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REACTIVE OXYGEN SPECIES (SUPEROXIDE) IN HUMAN INNER EAR PERILYMPH: PRELIMINARY RESULTS

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SUMMARY

Background

It is known that inner ear hair cell damage and death may occur as a result of reactive oxygen species (ROS) - mediated damage (1, 2, 3, 4). Oxygen radicals may trigger a series of events leading to cell death/apoptosis, including breaking down of lipids and proteins as well as damages to DNA (5).

The aim of this study is to investigate the presence and the production of ROS within the human inner ear perilymph (IP).

Materials and Methods. In order to investigate the ROS production in human inner ear a total of 142 human perilymph samples have been investigated; 135 samples were obtained from patients affected by profound sensorineural hearing loss, during surgery for cochlear implantation, and 7 from control subjects, affected by otosclerosis, in case of spontaneous gusher after stapedectomy. ROS production was investigated by spectrophotometric analyses, namely cytochrome c reduction kinetics in presence and absence of superoxide dismutase and xanthine dehydrogenase/xanthine oxidase (XA/XO), by catalase test, by uric acid test and polyacrilamyde gel electrophoresis.

Results. Among the 135 samples obtained from patients affected by profound sensorineural hearing loss, 29 had volume too small for spectrophotometric analysis and 8 contained blood traces and were thus unsuitable for ROS analysis; a total of 98 samples from patients and 7 from controls were then analyzed for oxygen radical production by different techniques (Table 1).

A total of 58 samples of inner-ear perilymph (IP) from patients affected by profound sensorineural hearing loss and 7 from healthy controls were analyzed spectrophotometrically

by cytochrome c reduction kinetics. In 40 samples from patients and in 3 controls the total protein content was lower than 0.01 mg, thus they were excluded from the analysis. In the remaining 18 samples from patients and 4 controls the analysis was performed: the average superoxide production in patients was 27.34 umoles/mg of total protein in sample, while no significant production of superoxide was detected in controls (0.36). All 8 samples analysed for the cytochrome c reduction kinetics in presence of XA/XO system resulted positive, while no change in spectral profile was observed in control samples. Traces of hydrogen peroxide were detected in 4 samples analysed by catalase test. The uricase test was positive in 2 patients, revealing the presence of a significant amount of uric acid within the samples. Finally, the SDS-PAGE analysis allowed to detect the presence of the xanthine oxidase enzyme system in IP.

Conclusions. This paper provides the first evidence concerning the presence of ROS within the human inner ear perilymph. Based on the preliminary results obtained, it is possible to state that: i) superoxide may be produced in human inner ear perilymph in pathological conditions; ii) the production of superoxide within IP could be ascribed to the activity of the XA/XO enzyme system; iii) the activity of XA/XO is supported by the preliminary results of the uricase test as well as by SDS-PAGE analysis of IP samples.

INTRODUCTION

The reactive oxygen species (or ROS) are oxygen-based radicals or radical-derived molecules that may be produced in the human body in both physiological and pathological conditions (1, 2, 3, 4). In health, they are produced as regulatory mechanisms, intercellular signalling species, or bactericidal agents, under the control of antioxidant defence mechanisms, among which intracellular enzymes (such as glutathione peroxidases and superoxide dismutases) and low molecular mass compounds (such as vitamin E or ascorbic acid). Regardless of such defences, some basal oxidative damage may normally occur in all individuals, but oxidative stress arises when there is a marked imbalance between production and removal of ROS and nitrogen species. The imbalance may be caused either by overproduction of ROS or from depletion of antioxidant defences.

It is known that inner ear hair cell damage and death may occur as a result of ROS-mediated damage (6). The exact sources of ROS in the cochlea are not completely understood; the electron transport chain in the mitochondria is thought to be a major font of intracellular superoxide (7), since hair cells (HCs) are known to be highly energy-demanding and oxygen-consuming cells (7). ROS may trigger cell death by inflicting damage upon lipids, proteins and DNA (3). In HC, death may occur either through necrosis or apoptosis (8-14).

Several conditions of increased ROS production within the cochlea have been described (8-20), thus suggesting that ROS may be the cause of cochlear damage: the most relevant include noise exposure, drug assumption, cochlear ischaemia, infective and genetic diseases.

Noise exposure

Significant increases in two ROS species (superoxide and hydroxyl radical) have been detected in the cochlea following noise exposure (9). Additionally, pre-treatment with antioxidants or antioxidant enzymes, either ROS-scavenging or catalyzing reactions that convert ROS into less harmful molecules, makes HCs less susceptible to noise damage (10, 11). The increased superoxide levels may lead to direct damage to hair cells and/or increase the generation of more toxic ROS, including: hydrogen peroxide (through a reaction catalyzed by superoxide dismutase), the hydroxyl radical, and peroxynitrite (through a reaction of superoxide with nitric oxide) (9).

Drug assumption

At least two major classes of drugs currently employed in clinical treatments can cause sensorineural hearing loss. Aminoglycoside antibiotics have a major role in the treatment of life-threatening infections and platinum-based chemotherapeutic agents are highly effective in the treatment of malignant disease: both these classes of drugs can produce cochlear damage, and in both cases HCs death is caused by the production of free radicals (8).

Aminoglycoside antibiotics

The production of ROS is considered the initial step of aminoglycoside ototoxicity in a cascade of events that ultimately results in cell death. The formation of ROS by aminoglycosides apparently involves iron metabolism. Aminoglycosides can form complexes with iron (12) that react with unsaturated fatty acids to form superoxide (O2•-) radicals and lipid peroxides (13). Typically, O2•- is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) and detoxified into water and oxygen by catalase. However, in highly oxidative conditions, endogenous antioxidant pathways can become overwhelmed, and oxygen radicals can become abundant. Aminoglycosides such as gentamicin can also activate inducible nitric oxide synthase in inner ear tissues, triggering an increase in nitric oxide (14).

Under these conditions, O2•- can react with available nitric oxide to form the destructive peroxynitrite radical or it can directly initiate hair cell death. (8)

Platinum compounds

Administration of ototoxic doses of cisplatin to experimental animals results in both production of ROS and depletion of glutathione and antioxidant enzymes (SOD, catalase, glutathione peroxidase and glutathione reductase) in cochlear tissues (15). The superoxide radicals generated by cisplatin exposure can be transformed into hydrogen peroxide, which can be catalyzed by iron to form the highly reactive hydroxyl radical. This radical species can in turn react with polyunsaturated fatty acids to generate the toxic aldehyde 4-hydroxynonenal (4-HNE), inducing cellular damage (8).

Other conditions

Cochlear ischaemia

Ischemia/hypoperfusion, may also contribute to ROS generation within the cochlea: there are also reports concerning the use of ROS scavengers to protect cochlea from ischemic damage (16, 17).

Genetic hearing loss

There is a growing evidence that the oxidative stress could play a key role in cochlear damage underlying some genetic hearing loss. Recently, in Pendred Syndrome mouse model it has been shown that high rates of K^+ secretion in the stria vascularis can enhance oxygen radical stress as a result of increased energy metabolism (18).

Middle and Inner ear infections

Middle ear infections may cause inflammation of inner ear tissues by infiltrating the round window membrane. In patients affected by otitis media the absorption of toxins or bacteria through the round window membrane has been suggested as explanation of inner ear cellular damage mediated by oxidative stress (13). Other routes for penetration of toxins and bacteria in the inner ear are perilabyrinthine fistulae or through cerebrospinal fluid in bacterial meningitis.

Presbycusis

Most hearing loss occurring in old age is probably due to long-term deterioration of mitochondria in the different structures of the cochlea. In presbycusis, it is likely that ROS may damage mitochondrial DNA and other mitochondrial components such as proteins and lipids: this further compromises both oxidative phosphorylation and repair processes in mitochondria, setting up a vicious cycle of degradation (19).

Menière

Oxidative stress has also been reported to be involved in development of endolymphatic hydrops leading to cellular damage which induces apoptosis by activation of caspase-3. Apoptotic cell death might contribute to the sensorineural hearing loss found in Menière's disease, as recently proposed and demonstrated on guinea pig models (20).

AIMS

Aim of this study is to investigate the presence and production of reactive oxygen species (ROS) in human inner ear perilymph samples, obtained from patients affected by profound sensorineural hearing loss, during surgery for cochlear implantation, as well as in control subjects affected by otosclerosis, in case of spontaneous gusher after stapedectomy.

MATERIALS AND METHODS

Population studied

A total of 142 human subjects were involved in this study (65 males, 77 females, age range 2 months - 69.8 years and average 18.4 years). Informed consent was gathered from Patients before any surgical procedure, according to Italian laws and to University of Ferrara and S. Anna Hospital bioethical guidelines. Samples of human inner-ear perilymph (IP), each from a different individual, were collected for analysis during surgery: 135 samples were from patients affected by profound hearing loss with different hearing pathologies or otherwise unknown causes (see Table 1) (63 males, average age 11.99 years, and 72 females, average age 21.90 years) and 7 from control subjects affected by otosclerosis (2 males, average age 67.3 years, and 5 females, average age 59.21 years).

Among samples collected from patients, 29 had volume too small for spectrophotometric analysis (the minimum volume required was 25 ul) and 8 contained blood traces that made them unsuitable for ROS tests. A total of 98 samples from patients and 7 from controls were analyzed for oxygen radical production by different techniques.

Experimental procedure

The samples of human perilymph were obtained from patients affected by profound sensorineural hearing loss during surgery for cochlear implantation. After the cochleostomy, about 25 µl of human perilymph were sampled, using a sterile micro-syringe (Hamilton Co., Reno, Nevada). The obtained perilymph samples were transferred in sterile micropipettes containing 50 µl of normal saline solution and then immediately transferred to 4°C. The final volume of sample was about 75 µl. The samples form control subjects, affected by otosclerosis, were obtained in case of spontaneous gusher, after stapedectomy.

Unless differently indicated, all reactants were from Sigma Aldrich Chemical Co. (St. Louis, Missouri).

Spectrophotometric analyses

Cytochrome c reduction kinetics

ROS (superoxide) activity was measured in human perilymph samples by spectrophotometric analysis of cytochrome c reduction kinetics: in presence of $O_2^{\bullet-}$ the cytochrome reduces according to the reaction:

cytochrome c-Fe(III) + $O_2^{\bullet-}$ → O_2 + cytochrome c-Fe(II)

Each experimental sample was composed of 0.375 ml of normal saline solution, 0.1 ml cytochrome c from bovine heart (10^{-4} M, Sigma), 25 μl of human perilymph. All kinetics were recorded for 15 min in 1-ml quartz cuvettes at 550 nm in a Uvikon 860 spectrophotometer (Kontron Instruments, Milan, Italy). The reduced cytochrome concentration was obtained by dividing the values of absorbance at 550 nm by the molar extinction coefficient of cytochrome c (ε=29500) (21, 22, 23). Each absorbance value was the average of 10 measurements per second and kinetics was recorded for 20 minutes, comparing initial and final spectral profiles. Kinetics were repeated at least twice for each sample.

In order to confirm that the reduction of cytochrome was caused by superoxide, we added to the reaction mixture the enzyme superoxide dismutase (superoxide:superoxide oxidoreductase, SOD, EC 1.15.1.1, 250 units/ml), a specific scavenger of superoxide that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide (H_2O_2), according to the following reaction:

$2O_2^{\bullet-}$ 2H+ → $O_2 + H_2O_2$

Also, to verify the possible production of H_2O_2 in the reaction mixture, we added a small amount of catalase (hydrogen peroxide: hydrogen peroxide oxidoreductase, EC 1.11.1.6, 5 units/ml), a specific scavenger of hydrogen peroxide that turns hydrogen peroxide into water and oxygen, according to the following reaction

$2H_2O_2 \rightarrow 2H_2O + O_2$

The total protein content of each sample was measured according to Bradford (1976) (24).

Cytochrome c reduction kinetics in presence of XA/XO system

Superoxide, a biologically highly relevant ROS, can be produced by the activity of the enzyme system xanthine/xanthine oxidase (XA/XO). This ubiquitarious enzyme (xanthine: xanthine oxidoreductase, EC 1.1.3.22) exists in two forms, dehydrogenase (XDH) and oxidase (XO), but only XO produces superoxide by the following reactions:

XA + $2O_2$ + H_2O → uric acid + $2O_2$•- + 2 H+

XA + O_2 + H_2O → uric acid + H_2O_2

The enzyme produces superoxide and uric acid in presence of suitable substrates (xanthine, hypoxanthine or other purines). Besides its role in purine metabolism, the enzyme is considered the main cytoplasmic source of superoxide and hydrogen peroxide. The addition of

xanthine as a substrate to the reaction allows to confirm the presence and activity of the enzyme. In this series of experiments the reaction mixture contained 0.8 ml phosphate buffer (PBS, 0.05 M, pH 7.2) and 0.2 ml cytochrome c solution (10^{-4} M), with addition of 10 ul saturated xanthine solution (4 x 10^{-4} M) and xanthine oxidase (grade III from bovine milk, suspended in 1 ml $(NH_4)_2SO_4$ 2.3 M, 1 mM EDTA and 1 mM Na salicylate). The concentration of the enzyme is 26.8 mg protein/ml, 1.0 units/mg and 25 units/ml. One units of enzyme converts 1.0 umoles xanthine to uric acid and superoxide per min, at pH 7.5 and 25° C. The solution of xanthine oxidase was prepared as follows: 0.05 ml xanthine oxidase and 0.95 ml phosphate buffer 0.05 M, pH 7.2. Kinetics were recorded for 5 min at 550 nm and repeated at least twice for each sample analyzed (22, 23).

Uric acid production

Uric acid production was tested in perilymph samples by the uricase test (25). Uricase (urate oxidase, EC 1.7.33) converts uric acid in allantoin, CO_2 and H_2O_2 according to the following reaction:

$$\text{uric acid} + 2\ H_2O + O_2 \rightarrow \text{allantoin} + CO_2 + H_2O_2$$

Uricase from swine liver (urate oxidase, EC 1.7.33 type VIII-S, Sigma Chemical Co.) was suspended in a 50% glycerol solution containing 0.13 M sodium carbonate and 5 mM glycine buffer, pH 10.2, with concentration 2.5 mg protein/ml and 5.3 units/mg protein, namely 13.25 units/ml. One unit of uricase converts 1 umole per minute of uric acid in allantoin at pH 8.5 and 25° C. Before uricase addition, xanthine (1 x10-5 M) was added to samples to initiate reaction (final volume 0.8 ml). The amount of uric acid in samples was monitored for 20minutes at 293 nm, before and after addition of 0.053 units of uricase.

Polyacrylamyde gel electrophoresis (SDS-PAGE)

To investigate the protein components in human perilymph samples we analyzed the protein band profiles by polyacrylamyde gel electrophoresis in presence of sodium dodecylsulphate (SDS-PAGE) in denaturing conditions, according to Laemmli 1970 (26), in a 10-cm electrophoretic Protean cell (BioRad, Richmond, California). Samples were diluted with 0.02 ml buffer (0.0625 M Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol). A protein mixture (albumin, 66 KDa; egg albumin, 45 KDa; glyceraldehyde 3-phosphate dehydrogenase, 36 KDa; carbonic anhydrase, 29 KDa; trypsin inhibitor, 24 KDa; trypsin, 20 KDa; alpha-lactalbumin, 14.2 KDa), diluted in 4 M urea, 2% SDS, 100 mM dithyotreitol, 0.01 mM EDTA, 1 mM sodium azide (NaN_3) and 33% glycerol was employed as standard. Protein bands during migration were visualized by adding 0.25% bromophenol blue and 30% glycerol to each sample.

The separation gel was prepared in 10% polyacrylammide with 1.5 M Tris HCl pH 8.8; the running gel was prepared at 5% with 0.5 M Tris HCl pH 6.8. A minimum of 10 ul (standard) and a maximum of 60 ul were loaded in wells by a microsyringe (Hamilton Co., Reno, Nevada). The samples were run for 4 hours at 8 mA in electrophoretic buffer (0.025 M Tris HCl, 0.192 M glycine, 1% SDS, pH 8.3). At the end of run the gel was stained by 0.1% Coomassie Brilliant Blue in 10% acetic acid and 40% ethanol, and destained for 2 hours in the same solution without colour. Gel images were acquired by an Epson GT-8500 scanner (Seiko Epson Co., Nagano-ken, Japan) through the Epson TWAIN program.

RESULTS

Spectrophotometric analysis

Cytochrome c reduction kinetics

A total of 58 samples of inner-ear perilymph (IP) from patients with different pathologies and 7 from healthy controls were analyzed spectrophotometrically for superoxide production by cytochrome c reduction kinetics, as described in Materials and Methods. The superoxide content of perilymph samples collected and stored as previously described was normalized by the total protein content according to Bradford (1976) (24). The reliability of analysis of oxygen radical content is insured by a sufficient amount of total protein in the sample and by an adequate sample volume (A total protein content above 0.01 mg and a minimum volume of 75 ul are required for a reliable analysis of oxygen radical content). In 40 samples from patients and in 3 controls the total protein content was lower than 0.01 mg, thus they were excluded from the analysis. The remaining 18 samples (Table 2) from patients (10 males, 8 females, average age 26.06, age range 1.17-66.75) affected by profound hearing loss of various aetiology (Table 3) had a minimum protein content (average 0.04 mg), thus were analysed by cytochrome c reduction kinetics in presence of superoxide dismutase (SOD), as well as 4 control samples (1 male and 3 females, average age 64.68, age range 58.75-68.00). In aqueous solution the oxidized cytochrome reduces in presence of superoxide since the radical acts as a weak base by reducing ferric heme Fe^{3+} to ferrous Fe^{2+}. The typical spectral profile of reduced cytochrome is visible in Fig. 1 (in red the oxidized cytochrome profile and in blue the reduced one).

Fig. 2 shows, as an example, a kinetic profile of a IP sample from a patient affected by profound hearing loss by genetic cause (Cx 26+), respectively without (in blue) and with addition of superoxide dismutase (SOD) (red). Since SOD is a specific scavenger enzyme for

superoxide, the change in kinetic profile after addition of SOD support the hypothesis that superoxide may be produced in IP samples of patients with different hearing pathologies.

Fig. 3 shows a typical spectral profile of cytochrome at the end of a kinetic run, respectively without (blue) and with addition of SOD (red). The lowering in cytochrome reduction after addition of SOD allows to measure the amount of superoxide produced in the samples. The results are reported in Table 3. The average superoxide production in patients was 27.34 +/- 14.42 umoles/mg of total protein in sample, while no significant production of superoxide was detected in controls (0.36 +/- 0.13). When normalized per mg of total protein content of each sample, the superoxide content shows great variability with high standard deviation (Table 4). To analyze the results in more detail, the samples were divided into standard age classes, children (1-10 years), youngsters (11-30 years), adults and elderly (30 or more years) and the average superoxide production was calculated for each class. In these conditions the average superoxide production in patients from each age class, expressed in umoles/ mg of total protein content, was respectively 15.87 (children), 60.67 (youngsters) and 21.06 (adults and elderly).

Cytochrome c reduction kinetics in presence of XA/XO system

It is known that superoxide production in an acellular system is due to the activity of the xanthine dehydrogenase/xanthine oxidase enzyme system (xanthine: xanthine oxidoreductase, EC 1.1.3.22, XA/XO), that produces superoxide and uric acid from its main substrate (xanthine) or other purines. To verify the involvement of the xanthine/xanthine oxidase enzyme system (XA/XO) in superoxide production, in 8 IP samples from patients with hearing impairments and 4 controls the cytochrome c reduction kinetics was repeated in presence of xanthine as described in Materials and Methods. The spectral profiles of cytochrome without (blue) and with (red) addition of xanthine are shown in Fig. 4: again, the change in spectral profile (that is the increased reduction of cytochrome in presence of xanthine) supports the

hypothesis that the XA/XO enzyme system is involved in superoxide production detected in IP samples. No change in spectral profile is observed in control samples.

Some IP samples (4) were also analyzed for hydrogen peroxide production by catalase: traces of hydrogen peroxide were detected in all samples, but the amount of production of this radical species could not be reliably measured (data not shown).

The data on superoxide production were also sorted by pathology (Table 3). When pathology was identified, a clear association appeared between relevant superoxide production (29.88 +- 9.77 umoles/mg of protein) and genetic diseases. When pathology was unidentified, superoxide production appeared slightly lower.

Uricase test

The XA/XO system produces superoxide and uric acid from xanthine or other purines. The production of uric acid in 22 IP samples and 2 controls (previously investigated by cytochrome c reduction kinetics) was investigated by the uricase test (25), according to the procedure described in Materials and Methods. Most IP samples resulted negative to uricase, but in two of them the addition of uricase (1.3 units/ml) at minute 7 caused a sharp decline of the kinetic profile at 293 nm (Fig. 5), revealing the presence and production of a significant amount of uric acid in IP samples (see Table 5).

Although still preliminary, these results support the production of uric acid in IP samples and the activity of XA/XO system. It is possible to advance hypotheses about the fact that most IP samples previously positive to cytochrome reduction kinetics later yielded negative results to the uricase test. These results could be due either to inadequate preservation of IP samples between collection and use, or to the normal decline in enzyme activity over time, even in perfectly preserved samples. More analyses on freshly collected IP samples are required to clarify this point.

SDS-PAGE

The results obtained by spectrophotometric analyses support the production of superoxide radical in human IP samples through the activity of the XA/XO system. To investigated the presence of this enzyme system we analyzed 6 IP samples and 2 controls (see Table 6) by sodium dodecyl sulphate polyacrylammide gel electrophoresis (SDS-PAGE), according to what described in Materials and Methods, together with standard protein mixture and samples of purified xanthine oxidase, as previously described (23). An example of the results obtained by SDS-PAGE is shown in Fig. 7: from left to right, IP samples n. 1, control sample, IP samples n. 2 and 3, purified xanthine oxidase sample. A double band is clearly visible in the purified xanthine oxidase sample and in each IP sample (although at different intensity), but not in the control sample. The molecular weight of this double band is identified by the standard protein mixture (data not shown) as 50-60 kD, in agreement to what previously described in the literature (27).

The results of SDS-PAGE analysis agree with those obtained by cytochrome c reduction and together support the hypothesis that in human inner ear perilymph of patients affected by profound hearing loss of different aetiology there is a production of superoxide radicals, probably involving the activity of the XA/XO system.

DISCUSSION AND CONCLUSIONS

It has been reported that an increased level of oxygen radical species (ROS) may be involved in cochlear damage in several pathological conditions. It is therefore relevant to precisely identify the sources of cochlear ROS production as well as the pathways of ROS-mediated cochlear damage, in order to develop new approaches for prevention and treatment of inner ear pathologies.

Concerning the origin of increased ROS levels within the cochlea following a specific damage, the mitochondria and the electron transport chain appear to be one of the major sources of intracellular radicals (9). As part of aerobic respiration, the electron transport chain could represent a major source of superoxide. When mitochondria are using more and more oxygen to meet the increased cellular demands for energy (i.e. following noise exposure), more and more superoxide is generated as an unwanted product: the excess of superoxide may overcome the antioxidant defences and react with other molecules, in turn generating higher levels of other ROS in the cochlea (9).

In an extracellular environment, Henderson et al. 2006, reported that some enzymes, including xanthine oxidase and NADPH oxidase, can catalyze reaction of hydrogen ions (H+) with O_2, thus producing superoxide (9). Once generated, ROS are also responsible of direct cellular damage, breaking down lipid and protein molecules and damaging DNA, thus triggering cellular apoptosis or necrosis (9). Only understanding the biological steps in cellular death it will be possible to develop mew methods for prevention and treatment.

So far several models of treatment in case of ROS mediated damage have been proposed (9, 28-35). The use of antioxidant molecules, scavenging ROS and converting them into less dangerous molecules, has been attempted. A series of studies show that increasing cochlear antioxidant supplies can effectively prevent hair cells damage and hearing loss. Pharmacological interventions have been directed at i) interrupting the lipid peroxidation

process, thus preserving the integrity of cell membranes (i.e. using lazaroids) (28); ii) prevention or at least moderation of the noise-mediated inner ear damage by cochlear ischemia/reperfusion (i.e. with pentoxifylline and sarthran) (29, 30); iii) prevent or avoid cellular apoptosis. In the last case therapeutical attempts have been done by using apoptosis pathways inhibitors, such as JNK inhibitors, D-JNK-1, Riluzole and Src inhibitors or minocyclin. (31, 32, 33, 34, 35, 36, 37, 38, 39).

This paper provides the first evidence of the presence and production of an oxygen radical species, superoxide, within the human inner ear perilymph, by spectophotometric analysis of cytochrome c reduction. The production of superoxide was also confirmed by the use of superoxide dismutase (SOD) and by the uricase test. The presently available data have so far shown presence of increased oxygen radicals levels in mammalian (mainly rat, chinchilla and guinea pig) inner ear, following ototoxic insults as well as the potential protective strategies from the induced damages in the above experimental animals (28-35). Our results detect for the first time the presence and production of superoxide in human subjects affected by profound hearing loss and treated with cochlear implantation.

We can exclude that the increased levels of free radicals in our human inner ear perylimph samples could be attributable to the bone drilling or even by the noise generated by drilling (40, 41 42), for several reasons: i) a production of free radicals due to the friction consequent to drilling can be excluded since for this production it necessary to reach local temperatures of at least >100°C during drilling (43); ii) the surgical procedures have been performed in all cases by the same experienced operator: thus, if oxygen radicals were produced as a consequence of surgery, we should have detected them in the large majority of samples (actually we detected them only in 28 out of 142 samples analyzed); also, no differences in ROS production would have been noticed among positive samples.

Moreover, when data on superoxide production are sorted by identified pathology (Table 3), a clear association appears between a high superoxide production (29.88 +- 9.77 umoles/mg of protein) and genetic diseases. When pathology is unidentified, superoxide production appears slightly lower.

Another interesting point is related to the fact that in perilymph samples where superoxide was found and measurable, the production of this radical shows an interesting distribution related to age: in teenagers and young people (age range 11-30 years), the superoxide production is about four times higher than that in children and in the adults/elderly (Table 7).

Based on these preliminary results, it is possible to advance the following hypotheses:

1. superoxide may be produced in human inner ear perilymph (IP) in pathological conditions;
2. the production of superoxide within IP could be ascribed to the activity of the xanthine dehydrogenase/xanthine oxidase (XA/XO) enzyme system;
3. the activity of XA/XO is supported by the preliminary results of the uricase test, as well as by SDS-PAGE analysis of IP samples.
4. the superoxide production shows some age distribution pattern, being about four times higher in teenagers and young adults, in comparison to children and adults/elderly.
5. some relation appears between high superoxide production and genetic hearing impairment.

The further steps could be to analyze the content of reduced glutathione (GSH) and other anti-ROS molecules in IP, and isolate and quantify the active form of the enzyme by Western blot.

The last several years have brought significant advancements in our understanding of inner ear damage, focalizing on the importance of the cochlear free radical expression. Given the similarities in cochlear pathology for hearing losses from noise, ototoxic drugs and aging, it is not surprising that there maybe be common pathways among the pathogenesis of the cochlear damage (9). There is a growing evidence that oxygen radical mediated damage in the cochlea may be a common factor for hearing loss from aminoglycoside antibiotics, ototoxic anticancer drugs and aging (1, 44, 45, 46, 47). Understanding the molecular mechanism of the cochlear cellular damage could help us in providing (i) new tools for possible prevention of cochlear damage or (ii) new approaches for a local treatment.

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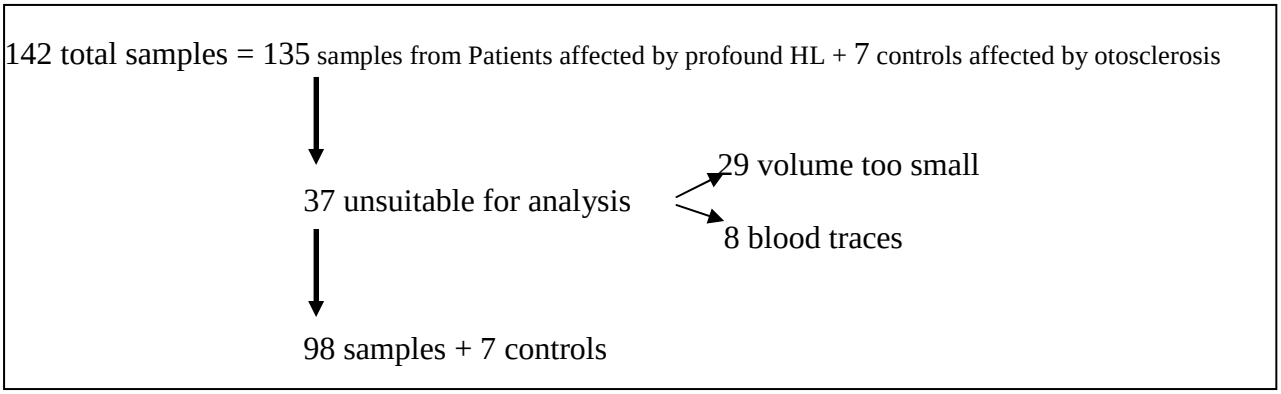
TABLES

Table 1. Population studied.



Tests performed:

A) 58 samples + 7 controls: *Cytocrome c reduction kinetics.*

B) 8 samples + 4 controls: *Cytocrome c reduction kinetics in presence of XA/XO system.*

C) 4 samples: *Catalase test.*

D) 22 samples + 2 controls: *Uricase test.*

E) 6 samples + 2 controls : *SDS-PAGE.*

Please note that control samples have been used for more than a single experimental procedure.

Table 2. Complete data of superoxide production in human inner ear perilymph samples analyzed by cytochrome c reduction kinetics. Unkn: unknown (unidentified pathology); nd: not detected; Cx: connexin mutation; EVA: enlarged vestibular aqueduct

N.	**Name**	**Age in years and decimal year fractions**	**Sex**	**Superoxide production (umoles/mg of total protein in sample)**	**H_2O_2 production (detected)**	**Pathology & notes**
1	P.C.	2.17	F	9.50	nd	Unkn
2	F. G.	28.92	M	46.30	nd	EVA
3	C. A.	1.42	M	19.30	nd	Cx homozygous
4	T. M.	11.83	F	78.40	nd	Unkn
5	N. G.	11.92	M	95.00	nd	Genetic
6	Z. L.	1.17	M	6.17	yes	Cx
7	C. F.	2.00	M	33.59	nd	Unkn
8	D. F.	2.58	M	16.75	yes	Unkn
9	B. A.	9.00	M	15.50	yes	Unkn
10	S. A.	65.00	F	17.00	yes	Unkn
11	C. N.	26.92	M	23.00	yes	Unkn
12	A. R.	43.33	M	18.75	yes	Familiar hypoacousia
13	P. L.	62.00	F	11.48	nd	Menière
14	V. A.	66.75	F	4.56	nd	Othosclerosis
15	B. L.	34.83	F	51.05	nd	Unkn
16	R. R.	3.67	M	10.00	nd	Unkn
17	B. G.	40.00	F	23.50	nd	Unkn
18	G. N.	1.50	F	12.29	nd	Genetic
19	B. E.	66.92	M	0.11	nd	Othosclerosis (control)
20	F. M.	65.08	F	1.11	nd	Othosclerosis (control)
21	N. E.	58.75	F	0.11	nd	Othosclerosis (control)
22	P. A.	68.00	F	0.11	nd	Othosclerosis (control)

Table 3. Production of superoxide in human inner ear perilymph samples according to pathology. Unknown: unidentified pathology; Cx: connexin mutation; EVA: enlarged vestibular aqueduct

Pathology	**N.**	**Average superoxide production (umoles/mg of total protein)**
Genetic (Cx, EVA, familiar hypoacousia, Menière)	7	29.88 +- 9.77
Unknown	10	27.83 +- 10.53
Othosclerosis (control)	5	1.20 +- 0.75

Table 4. Superoxide production in human inner ear perilymph samples, analysed by cytochrome c reduction kinetics: general results.

- 18 patients, 10 males and 8 females, average age 23.06, age range 1.17-66.75 - 4 controls, 1 male and 3 females, average age 64.68 +- 4.14,age range 58.75-68.00 Average superoxide production in patients 27.34 +/- 14.42 umoles/mg of total protein in sample Average superoxide production in controls 0.36 +- 0.13

Table 5. Comparison of positive results of uric acid production in two inner ear perilymph samples and one control.

Name	Age	Sex	Uric acid production (umoles)	Total protein in sample (mg)	Uric acid production/mg of protein	Pathology
M. G.	1.83	M	3.48	0.085	40.94	Cx 26+
S. C.	65.92	F	1.52	0.040	38.00	Unk
B. E.	66.92	M	0.02	0.071	0.28	Othosclerosis
P. K.	37.50	F	0.05	0.063	0.79	Othosclerosis

Table 6. Data of inner ear perilymph samples analyzed by SDS-PAGE according to the procedure described in Materials and Methods (6 patients and 1 control, all females, average age 35.75 +- 5.32, age range 27.58-43.58)

Name	Age (in years and decimal fractions of years)	Pathology
C. A.	27.58	Unknown
B. L.	43.58	Unknown
R. R.	42.33	Unknown
P. F.	33.17	Unknown
C. T.	34.00	Unknown
T. L.	32.00	Unknown
P. K.	37.50	Othosclerosis

Table 7. Production of superoxide radicals measured as amount of reduced cytochrome (red cyt, umoles /mg of protein) in human inner ear perilymph samples of patients from different age ranges.

Age range	**N.**	**Superoxide (umoles/mg prot)**
0-10	8	15.87 +- 7.96
11-30	4	60.67 +- 27.92
Over 30	6	21.06 +- 14.67
Controls	4	0.36 +- 0.13

FIGURES

Fig. 1. Typical spectral profile changes during cytochrome c reduction: oxidized cytochrome (red); reduced cytochrome (blue).

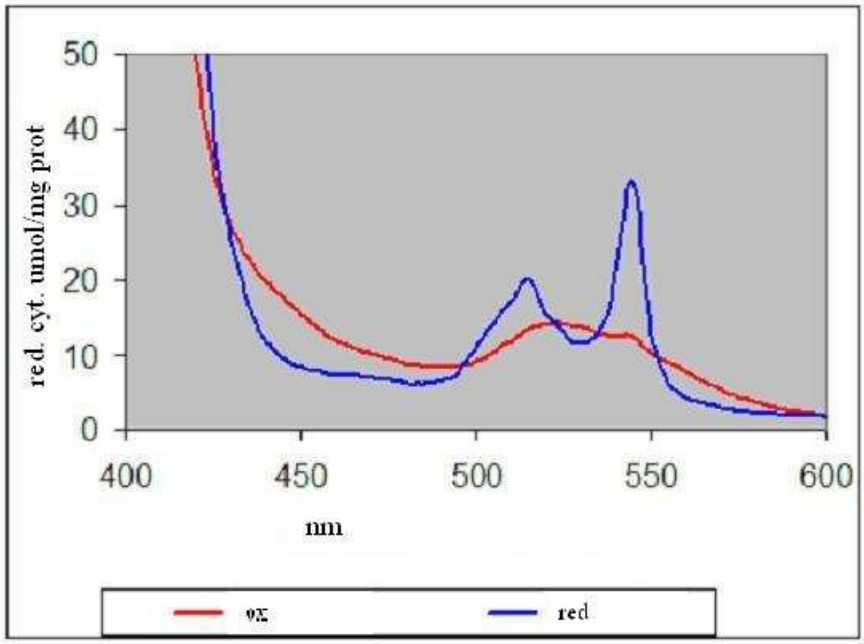


Fig. 2. Kinetic profile of a IP sample from a patient affected by profound hearing loss by genetic cause (Cx 26+), respectively without (blue) and with (red) addition of superoxide dismutase (SOD).

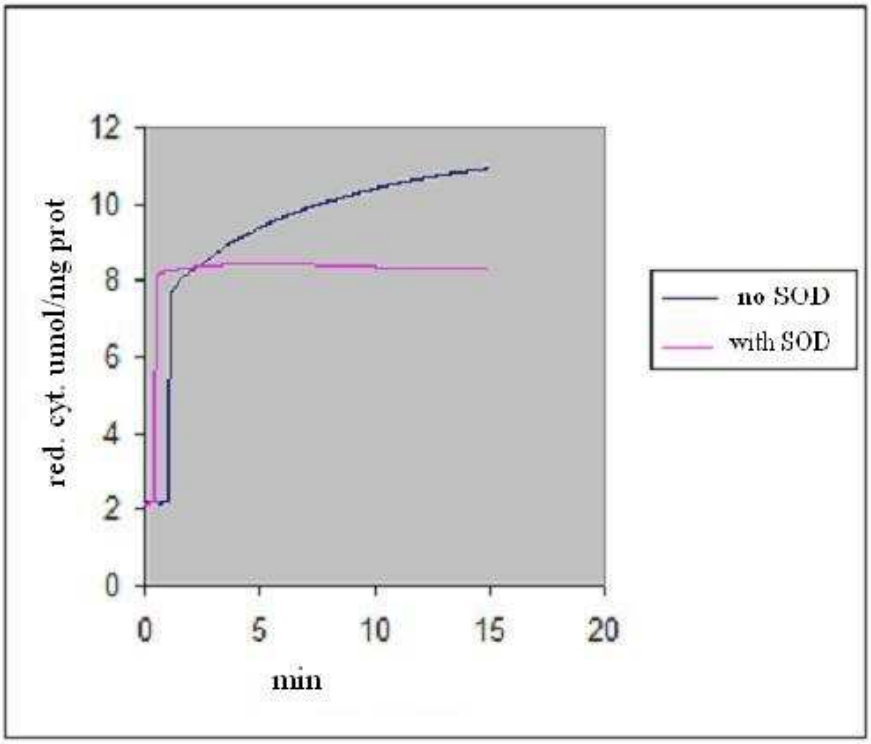


Fig. 3. Spectral profiles of cytochrome at the end of the kinetic run in Fig. 2, respectively without (blue) and with addition of SOD (red).

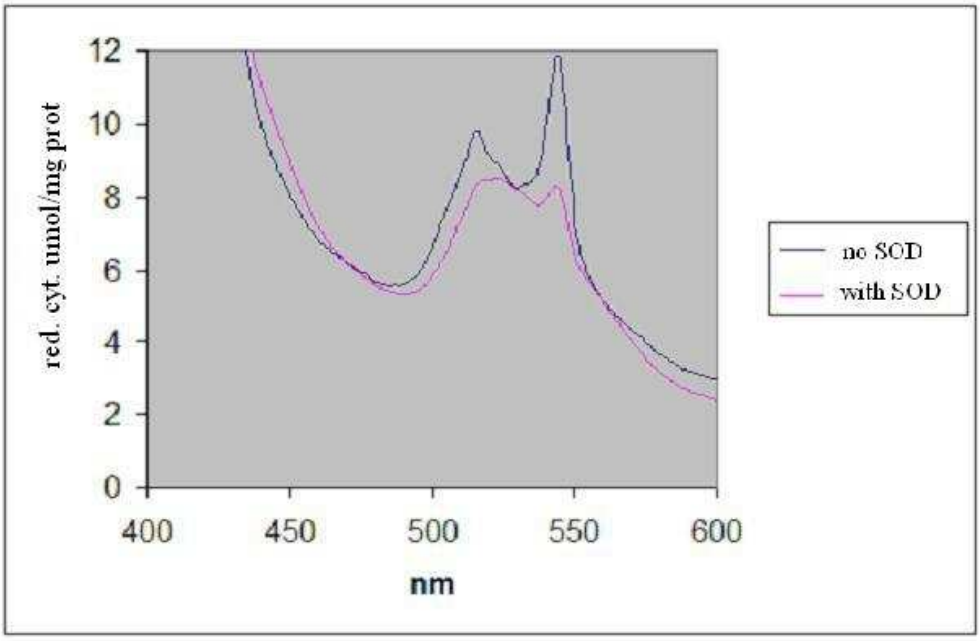


Fig. 4. Spectral profiles of cytochrome c reduction in presence of a IP sample from a patient affected by profound hearing loss, without (blue) and with (red) addition of xanthine (XA).

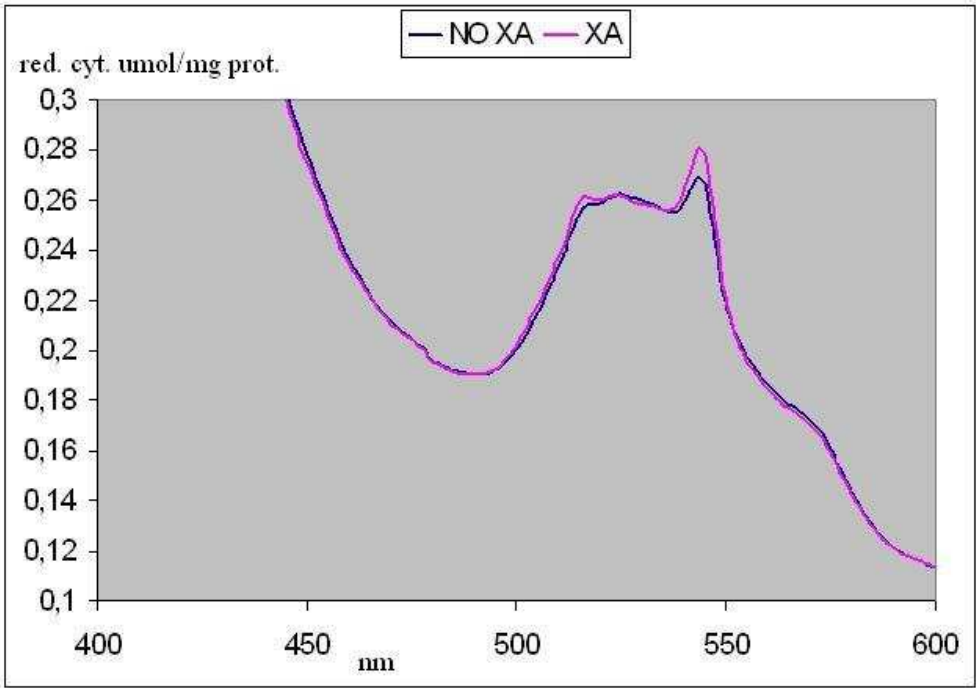


Fig. 5. Uricase test. Typical kinetic profile of uric acid production in presence of a IP sample from a patient affected by profound hearing loss: the addition of uricase (1.3 units/ml) at minute 7 caused a sharp decline of the kinetic profile at 293 nm.

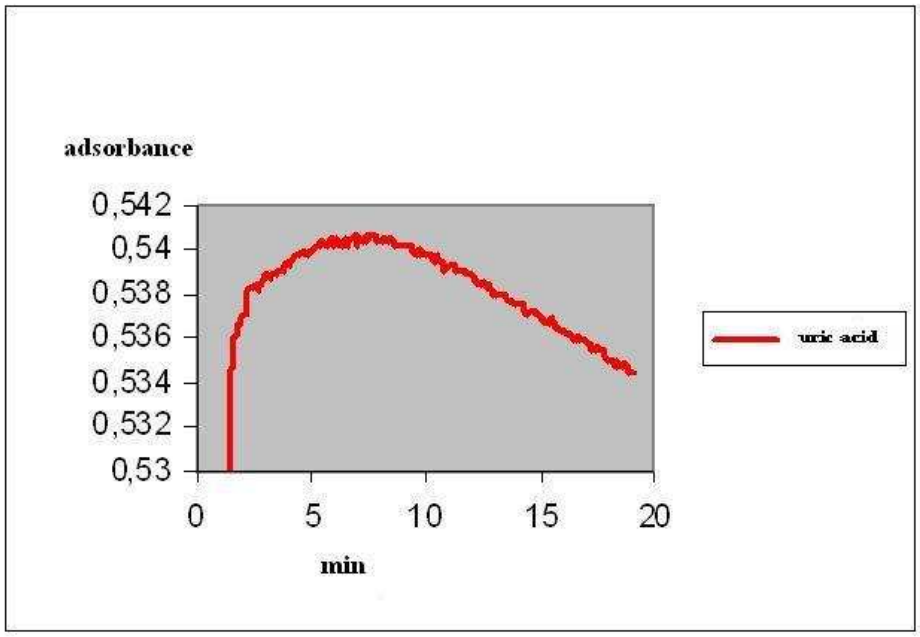


Fig. 6. Changes in spectral profiles of uric acid production in presence of a IP sample from a patient affected by profound hearing loss, before and after addition of uricase(see Fig. 5). The arrow indicates the lowering of uric acid production after uricase addition.

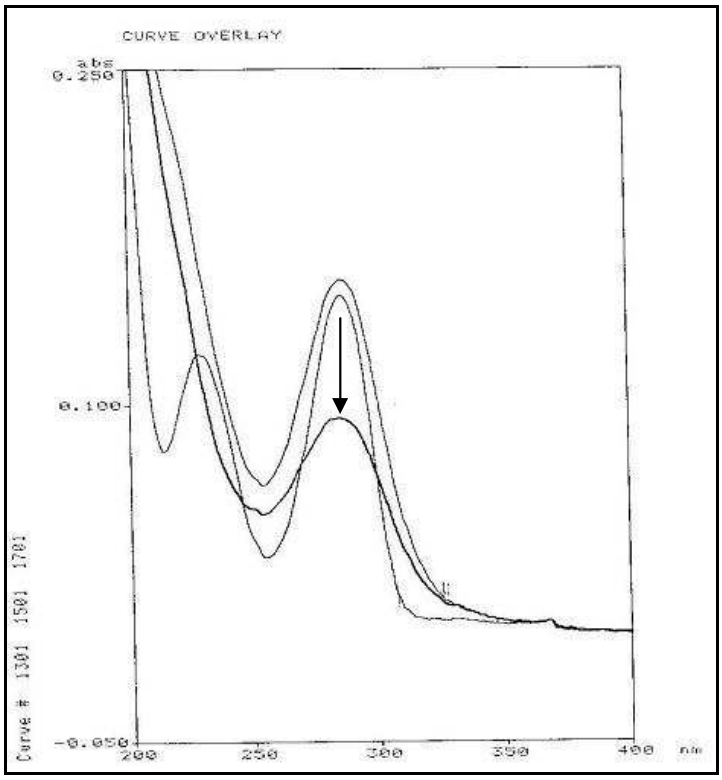
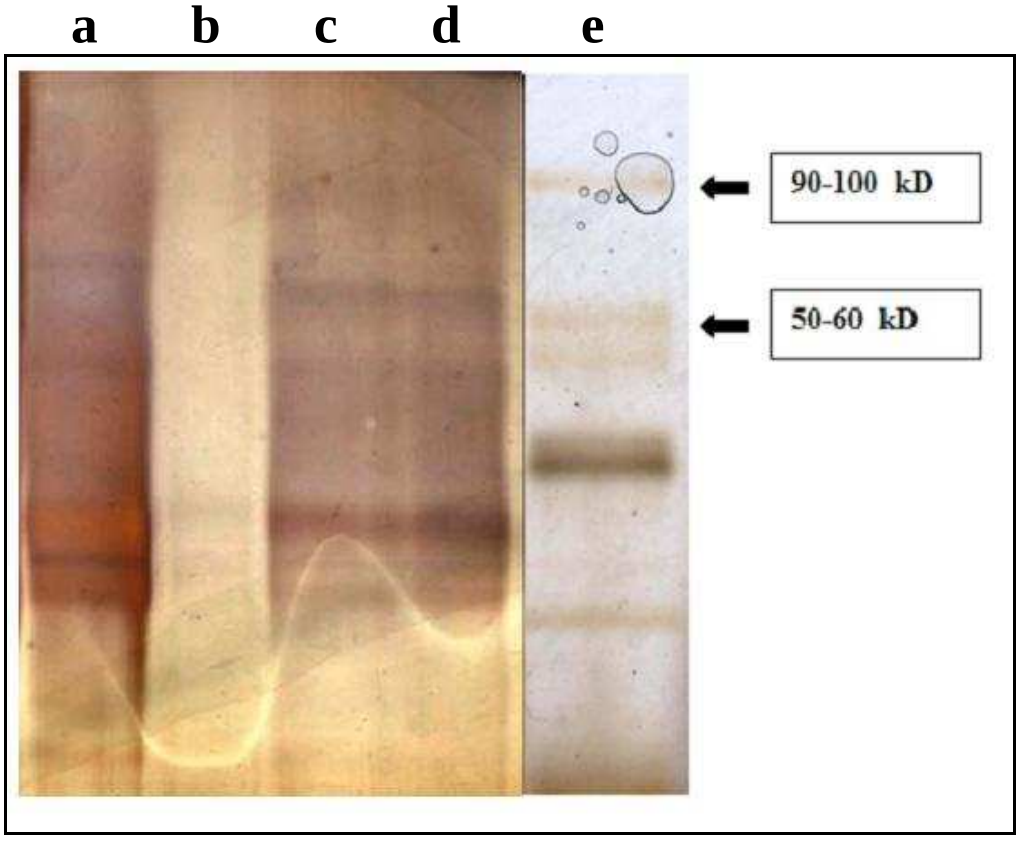


Fig. 7. SDS PAGE analysis. From left to right: IP samples n. 1 (**a**), control sample (**b**), IP samples n. 2 and 3 (**c** and **d**), purified xanthine oxidase sample (**e**). A double band is clearly visible in the purified xanthine oxidase sample and in each IP sample (although at different intensity), but not in the control sample.



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