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MEDICAL HYPOTHESIS

Oxidative stress in the animal model: the possible protective role of milk serum protein

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Abstract In the field of biology, free radicals which are derived from the incomplete reduction of oxygen take on great importance; they belong to the so called reactive oxygen species, whose production in the organism is an inevitable consequence of various external or internal factors to which it is exposed. Once free radicals are generated they are often capable of giving rise to chain reactions. A lot of biological molecules are susceptible to the attack by free radicals including lipids, proteins, carbohydrates and nucleic acids. Molecular alterations caused by the radical reactions have been frequently studied and are considered as pathogenetically main passages in the development of many diseases and ageing. In order to face a radical attack, living organisms have developed several biological defensive systems against it: the main ones are represented by anti oxidizing molecules and by enzymatic anti oxidizing systems. Among the various defence systems, glutathione stands out as the principal guarantor of homoeostatic intra-cellular oxidation–reduction. One of glutathione's most important functions is to act as cysteine "tank"; this amino acid is extremely unstable in the extracellular environment and it rapidly auto-oxidates. Whey proteins (WP) are particularly rich in cysteine (cys) and in glutamine (glu) and therefore potentially capable of increasing the organism's antioxidant defences. It is thought that the principal mechanism which allows WPs to exert their properties is through the contribution of cys and glu, which is rich in these proteins and is used intracellularly for the synthesis of glutathione. A diet based on milk serum proteins which supplies a superior quantity of cys, allows for a greater synthesis of hepatic glutathione in oxidative stress conditions. The use of ultra-filtrated WP could represent a useful tool in the control of oxidative stress in numerous pathological situations.

Keywords Whey proteins (WP) · Glutathione · Antioxidant defences · Oxidative stress · ROS (reactive oxygen species) · Free radicals

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Free radicals

Free radicals are by definition molecules or fragments of molecules that contain an odd electron on their outer orbit. In many instances their extreme reactivity is characteristic as they often pair up the odd electron with another one belonging to another molecule [1, 2]. They can behave like acceptors of electrons (oxidants) or as donors (reductors). Free radicals' stability is extremely variable. There are free radicals which only live for nanoseconds ($CH_3^{\bullet}$), others milliseconds ($O_2^{-\bullet}$) and others which are stable for a long time as they are capable of de-localizing the odd electron by creating resonant structures, the free radicals reactivity depends upon these characteristics.

In the field of biology those free radicals which are derived from the incomplete reduction of oxygen take on great importance. They belong to the so called ROS (reactive oxygen species), which are represented by radical hydroxyl ($OH^{\bullet}$), super oxide anion ($O_2^{-\bullet}$) as well as the oxygen singlet ($^1O_2$) and hydrogen peroxide (H_2O_2); despite the fact that the

latter two have a complete electronic configuration they present a reactivity which is similar to free radicals due to spin or molecular structure.

The production of ROS in the organism is an inevitable consequence of various external or internal factors to which it is exposed. The main external cause for the formation of free radicals in the organism is exposure to ionizing radiations [3], which are statistically derived, above all, from natural sources (cosmos, earth). Such radiations are in fact capable of ionizing the water molecule, generating radical reactive species of different natures, but principally $OH^{\bullet}$, $H^{\bullet}$, solvent electrons by water and $^{1}O_2$.

The internal production of free radicals depends on enzymatic and non-enzymatic sources. Enzymatic sources are represented by various enzymes (aldehyde oxidase, enzymes from prostaglandin synthesis, NADPH oxidase, xanthine oxidase, NO synthesis) [4] which directly produce free radicals as reaction products or by-products. But above all, the production of ROS depends on the chain of electronic transport present in mitochondria and endoplasmic reticules. Certainly, one of the sites of major production of ROS is represented by the mitochondria, [5, 6], organelles in which enzymatic transport reactions of electrons and oxidative phosphorilation of the ATP (mitochondrial respiration) take place. The complete reduction of oxygen to water is not always obtained and it is known that a percentage (the actual amount is under discussion, but it could be around 5%) of oxygen is only partially reduced to form a super oxide anion. The electronic microsomal chain of transport associated with the biotransformation of xenobiotics connected to the cytochrome P450 [7] is also capable, once again due to incomplete reduction, of giving origin to free radicals during such metabolization; thus exposure to xenobiotics may not only result in eventual toxic activity of the same, but also in further damage due to the generation of free radicals during their metabolisation.

A certain number of non-ezymatic sources also exist. Let's remember the oxidation of oxygenated haemoglobin to meta-haemoglobin, a spontaneous reaction that takes place para-physiologically in all organisms and generates super oxide anion [8]; furthermore, several organic substances, such as monosacharides, lipids or even absorbic acid can go into so called spontaneous auto-oxidation with the production of super oxide anion [9, 10].

The consequences of the production of free radicals

Once free radicals are generated they are often capable of giving rise to chain reactions, i.e., reactions that create new radicals which in turn trigger new reactions. In chain reactions, three phases can be clearly distinguished: an initial phase, one of propagation and an end phase when the free radicals are consumed without out being generated. In biology, a typical example is shown by lipid peroxidation [11]. During this process, on one hand the morphological and functional characteristics of lipids are modified and on the other a series of molecules are produced, among which, different aldehydes (i.e.: malonaldehyde and 4-hydroxy-2-nonenal) [12], capable of producing damage to the biological macro-molecules even at a distance from the formation site.

More recently, another example of radical chain reactions in the biological field has been observed: protein peroxidation [13]; this phenomenon is based upon the capacity of ROS for interacting with protein structures and generating hydro peroxides on the polypeptide chain [14]. These structures are only relatively stable and in the presence of metallic transition ions they are capable of creating other radicals which in turn are responsible for further intra- and inter-molecular chain reactions: it has been shown that a single hydroxyl is capable of causing changes to 15 amino acids of a peptide chain [15]. This would generate various by-products among which oxidated amino acids, carbonyls and fragmentation products [16]; the result is therefore the loss of the native structure and thus the functioning of the molecule in question.

So, as well as lipids other biological molecules are susceptible to an attack from free radicals including proteins as well as carbohydrates and nucleic acids. Following this attack modifications of different types may occur: chemical changes to the monomeric base units (azote bases, amino acids and monosaccharide units), denaturation, aggregation or fragmentation phenomena; mutations, structural changes, anomalies or functional deficits can thus be derived. Molecular alterations caused the radical reactions have been frequently studied and are considered as pathogenetically fundamental passages in the development of many diseases and ageing.

Antioxidants processes

In order to face a radical attack living organisms have developed several biological defensive systems against it: the main ones are represented by anti oxidizing molecules (e.g., tocopherol, carotene, uric acid, glutathione, ascorbic acid) and by enzymatic anti oxidizing systems (e.g., catalysis, super oxide dis mutation, glutathione peroxidase). Among the various defence systems glutathione stands out as the principal guarantor of homoeostatic intracellular oxidation–reduction [17], also in virtu of its high concentrations within the cells. Glutathione is a tripeptide which is important in the maintenance of homoeostasis and the protection of cells against antioxidant, toxic and

carcinogenic agents; it is composed of glutamate, cysteine and glycine (γ-glutamyl-cysteinyl-glycine), and it is omnipresent in all mammal cells; it is particularly concentrated in the liver, kidneys, leukocytes, the spleen and erythrocytes where it is found in millimolar concentrations. It can exist in a reduced form (GSH) or oxidized (GSSG), which is made up of two glutathione molecules joined by a disulphide bridge.

About 90% of GSH is found in cellular cytosol at a remarkably higher concentration compared to GSSG; a lower percentage is found in mitochondrial and endoplasmic reticules. The glutathione molecule has two particular structural characteristics: the γ-glutamylic ligand and the free sulphidrilic group; such portions, respectively, favour intra-cellular stability of the GSH and are involved in its multiple functions [18].

Peptide ligand involvement between cysteine and the glutamate γ-carbocyclic group (instead of the traditional α-carbocyclic) protects the glutathione from degradation by intra-cellular peptidase; in fact the only enzyme capable of breaking the γ-glutamylic ligand is γ-glutamyl transpeptidase (γGT), which is localised on the external surface of cellular membranes. Further protection is provided by carboxy-terminal glycine [17]. To conclude, GSH resists intra-cellular degradation and may only be metabolised outside of the cell.

Moreover, the cysteine residue sulphidrilic group carries out its important function by participating in multiple reactions: GSH carries out many functions of vital importance:

Detoxifying action: GSH can be the conjugating agent of xenobiotics with the subsequent formation of mercapturic acid. These reactions can take place spontaneously or they may be catalysed by the GSH S-transferase enzyme which favours the hooking up between the cysteine SH reactive group and the xenobiotic.

Maintenance of the sulphidic groups of proteins in a reduced form by preventing oxidation or inducing the reduction of the disolfuro ligands induced by oxidative stress. In order to do this, GSH faces exchange reactions among groups of thiols and disulphur catalysed by thiol-transferase as shown below:

$$\text{protein-SSG} + \text{GSH} \leftrightarrow \text{protein-SH} + \text{GSSG}$$

Since this reaction can be reversed, the balance is established by the state of the cell's redox which depends on the concentration of GSH and GSSG. Normally the GSSG content in the cell is maintained at an extremely low level in order to limit the exchange reaction.

Antioxidant function: As a consequence of the aerobic metabolism, all organisms are subject to a certain level of physiological oxidative stress. The intermediates that form, for example, super oxide anion ($O_2^{-\bullet}$) and hydrogen peroxide which can bring about the further production of other dangerous radicals of oxygen which can induce lipid peroxidation and cellular damage. The H_2O_2 that forms is reduced by GSH thanks to the GSH peroxidase enzyme action, while GSH is oxidized to GSSG. Organic peroxides can be reduced both by the GSH peroxidase and by the S-transferase, while the catalase which is only present in the perox peroxisome reacts with the H_2O_2 converting it into O_2 and H_2O.

The presence of GSH in the mitochondria is particularly important in that there is no catalytic action, and as recent studies have shown, mitochondrial GSH plays a critical role in the defence against physiological and pathological oxidative stress. Catalysis and peroxide GSH play a complementary role; moreover, peroxide GSH interacts with other hydro peroxides and reduces them to corresponding alcohols:

$$2\,\text{GSH} + \text{ROOH} \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{GSSG}$$

Such an enzyme particularly acts on fatty acid hydro peroxides, and thus it is fundamental in protecting against the oxidation of lipid membranes.

It seems to be clear, like the relationship between GSSG and GSH that it is a useful indicator of intra-cellular oxidative stress [19]; this relationship is vital for cell homoeostasis as it regulates activity and survival and for this reason it is subjected to strict homoeostatic control. A severe depletion of GSH in cells could bring about greater vulnerability to an oxidative attack [20] and trigger a process of programmed suicide known as apoptosis [21].

Glutathione in its oxidative form can be re-converted into GSH thanks to the GSSG reductase enzyme which through the NADPH co-enzyme forms a redox cycle [17].

Cysteine tank: This is one of glutathione's most important functions in that cysteine is extremely unstable in the extra-cellular environment and it rapidly auto-oxidates into cysteine through a process which produces potentially toxic oxygenated free radicals.

The γ-glutamylic cycle described by Meister at the beginning of 1970 [18], guarantees that GSH is a continuous reserve of cysteine. In this cycle GSH is released outside of the cell through a carrier-mediated transporter and the γ-glutamyl transferase (γGT) enzyme transfers the γ-glutamylic portion of the GSH to an amino acid (the best acceptor could be cysteine itself) forming the γ-glutamyl amino acid on one side and cysteinil glycine on the other. γ-glutamyl amino acid can then be returned to the inside of the cell to complete the cycle. Once inside, it can be further metabolised and release the amino acid and 5-oxoproline; the latter can be converted into glutamate and if necessary used for the synthesis of glutathione. Conversely, cysteinil glycine is a substrate of dipeptidase which is split to form cystein and glycine. The sulphurate amino acid is rapidly captured by most if not all the cells. Within these cells

almost all the cystein is incorporated with the GSH; a small part in the proteins (according to the cell's requirements); and a part, degredated to sulphate and taurine. For the majority of cells this mechanism provides a continuous supply of cystein; thus the γ-glutamylic cycle represents an efficient cystein deposit [17].

Co-enzyme: Finally, GSH intervenes as a co-enzyme in many reactions. The first to be discovered, for example, was the glyoxylase reaction, where methylglyoxal is converted into D-lactate. Other enzymes also provide for the involvement of glutathione: For example, formaldehyde dehydrogenase and prostaglandin isomerase, just to cite a few.

Free radicals and disease

Despite the numerous lines of defence, protection against free radicals is never complete: more or less severe random damage continually takes place within living organisms; furthermore, considering that the sites responsible for the greatest production of oxygen radicals are localised on biological membranes (electronic transport chains), it is likely that the components of the membrane themselves (phospholipids and proteins) are among the principal targets.

One of the fundamental roles of free radicals has been studied and recognised in numerous pathological situations regarding several organs; among these for instance are:

- Inflammation [22]
- Ischemia–reperfusion syndromes [23]
- Atherosclerosis [24]
- Degenerative cerebral syndromes, particularly Alzheimer's [25]
- Neoplasms [26]
- Diabetes [27, 28]
- Renal insufficiency [29]
- Ageing "physiological organisms" [30]

Interactions between free radicals and glycation

Over the last few years an ever increasing number of experiments aimed at studying the alterations induced by free radicals and the end-products of advanced glycation [31] have highlighted specific inter-relations between these two non-enzymatic modification mechanisms [32]. Specifically, the ability of ROS in activating glucose and other alpha-hydroxy aldehydes (or alpha-hydroxy ketones) rendering them more reactive and favouring the attack of biological macro-molecules; ultimately, ROS are capable of accelerating Maillard's reaction. Equally, the reducing sugars, Amadori's products and other intermediaries of Maillard's reaction can, in the presence of metallic transition iones, lead to auto-oxidation generating oxygen free radicals [9].

On the basis of these data, oxidative phenomena and glycation appear not only as two possible mechanisms of parallel modification, but also as a single biochemical mechanism involved in the pathogenesis of the typical alterations which occur in the ageing process and its various related diseases.

Study models

The study of oxidative stress in the animal model can adopt different methodologies, using and/or emphasising the radical sources mentioned above, some example can be:

- Irradiation, which in theory allows for a rather precise assessment of the quantity of radicals that form in the irradiated structure [33].
- Exposure to xenobiotics capable of being metabolised by the P450 cytochrome system generating free radicals; many substances have shown themselves to be capable of generating radicals, and among these we can note the halogenated hydrocarbons of which the head of the family, carbon tetrachloride, of notable toxicity particularly to the liver and potentially carcinogenic, correlated in fact to its metabolisation [34].
- Exposure to hyperbaric oxygen which accentuates the toxic potential of oxygen correlated to the generation of ROS [35].

Another approach is represented by the induction of a state of depletion of the anti oxidizing defence which by unbalancing the equilibrium oxide-reductive causes a relative excess in the production of radicals. Diets without vitamin E can be used [36], or substances which deplete the organism of antioxidants like BSO capable of blocking the synthesis of glutathione [37].

Studies carried out on animal models can be contradictory and may be full of antioxidants, for example, with the administration of vitamin E supplements [38]. In this way, the use of whey proteins (WP) rich in cysteine and therefore potentially capable of increasing the organism's antioxidant defences could make sense.

There is plenty of evidence in current literature which correlates the consumption of WP with physical performance levels, an improved oxidative balance and positive results in the treatment of various diseases such as cancers, hepatitis, cardiovascular diseases and diabetes [39].

It is thought that the principal mechanism which allows WPs to exert their properties is through the contribution of

cysteine, which is rich in these proteins and is used intracellularly for the synthesis of glutathione [40].

Our group's recent studies suggest that a diet based on milk serum proteins which supply a superior quantity of cysteine, allow for a greater synthesis of hepatic glutathione in oxidative stress conditions, such as, CC14 intoxication, so that, after the stress, instead of noting a fall in total glutathione we note an increase [41, 42]. This may indicate an increase in anti oxidating defence capability brought about by the milk serum proteins; it could depend on the greater quantity of cysteine in the milk serum proteins compared to caesium, but also by the quicker manner in which these proteins are adopted.

The reduction of the haematic content of glutathione in rats on a WP diet was surprising and deserves further investigation. Nevertheless, after CC14 intoxication, an effect similar to that observed in the liver has been observed: GSH decreases in rats on a CAS diet while it increases in rats on a WP diet. From this perspective, the interpretations suggested in hepatic GSH can be repeated: whey proteins appear to allow for a more efficient synthesis of GSH following an oxidative stimulus, so much so that its concentration increases regardless of the possible consumption due to intoxication.

It is believed that oxidative stress increases ageing in obesity and diabetes. The use of ultra-filtrated whey proteins could represent a useful tool in the control of oxidative stress related to such conditions which are in great epidemiological expansion. Nevertheless, the long-term dietary integration of WP in man will need to be assessed further.

Conflict of interest None.

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