-treatment). All the samples were allowed to incubate at 37°C for 6 and 20 h before being collected and analysed, based on visual inspection. In fact, after 6 h in both the conditions investigated (co- and pre-treatment), only sera added with HGA or with ASC + HGA were visibly darker. After 20 h incubation, all the samples produced an intense brownish discolouration, which was, however, darker in serum supplemented with HGA or with ASC + HGA than in other samples (see supplementary figures 1 and 2 available as supplementary data at *Rheumatology* Online).

Analysis of HGA-induced melanin-like fluorescence

Serum proteins incubated for 6 and 20 h with HGA were resolved through SDS-PAGE. Before Coomassie staining (see supplementary figure 3 available as supplementary data at *Rheumatology* Online), gel images were acquired with the Molecular Imager Versadoc in order to visualize fluorescent melanin-like bands after a brief UV illumination. From one to five specific fluorescent bands

were visible on gels, depending on co- or pre-treatment and time of incubation. A quantitative analysis (see supplementary figure 4 available as supplementary data at *Rheumatology* Online) was carried out on the intensity of fluorescent bands, chosen as the reference parameter.

Natural melanins are normally found in plasma of healthy donors [14, 21]; in fact, we found basal fluorescent bands even in healthy serum without any exogenous supplementation. After 6 h incubation, the addition of HGA to serum caused a 5-fold increase in pigment fluorescence (75, 55 and 25 kDa band) with respect to the control (Fig. 1A and B). With respect to HGA alone, a significant reduction in pigment fluorescence was found in serum samples co-treated with HGA and NAC or ASC + NAC (with enhanced effects with respect to NAC singly tested). No significant effect could be attributed to ASC when co-administered with HGA. PHY, TAU and FER reduced ochronotic pigment fluorescence as well, whereas beneficial effects of LIP, compared with its vehicle control, could not be highlighted in the conditions tested because the vehicle DMSO itself caused a reduction in pigment fluorescence (Fig. 1A).

In anti-oxidant pre-treated 6 h samples (Fig. 1B), the number and intensity of HGA-induced fluorescent bands were generally lower than in co-treated samples. All the tested compounds had significant beneficial effects. Particularly, all the compounds tested made the fluorescent 75 and 25 kDa bands disappear.

After 20 h incubation, total band intensity in HGA-treated serum was almost doubled when compared with the 6 h treatment (Fig. 1C and D). Two additional fluorescent bands could be detected (27 and 17 kDa) only in HGA and anti-oxidant co-treated samples (Fig. 1C). In general, at this sampling time, the effects of tested anti-oxidants were moderate or almost null. However, significant beneficial effects of NAC or ASC + NAC in reducing HGA-induced pigment fluorescence and more important effects of TAU and LIP could be observed. Differences between co- and pre-treated samples were not as evident as after 6 h incubation with HGA, although pre-treatment of serum with ASC + NAC and PHY seemed effective in reducing fluorescence (Fig. 1C and D).

Analysis of protein carbonylation

HGA-induced oxidative damage of serum proteins was assessed through western blot analysis of carbonylation. Although carried out at both the sampling times investigated, we were able to analyse quantitatively only the results produced with 6 h-treated samples, as the signals of 20 h samples were too high to be properly quantified (data not shown). The quantitative analysis is reported in supplementary figure 5 available as supplementary data at *Rheumatology* Online. The addition of HGA to human serum greatly enhanced the formation of protein carbonyls after 6 h treatment, reaching levels from 2- to 3-fold higher than in serum without any supplementation (Fig. 2A and B).

Fig. 1 HGA-induced melanin-like fluorescence on unstained SDS-PAGE gels of human serum samples after 6 h (A) and 20 h (C) incubation at 37°C with 0.33 mM HGA and/or 10 µM anti-oxidants simultaneously added or after 6 h (B) and 20 h (D) incubation at 37°C with 0.33 mM HGA after an initial 1 h pre-treatment with 10 µM anti-oxidants. Controls: untreated serum and serum + 0.33 mM HGA. Vehicle controls for LIP: serum + 6.6% (v/v) DMSO with or without 0.33 mM HGA. Quantitative analysis is reported in supplementary figure 4 available as supplementary data at *Rheumatology* Online.

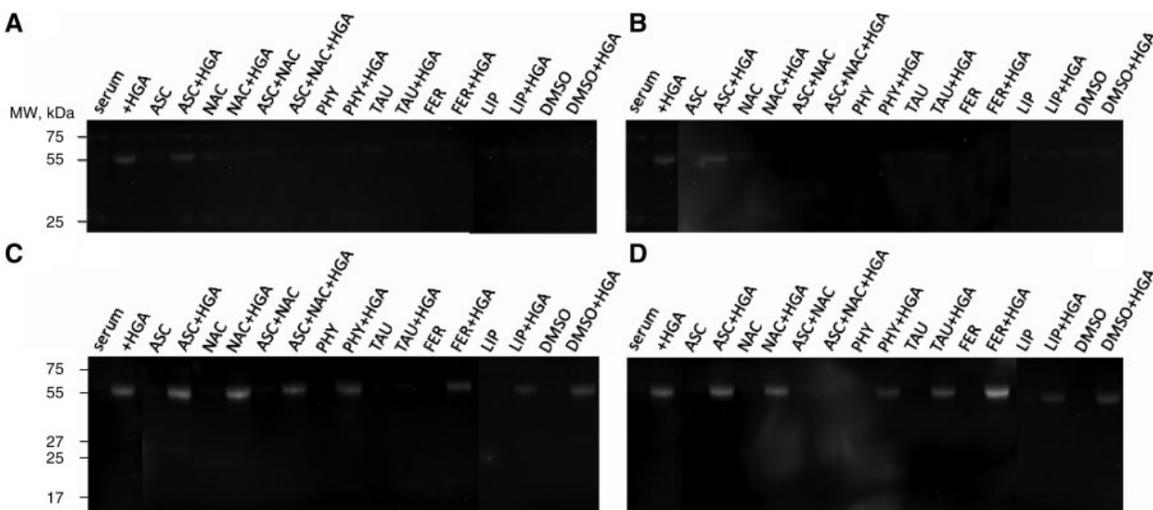
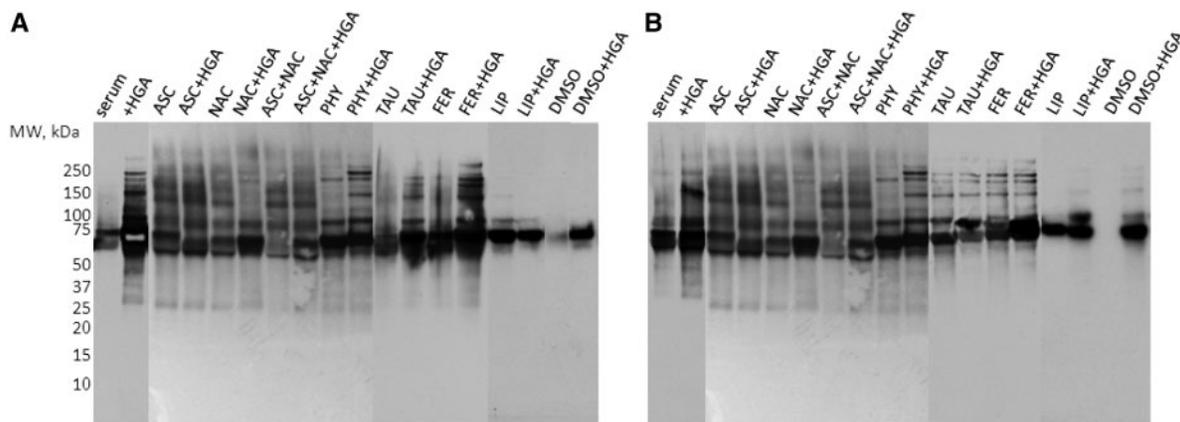


Fig. 2 Western blot detection of carbonylated proteins in human serum samples after 6 h incubation at 37°C with 0.33 mM HGA and/or 10 µM anti-oxidants simultaneously added (A), or after 6 h incubation at 37°C with 0.33 mM HGA after an initial 1 h pre-treatment with 10 µM anti-oxidants (B). Controls: untreated serum and serum + 0.33 mM HGA. Vehicle controls for LIP: serum + 6.6% (v/v) DMSO with or without 0.33 mM HGA. Quantitative analysis is reported in supplementary figure 5 available as supplementary data at *Rheumatology* Online.



The co-treatment with anti-oxidants was beneficial in all the conditions tested except for FER, which had no significant quantitative effect. ASC, NAC and ASC+NAC seemed more effective in protecting from carbonylation proteins ranging from 50 to 150 kDa (Fig. 2A).

The reduction of protein carbonyls observed when HGA was applied after 1 h pre-treatment with ASC, NAC or ASC combined with ASC was similar to that

observed in co-treatment experiments and directed towards the same range of proteins (50–150 kDa; Fig. 2B). On the contrary, pre-treatment with TAU and FER seemed more effective than co-treatment, particularly for 25–75 kDa proteins (Fig. 2B). Also, we were able to detect a reduction in protein carbonyls when the vehicle DMSO was applied together with HGA, thus making the discrimination of the effects of LIP difficult.

Analysis of protein thiols

We also analysed protein thiol residues that could be oxidized by HGA. The assay adopted relies on the selective labelling of non-oxidized thiols with BIAM followed by streptavidin immuno-staining; as a consequence, oxidation can be indirectly evaluated as a lower signal in the western blots. The quantitative analysis of BIAM-labelled protein thiols is reported in supplementary figure 6 available as supplementary data at *Rheumatology* Online.

The addition of HGA to human serum greatly reduced the intensities of immuno-reactive bands in all the molecular weight (MW) ranges and at a significant degree after 6 and 20 h incubation, thus confirming the HGA-induced oxidation of serum protein thiols (Fig. 3A–D).

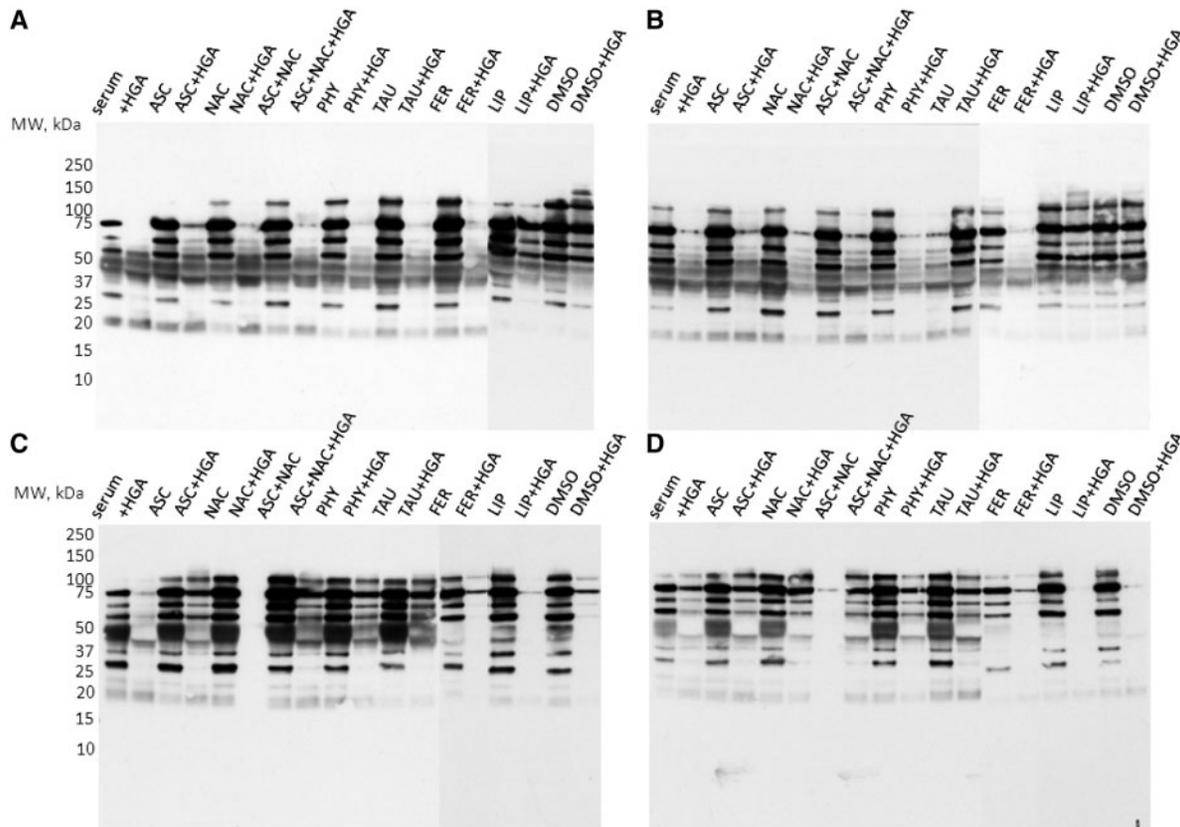
The co-treatment with anti-oxidants within 6 h incubation did not allow us to highlight the protection of proteins from oxidation (Fig. 3A), whereas after 20 h beneficial effects were found for ASC, ASC+NAC, PHY, TAU, FER and for the DMSO vehicle alone (Fig. 3C). The action of ASC was limited to 150–250 kDa proteins, whereas ASC+NAC, PHY, TAU and FER protected proteins in a broad MW range (50–250 kDa).

Pre-treatment with TAU and LIP for 1 h was more effective than in co-treatment experiments in protecting proteins from HGA-induced thiol oxidation after 6 h incubation, and the action of TAU was especially directed towards 75–150 and 25–50 kDa proteins (Fig. 3B). The anti-oxidant properties of ASC, NAC and ASC+NAC in pre-treatment experiments were evident only after 20 h incubation with HGA (Fig. 3D). In particular, ASC+NAC protected a broad range of proteins between 25 and 150 kDa.

Discussion

AKU has been extensively described from the clinical point of view. However, the molecular bases of the disease are still quite obscure, since it is difficult to obtain pathological samples (AKU is rare, affecting 1:250 000–1:1 000 000) [1] and adequate *in vitro* or *in vivo* models to study AKU are still lacking. As an example, the recently introduced AKU model of mice deficient in HGO does not allow the study of ochronosis since mice do not develop this articular manifestation of AKU [22]. In the present work, we developed a model to study alkaptonuric

Fig. 3 Western blot detection of BIAM-labelled protein thiols in human serum samples after 6 h (A) and 20 h (C) incubation at 37°C with 0.33 mM HGA and/or 10 µM anti-oxidants simultaneously added, or after 6 h (B) and 20 h (D) incubation at 37°C with 0.33 mM HGA after an initial 1 h pre-treatment with 10 µM anti-oxidants. Controls: untreated serum and serum + 0.33 mM HGA. Vehicle controls for LIP: serum + 6.6% (v/v) DMSO with or without 0.33 mM HGA. Quantitative analysis is reported in supplementary figure 6 available as supplementary data at *Rheumatology* Online.



ochronosis based on human serum treated with HGA. The HGA concentration we tested in our model, 0.33 mM, is in the range of circulating HGA in AKU patients' serum (between 50 and 400 μ M) [16], and we had already found it to be optimal in leading to the formation of ochronotic pigment in a reasonable time with only minimal effects on cell viability (data not shown).

Alkaptonuric ochronosis can be treated symptomatically during the early stages, whereas for end stages total joint replacement may be required. A first attempt to treat AKU was made by administering ASC to prevent HGA oxidation [23], but the efficacy of this treatment is still controversial [1]. In fact, under certain conditions, ASC can act as a pro-oxidant and auto-co-oxidate with HGA, leading to the production of additional reactive oxygen species (ROS) [24].

A low protein diet, with or without ASC supplementation, was also proposed [25–27]; however, it is difficult to maintain, and beneficial effects have not been proved undoubtedly [28]. Based on evidence produced in both mice [29] and human beings [1, 30], the herbicide 2-(2-nitro-4-trifluoromethylbenzyl)-1,3-cyclohexandione (nitisinone, Orfadin), which is used to treat hereditary Type I tyrosinaemia as it inhibits the enzyme *p*-hydrophenylpyruvate dioxygenase, was proposed to treat AKU. Nevertheless, further studies are needed to support this therapeutic strategy.

Of undoubted importance is the fact that oxidative stress is a key mechanism for the development of alkaptonuric ochronosis and, consequently, ochronotic arthropathy. HGA undergoes spontaneous oxidation, yielding a quinone intermediate (BQA), which is very toxic itself and contributes to the production of ROS, such as O_2^- and H_2O_2 . [24] Furthermore, either BQA or its semi-quinone catalyses a rapid co-oxidation of ASC, whose level in serum and extracellular fluids is ordinarily 40–150 μ M. Again, O_2^- and H_2O_2 are generated during the reaction [24]. The participation of metal ions, and especially ferrous iron in the oxidation of HGA and the potentiating action of ASC in this reaction, has already been confirmed [31]. Both HGA and BQA can thus deplete systemic or local anti-oxidants, resulting in oxidative damage of macromolecules, especially proteins, lipids and nucleic acids. This can initiate a variety of reactions, promoting inflammation and contributing to tissue damage [32].

Although alkaptonuric ochronosis develops around the third to fourth decade of life, its real onset may be the consequence of repeated oxidative damage of target tissues initiated by HGA auto-oxidation. This implies that, at some point in the development of ochronosis, anti-oxidant defences may be overwhelmed by ROS or their by-products originated by the circulating HGA and BQA excess.

Anti-oxidants may act by mitigating the oxidative stress that would otherwise initiate the cascade of reactions leading to ochronosis. It is thus conceivable that a proper anti-oxidant therapy, especially if preventative, may be helpful in delaying the disease progression. To this aim, it is crucial to identify, among the wide range of

anti-oxidant compounds, those with the highest efficacy, in the correct form, at the appropriate dose and to be used for the right length of time [33].

In this light, with our proposed *in vitro* serum model, we were provided with the opportunity to test rapidly various anti-oxidants potentially to be used to treat alkaptonuric ochronosis. We evaluated the production of HGA-induced melanin-like pigments together with the oxidation of proteins, which are the most important targets of oxidative insults, as the parameters to monitor the efficacy of anti-oxidants to counteract them. We chose to investigate protein carbonylation, a widely accepted biomarker of oxidative stress, since this modification is non-enzymatic, stable and easily detectable [18], as well as protein thiols, since these groups have already been identified as being subject to the oxidation mediated by quinones, such as BQA [31]. Also, blood has a critical role in maintaining redox balance because it transports and redistributes anti-oxidants to every part of the body; its anti-oxidant status is the result of multiple interactions among different compounds and metabolites, the non-protein thiol compound glutathione (GSH) being one of the most important defences. Proteins' thiol groups are responsible for the so-called plasma anti-oxidant capacity, which gives a measurement of free radicals scavenged by a test solution rather than taking into account the specific contribution of single anti-oxidants present in the mixture [34]. Protein thiols participate in the overall redox balance and modulate oxidative stress generating reversible semi-oxidized species (mixed disulphides with non-protein low-molecular weight thiols) [34].

Although HGA-induced melanin-like fluorescent compounds have already been reported in various models, including plasma [14, 21, 32] and microorganisms [35–38], to our knowledge we were the first to propose this original and sensitive method to analyse qualitatively and quantitatively the presence of ochronotic pigment through SDS-PAGE and determination of the intrinsic fluorescence of specific protein bands. Our method being non-destructive also gives the possibility of detecting *in situ* the melanin-like fluorescence on gel before further staining on the same samples.

We found that, generally, pre-treatment with anti-oxidants is more efficacious in reducing the production of HGA-induced ochronotic pigment. The compounds with the highest efficacy were PHY, TAU and LIP, although favourable effects were also detected with the vehicle DMSO alone. As for protein oxidation, effects seemed generally to be regardless of co- or pre-treatment with anti-oxidants and HGA, and the favourable actions of NAC, ASC+NAC, PHY and TAU in protecting proteins from carbonylation, and of NAC and ASC+NAC could be detected in protecting protein thiols (see supplementary figure 7 available as supplementary data at *Rheumatology* Online).

It is noteworthy to underline how NAC combined with ASC could be more efficacious than NAC or ASC when singly tested, thus confirming previous observations [39] and also previous work of ours on human articular

chondrocytes treated with HGA (data not shown). Moreover, the mechanisms of action of those anti-oxidants found to be most active in our conditions also allowed us to find indirect confirmations of HGA-induced oxidative stress and toxicity. Especially, the effects of TAU as a protector against oxidative damage caused by reactive quinones (such as BQA originated from HGA) and oxygen radicals [40] as well as its ability to decrease protein carbonylation [41, 42] were confirmed in our model. More generally, however, TAU has also been demonstrated to increase superoxide dismutase activity [42], decrease lipid peroxidation, restore depleted GSH and stimulate the activity of GSH-metabolizing enzymes [41].

LIP contains two thiol groups, which may be oxidized or reduced; it is part of a redox pair, being the oxidized partner of dihydrolipoic acid, the reduced form [43]. It can scavenge directly ROS, chelate metals, reduce ROS production and lipid peroxidation [44], and recycle other anti-oxidants (GSH, vitamin E and ASC) [45]. Importantly, LIP has been proposed as a new adjunctive therapeutic agent for RA [46].

PHY acts as a metal chelator *in vitro*, and it is also considered to inhibit the generation of ROS [47]. PHY was shown to inhibit •OH hydroxyl radical formation and decrease lipid peroxidation catalysed by iron and ASC in human erythrocytes [48].

It is known that DMSO can have anti-oxidant properties as well [43], and it was introduced as a therapeutic agent to the scientific community in 1963. It can scavenge hydroxyl radicals, a trigger for inflammation, and protect HA against depolymerization due to several types of stress, including oxidation. Possible efficacy exists for DMSO in the treatment of various pathologies, including arthritis [43, 49]. Evidence of DMSO anti-oxidant properties have also been produced in chondrocytes, where DMSO proved to scavenge hydroxyl radicals [43, 50] and prevent cell damage (restoring at the same time the synthesis of proteoglycans) [50]. Importantly, DMSO was also recently demonstrated to be effective in reducing hydroquinone toxicity [51]. Nonetheless, DMSO is not devoid of adverse effects and caution should always be used in considering its beneficial effects.

The need to explore new anti-oxidants is felt for many pathologies [33], including AKU. Overall, our simple, rapid, sensitive, fast and accessible model allowed the efficacy of PHY, TAU, LIP and FER in counteracting the production of ochronotic pigment and protein oxidation induced by HGA and its metabolites to be proved, so that future work on more sophisticated models could be more easily driven to investigate new anti-oxidant therapeutic strategies to treat alcaptonuric ochronosis. Notably, future work will be dedicated to the identification of those serum proteins responsible for the HGA-induced production of HGA-induced melanin-like fluorescent pigment. The production of ochronotic pigment and oxidative stress being the base of AKU aetiopathogenesis, finding new molecules that are able to prevent, reduce or slow down these processes, establishes the base to set up adequate therapies to treat AKU.

Rheumatology key messages

- The identification of anti-oxidants as potential therapeutics will be beneficial for the treatment of AKU.
- We proposed a sensitive, non-destructive method to evaluate HGA-induced melanin-like fluorescence qualitatively and quantitatively.

Acknowledgements

The authors are grateful to Dr Lara Rossini and Dr Michele Sotgiu.

Funding: This work was supported by Fondazione Monte dei Paschi di Siena Esercizio 2008 and Esercizio 2009 and Toscana Life Sciences Foundation Orphan 0108 grants.

Disclosure statement: The authors have declared no conflict of interest.

Supplementary data

Supplementary data are available at *Rheumatology* Online.

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