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Original article

Antioxidants inhibit SAA formation and pro-inflammatory cytokine release in a human cell model of alkaptonuria

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Abstract

Objective. Alkaptonuria (AKU) is an ultra-rare autosomal recessive disease that currently lacks an appropriate therapy. Recently we provided experimental evidence that AKU is a secondary serum amyloid A (SAA)-based amyloidosis. The aim of the present work was to evaluate the use of antioxidants to inhibit SAA amyloid and pro-inflammatory cytokine release in AKU.

Methods. We adopted a human chondrocytic cell AKU model to evaluate the anti-amyloid capacity of a set of antioxidants that had previously been shown to counteract ochronosis in a serum AKU model. Amyloid presence was evaluated by Congo red staining. Homogentisic acid-induced SAA production and pro-inflammatory cytokine release (overexpressed in AKU patients) were evaluated by ELISA and multiplex systems, respectively. Lipid peroxidation was evaluated by means of a fluorescence-based assay.

Results. Our AKU model allowed us to prove the efficacy of ascorbic acid combined with *N*-acetylcysteine, taurine, phytic acid and lipoic acid in significantly inhibiting SAA production, pro-inflammatory cytokine release and membrane lipid peroxidation.

Conclusion. All the tested antioxidant compounds were able to reduce the production of amyloid and may be the basis for establishing new therapies for AKU amyloidosis.

Key words: ascorbic acid, *N*-acetylcysteine, taurine, phytic acid, lipoic acid, homogentisic acid, serum amyloid A, inflammation, chondrocyte, lipid peroxidation.

CLINICAL SCIENCE

Introduction

Alkaptonuria (AKU; MIM no. 203500) is an ultra-rare (1:250 000–1 000 000 incidence) autosomal recessive inborn error of catabolism of the aromatic amino acids phenylalanine and tyrosine due to deficient activity of the enzyme homogentisate 1,2-dioxygenase (HGO; EC 1.13.11.5). This leads to the accumulation of homogentisic acid (HGA; 2,5-dihydroxyphenylacetic acid). HGA oxidizes to benzoquinone acetic acid (BQA), which in turn forms melanin-based polymers, deposited in the connective tissue of various organs, causing a pigmentation known as ochronosis, leading to dramatic tissue degeneration. A severe form of arthropathy is the most common clinical presentation of AKU but patients often suffer from cardiovascular disease (frequent cause of death) and kidney disease as well. There is no effective cure for AKU at the moment and treatment is symptomatic.

The oxidation of HGA into BQA produces free radical species that are associated with tissue oxidative damage and are thought to cause degeneration by inciting inflammation [1].

We introduced novel human ochronotic cell, tissue and serum models and undertook preclinical testing of

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potential antioxidant therapies for AKU [2–10]. We previously found that HGA-induced production of ochronotic pigment is accompanied by lipid peroxidation (LPO), decreased activity of the enzyme glutathione peroxidase and massive depletion of thiol groups, together with increased protein carbonylation and thiol oxidation, suggesting protein aggregation as one of the possible outcomes of such an oxidative stress [3, 5–7].

More recently we provided experimental evidence that AKU is a secondary serum amyloid A (SAA)-based amyloidosis, thus opening novel perspectives for its treatment [11]. Interestingly, we observed a co-localization of SAA-amyloid and ochronotic pigment [11].

In the present work we further exploited our human chondrocytic cell AKU model to evaluate the anti-amyloid capacity of antioxidants that we had previously shown to be able to counteract ochronosis in a serum AKU model [3]. We found that all the tested antioxidant compounds (ascorbic acid, *N*-acetylcysteine, taurine, lipoic acid and phytic acid) were able to significantly reduce the production of amyloid, inhibit HGA-induced pro-inflammatory cytokines and may be the basis for establishing new therapies for AKU.

Patients and methods

The study was conducted following approval of the local university hospital ethics committee (Comitato Etico Locale dell'Azienda Ospedaliera Universitaria Senese). All patients gave written informed consent prior to inclusion in the study. All reagents were from Sigma-Aldrich (St Louis, MO, USA), if not otherwise specified.

AKU cell models and antioxidant treatment

The cell model used for this work was previously developed by us and consists of human chondrocytes treated with 0.33 mM HGA up to the development of ochronosis [2, 4]. First passage chondrocytes were seeded into 24-well plates (4×10^4 cells/well) and grown until confluence. Chondrocytes were pre-incubated for 24 h in culture medium in the absence or presence of various concentrations of antioxidants previously determined by us to be effective in reducing ochronotic pigment formation in a serum model of AKU [3]. Stock solutions of 100 mM ascorbic acid (Asc), *N*-acetylcysteine (Nac) and taurine (Tau) and a stock solution of 15 mM phytic acid (Phy) were prepared in sterile PBS. Lipoic acid (Lip) (15 mM) was prepared in sterile dimethyl sulphoxide. All the solutions were freshly prepared immediately before use, protected from sunlight and serially diluted with fresh culture medium to obtain the desired final concentrations. For the first screenings, 10^{-5} M antioxidants and 0.33 mM HGA (final concentration) were tested; in a second phase we focused our research on Nac + Asc and tested such antioxidants at 10^{-6} and 10^{-7} M (each) in combination. Cells not challenged with exogenously added HGA were used as controls.

AKU chondrocytes

Alkaptonuric chondrocytes for the determination of released pro-inflammatory cytokines were obtained from articular cartilage of AKU patients and cultured, as described previously [5, 8, 10].

Congo Red staining

After 8 days of culture, cells were fixed with 70% ethanol for 15 min and observed by microscopy after Congo Red (CR) staining, following a modified protocol [12, 13] to allow a perfect distinction between collagen and amyloid. Samples were incubated in 1% CR for 40 min, washed in water, incubated for 10 s in 1 ml of 1% sodium hydroxide in 100 ml of 50% ethanol, incubated for 30 s in Mayer's haematoxylin, sequentially washed in 50%, 75% and 95% ethanol, mounted and observed under a polarized light microscope (Zeiss Axio Lab.A1, Arese, Milano, Italy). Moreover, CR birefringence was quantified in the polarized images by Photoshop CSII software (Adobe Systems, Mountain View, CA, USA). Three 20× fields were chosen as to best reflect the overall CR birefringence of cells contained on the entire slide. Total black area was automatically selected. Subsequently the selection was inverted to evidence birefringence areas. The number of birefringence pixels was extrapolated and normalized with the total cell number in each field. Finally, based on the ratio birefringent pixels/cells, the percentage of birefringence inhibition of each antioxidant in HGA-treated cells was evaluated. We also evaluated the birefringence generated by the cell exposition to each antioxidant compound without HGA treatment.

SAA release measurement

SAA levels were measured in cell culture supernatant by ELISA (Invitrogen-Life Technologies, Carlsbad, CA, USA) after 8 days of culture with or without treatments.

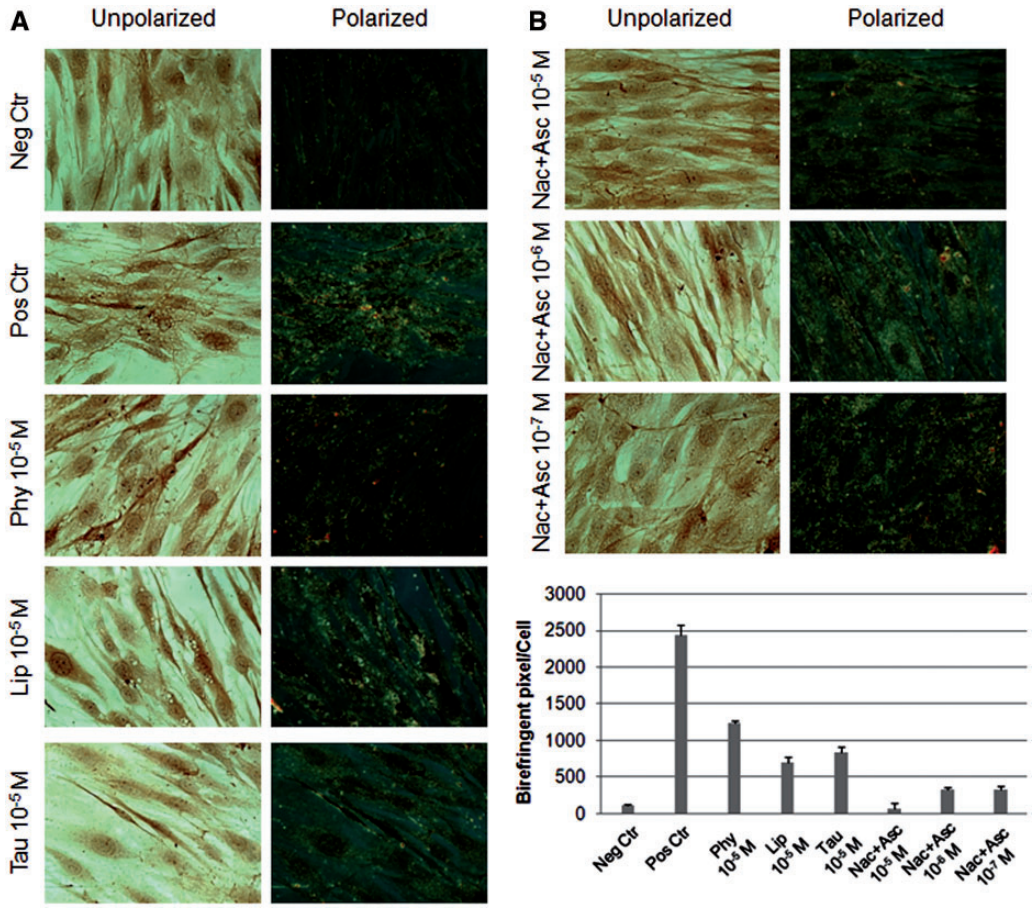
Pro-inflammatory cytokine measurement

The release of a panel of pro-inflammatory cytokines was evaluated in cell culture supernatants by Bioplex (Bio-Rad, Milan, Italy) after 8 days of culture with or without treatment.

LPO assay

Human chondrocytes were incubated with a fluorescent fatty acid probe (50 μM), 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid (C11-BODIPY$^{581/591}$, Molecular Probes, Eugene, OR, USA) at 37°C for 30 min. C11-BODIPY$^{581/591}$ is able to intercalate in the phospholipids bilayer and LPO was localized by observing the changes in C11BODIPY$^{581/591}$ fluorescence. The intact probe fluoresces red when it is intercalated into the membrane (λ excitation = 590 nm and λ emission = 635 nm) and shifts to green (λ excitation = 485 nm and λ emission = 535 nm) after oxidative radicals attack. Excess probe was removed by washing the cells twice with PBS. After incubation with the probe, samples were treated with HGA, Nac + Asc or Nac + Asc + HGA at 37°C and 5% CO_2 for 2 h. The staining

FIG. 1 Antioxidant treatment inhibits HGA-induced amyloid formation in human chondrocytes.



(**A**, **B**) HGA-treated human chondrocytes were pretreated with antioxidants and amyloid presence was revealed by CR staining. Magnification ×20. (**B**) Dose-response effect of treatment with *N*-acetylcysteine and ascorbic acid. Magnification ×20. (**C**) Semi-quantitative analysis of birefringence reduction. Data are the mean of three independent experiments each performed in triplicate. $P < 0.05$. All data were significant.

was examined by Leitz Aristoplan microscope fluorescence (Leica, Wetzlar, Germany). Representative images were taken in specific areas of samples using Leica Q Fluoro Standard, Leica Chantal software. The percentage of cells showing a high green fluorescence was calculated against the total number of cells in each sample.

Statistical analysis

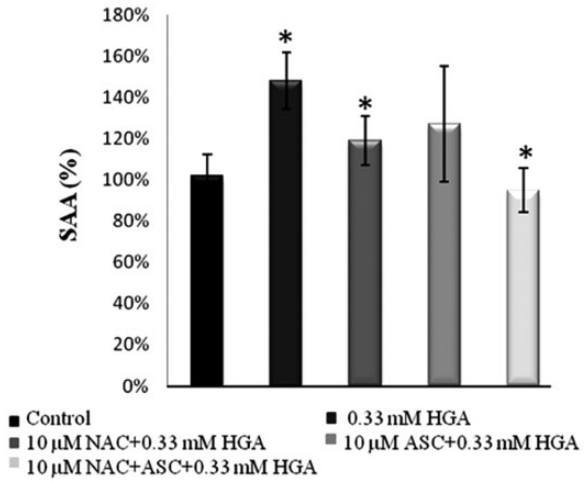
Student's *t*-test was used when appropriate. Two-tailed analysis with $P < 0.05$ was considered significant. Correlation analysis was performed using Pearson's correlation.

Results

We evaluated HGA-induced amyloid production in human chondrocytes in the absence or presence of antioxidants such as Asc, Nac, Asc combined with Nac, Phy, Tau and Lip. Our *in vitro* AKU model allowed a semi-quantitative analysis of the production of amyloid due to HGA addition [11] and its reduction due to treatments with different concentrations of antioxidant compounds. CR birefringence was chosen as a reference parameter to evaluate amyloid presence and quantity in cultured AKU chondrocytes model. All compounds tested at 10^{-5} M concentration were able to significantly inhibit amyloid formation (Fig. 1A). In particular, 50%, 66% and 71% decreases in CR birefringence were observed for Phy, Tau and Lip, respectively. We had previously evaluated the beneficial effect of the combination of Nac + Asc in counteracting ochronosis [3]. In the present work, Nac + Asc confirmed their dose-dependent synergistic activity ranging from 86% to 97% inhibition of amyloid formation (Fig. 1B). The Nac + Asc combination was then adopted as the elective antioxidant for further investigation.

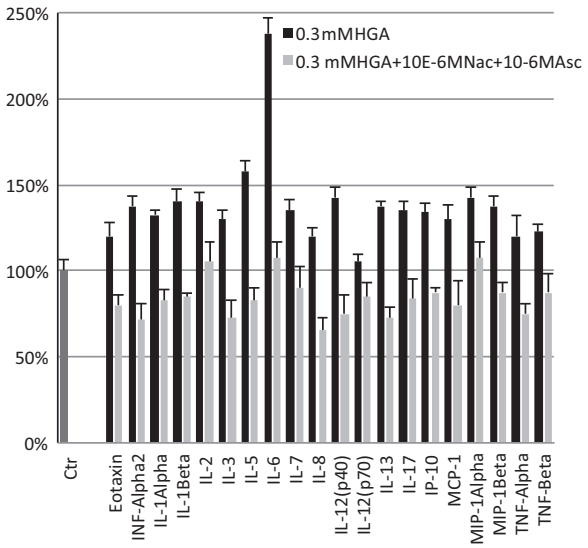
HGA-treated chondrocytes express SAA and release it in culture supernatant, as revealed in our AKU cell model (Fig. 2). HGA treatment increased SAA levels of 48% with respect to controls. Pretreatment with the

Fig. 2 HGA-treated human chondrocytes overexpress and release SAA, and Nac + Asc treatment restores control levels.



Data are the mean of three independent experiments each performed in triplicate. $P < 0.05$.

Fig. 3 Pro-inflammatory cytokines are overexpressed in HGA-treated human chondrocytes, and Nac + Asc treatment restores control levels.

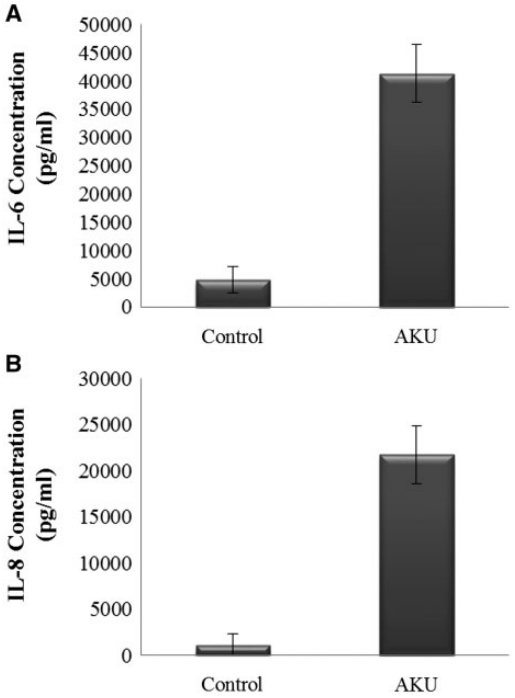


Data are the mean of three independent experiments each performed in triplicate. $P < 0.05$.

combination of Nac + Asc was able to decrease SAA levels to those of controls (Fig. 2).

Chondrocytes treated with 0.33 mM HGA also expressed and released several pro-inflammatory cytokines (Fig. 3). Although we are the first to show the expression of such pro-inflammatory cytokines under AKU conditions

Fig. 4 Pro-inflammatory cytokines IL-6 and IL-8 are overexpressed in chondrocytes from AKU patients.



(**A**) IL-6 is overexpressed in chondrocytes from AKU patients compared with non-diseased cells. Data are reported as the average concentration from three patients expressed in pg/ml and are the mean of three independent experiments each performed in triplicate. $P < 0.05$. (**B**) IL-8 is overexpressed in chondrocytes from AKU patients compared with non-diseased cells. Data are reported as the average concentration from three patients expressed in pg/ml and are the mean of three independent experiments each performed in triplicate. $P < 0.05$.

(Figs. 3 and 4) [11], the role of these molecules in other rheumatic diseases, and particularly in cartilage degeneration, has been clearly reported. IL-6 is involved in cartilage degradation and enhances the production of SAA. IL-7 is involved in cartilage destruction not only indirectly via inflammatory cells, but also directly via IL-7R-expressing chondrocytes [14]. Production of IL-8, MCP-1 and MIP-1a by synoviocytes from OA patients has been demonstrated both *in vivo* and *in vitro* [15]. Although *in vivo* the role of IL-12 is less clear, *in vitro* studies suggest that IL-12 has a pro-inflammatory function in arthritis. IL-2 induces the secretion of IL-1, TNF-α and TNF-β. Cytokines such as IL-1 and TNF-α produced by activated synoviocytes, mononuclear cells or articular cartilage itself significantly upregulate metalloproteinase gene expression and the neutralization of IL-1 and/or TNF-α upregulation of metalloproteinase gene expression appears to be a logical development in the potential medical therapy of OA. IL-17 shows properties similar to those of IL-1β and TNF-α. They induce *in vitro* the synthesis of

nitric oxide and metalloproteases by chondrocytes and the production of IL-6 and IL-8 by fibroblasts [16]. Eotaxin-1 plays an important role in cartilage degradation in OA [17]. The combined treatment with 10^{-6} M Nac + Asc proved to be able to significantly decrease pro-inflammatory cytokine levels or even restore control levels (Fig. 3).

Indeed, once evaluated in chondrocytes extracted from cartilage of AKU patients [5], we found overexpression and release of high levels of pro-inflammatory cytokines IL-6 (9-fold change with respect to control, Fig. 4A) and IL-8 (20-fold change with respect to control, Fig. 4B).

In human chondrocytes we observed that LPO localization was extended to the whole plasma membrane (Fig. 5A). The percentage of high green fluorescence, an index of LPO, observed in samples treated with HGA (0.33 mM) confirmed that this compound induced LPO on chondrocyte plasma membranes after 2 h of treatment (83.3% ± 7.5%); this percentage was higher compared with that of cells treated with HGA in the presence of Nac + Asc (HGA + Nac + Asc, 40.6% ± 4.5%). Samples treated with antioxidants (Nac + Asc) and without any treatment (control) showed a low percentage of high green fluorescence, 21% ± 6% and 15.3% ± 2.5%, respectively (Fig. 5).

Discussion

There is no appropriate therapy for AKU at the moment. Alkaptonuric ochronosis can be treated symptomatically during the early stages, whereas for end stages total joint replacement may be required. We have recently shown that SAA amyloidosis is a secondary complication of AKU, due to a chronic inflammatory status derived from HGA-BQA-melanin-induced oxidative stress [7, 11]. We also proved that MTX has excellent efficacy in inhibiting the production of amyloid in our AKU model chondrocytes, suggesting the introduction of its use in AKU therapy [11]. This and other alternative treatments (i.e. administration of antioxidants) would be useful, especially for those symptomatic AKU patients for whom the therapy with nitisinone (the only orphan drug so far recognized for AKU) failed in a clinical trial [18].

Oxidative stress is a key mechanism for the development of alkaptonuric ochronosis and, consequently, ochronotic arthropathy and for the development of any form of amyloidosis as well.

Oxidized proteins are generally more prone to misfolding and aggregation [19]. It is well known that HGA undergoes spontaneous oxidation into BQA, and that such a process is a source of ROS, further promoting oxidative stress [20]. In our previous work we highlighted that BQA-mediated toxicity may provide a mechanistic link between oxidative stress and structural/functional organization of macromolecules and the production of ochronotic pigment through the propagation of oxidative stress and enhancement of protein aggregation [7].

Both HGA and BQA can also deplete systemic or local antioxidants, causing oxidative damage of macromolecules; 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, antioxidant defences may be overwhelmed by ROS or their by-products originated by the circulating HGA and BQA excess. In this case, a suitable antioxidant therapy, especially if preventative, may be helpful in delaying disease progression. To this end, the identification of the right compound to be used and its optimal concentration are of crucial importance.

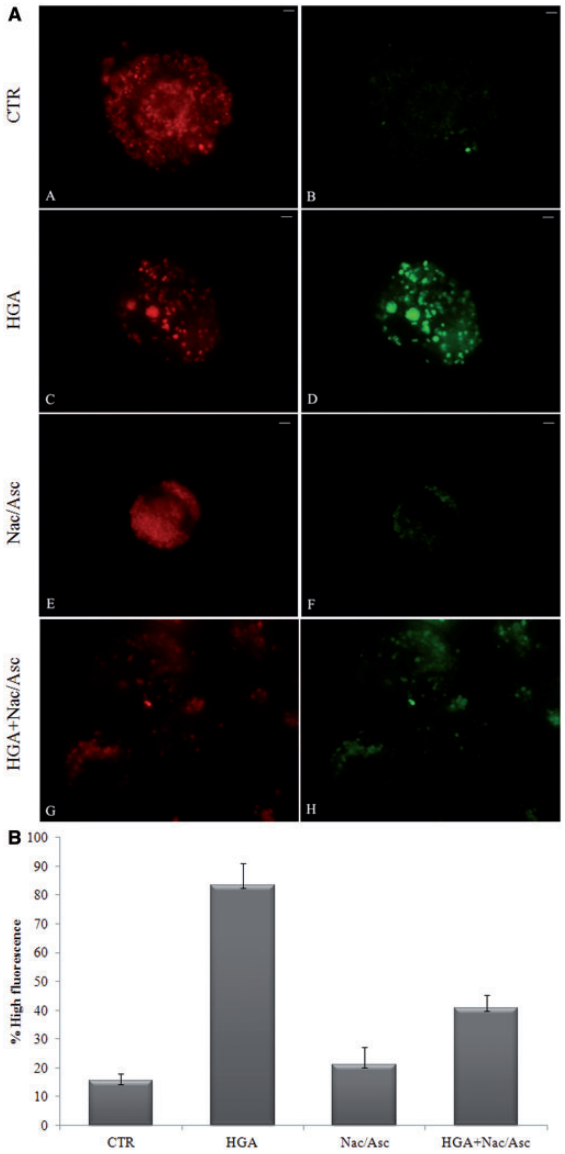
We previously reported that AKU cells are markedly oxidized and try to react to this type of stress [3–7]. We also proposed the use of antioxidants to prevent the oxidation of HGA into BQA that induces the production of ROS, thus causing tissue damage by inciting inflammation [3, 4, 6, 7]. Treatment of AKU patients with Asc to prevent HGA oxidation is very controversial, since Asc can act as a pro-oxidant and auto-co-oxidate with HGA, leading to the production of additional ROS [20].

It has been demonstrated that Nac, whose safety is supported by more than 40 years of clinical use, inhibits HGA polymerization [21, 22], and we also proved the efficacy of the co-administration of Nac and Asc to counteract the negative effects of HGA for the treatment of ochronotic arthropathy [3, 4]. In fact, Nac and Asc have additive properties and Nac is able to prevent the pro-oxidative effects of Asc [23]. In a previous work, we showed in an *in vitro* serum model that the most effective antioxidant compounds in reducing the production of HGA-induced ochronotic pigment and in protecting protein thiols and proteins from carbonylation are Phy, Tau, Lip and Nac + Asc [3].

Membrane unsaturated fatty acids are susceptible to oxidative damage and the resultant LPO can alter membrane fluidity and permeability. We have already indicated in an *in vitro* serum model that HGA can induce LPO [7]. Interestingly, our previous study [11] found amyloid deposits on the surface of AKU cartilage that is directly bathed in SF and/or in the perivascular area of the synovia. These observations indicate the possibility that oxidative damage and consequent hyperpermeability in synovial vessels may promote plasma proteins such as SAA to pass through the vessels and deposit in the articular tissues. The low molecular weight of SAA, its high affinity for the extracellular matrix, namely collagen, and its high plasma concentration in AKU may promote such a passage. If this hypothesis is true, redox therapy or strategies aimed at blocking oxidative stimulant formation could be useful in the prevention and treatment of AKU and of the related amyloidosis. Here we presented data indicating lipid peroxidative damage of chondrocyte plasma membrane due to HGA that may be counteracted by tested antioxidants.

In the present work we also showed that HGA-treated human chondrocytes released SAA and that Nac + Asc can have a beneficial effect in restoring normal levels. In

Fig. 5 LPO assay.



(**A**) Fluorescence micrographs of human chondrocytes treated with HGA, *N*-acetylcysteine + ascorbic acid (Nac/Asc), HGA in the presence of *N*-acetylcysteine + ascorbic acid (HGA + Nac/Asc); CTR: human chondrocytes without any treatment. For each sample, two micrographs were acquired (only specific areas are shown): red colour showing the intercalated probe in the membrane and green colour showing the intensity of LPO. Bar = 5 μm. (**B**) Mean and S.D. of the percentage of human chondrocytes (calculated against the total number of cells) that showed a high green fluorescence (C11-BODIPY$^{581/591}$ probe), an index of LPO, are reported.

the evaluation of antioxidant treatments in an *in vitro* model of alkaptonuric ochronosis we also established the efficacy of Nac + Asc, Tau, Phy and Lip in counteracting the production of HGA-related amyloid deposits [11].

HGA-treated cells overexpressed various cytokines, including IL-8, IL-6 and TNF-α [11]. We have previously shown that AKU patients have high levels of plasma SAA, suggesting SAA to be involved in the pathogenesis of AKU [11]. However, it remained unclear whether SAA was involved in the overproduction of pro-inflammatory cytokines in AKU tissues. SAA is implicated in rheumatoid inflammatory processes through stimulating chondrocytes to produce IL-6 that in turn is recognized as the main inducer of most acute-phase proteins, including SAA. Therefore SAA-induced IL-6 production may enhance the sustained SAA production in an autocrine manner that perpetuates the rheumatoid inflammation. Here we showed that chondrocytes extracted from AKU patients' cartilage produce high levels of IL-8 and IL-6, suggesting that these ILs could play a role in AKU progression. Taken together, the results obtained in this study demonstrate that it is possible to take advantage of the adoption of molecules that combine a range of important antioxidant and anti-amyloidogenic properties for the treatment of AKU.

Rheumatology key messages

- The identification of antioxidants as potential therapeutics will be beneficial for AKU amyloidosis treatment.
- Antioxidants inhibit SAA production and pro-inflammatory cytokine release in alkaptonuria.

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