

# **Oxidative stress and antioxidants: how to assess a risk or a prevention?**

## **Theoretical consideration and application to SOD-Gliadin.**

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### I) Introduction

Since the earliest beginning of Life, living organisms are continuously exposed to a major risk of oxidative damage, which is mostly related to oxygen. Other sources, such as light, radiations or chemicals, are also involved. The extreme toxicity of oxygen is related to its unique capacity of generating free radicals, which have long been recognized as harmful compounds on biological molecules. Actually, Life appeared before oxygen, since its early emergence resulted from a long period of microorganism and plant photosynthesis. The increase in oxygen partial tension in earth atmosphere was extremely deleterious for all biological molecules and the massive contemporary vanishing of living species was related to oxygen toxicity. Among several antioxidant strategies developed through the process of Evolution, mitochondrion ancestors played probably a major role in adapting Life to survive to such massive oxidative stress. Because of this long and mandatory metabolic evolution, a highly complex interaction between life (mitochondrial ATP synthesis) and death (oxidative toxicity) has been developed. The most harmful free radicals or reactive oxygen species (ROS) have in common a very short half-life indicating that they can very quickly exchange electrons with any kind of biological molecule. Therefore, these molecules are difficult to be directly

evidenced in normal biological conditions and detection of oxidative stress is mostly achieved by assessing the result of this process: *i.e.* through the detection of damaged (oxidized) molecules (DNA, lipids, proteins etc.). On the other hand, the balance between pro and antioxidant activities is so finely tuned that any increase in antioxidant defense can be viewed either as the development of a good protection against oxidative stress or as an adapted response to previous oxidative damage. Hence an increase in the antioxidant defense indicates actually the result of an exposure to higher oxidative conditions. Therefore, assessing antioxidant capacity cannot be obtained by using a single parameter, whatever its intrinsic value and it is probably more informative to perform a dynamic assessment to a response of a given stress than a basal biological compound determination.

### II) Metabolic basis of oxidative stress.

A free radical is an atom or a molecule, which contains one (or more) unpaired electron(s) (single-electron) on its outer layer. When generated from oxygen, free radicals are also called Reactive Oxygen Species (ROS, Figure 1). Different mechanisms of production occur at the level of (i) mitochondrial respiratory chain, (ii) NADPH oxidase, (iii) multienzymatic complex cytochrome P450, (iv) xanthine oxidase, and also (v) NO synthesis from

arginine (Figure 2). In addition, metallic ions such as iron and copper are very potent catalysts leading to ROS production (hydroxyl radical) from hydrogen peroxide (Fenton's reaction). Several members of the ROS family are involved (i) in cellular sensing and signaling pathways, (ii) in the regulation of the vascular tone, (iii) in the immune response, (iv) in the fight against exogenous organisms and (v) in the destruction of several exogenous or endogenous molecules.

However, ROS (mostly hydroxyl radical:  $\text{OH}^\circ$  and peroxonitrite:  $\text{ONOO}^-$ ) are also extremely harmful for the biological molecules, explaining the major danger of oxidative stress for any living organism. If such toxicity can be developed against all biological molecules, some are especially sensitive to this peculiar stress: DNA, membrane-phospholipids, lipoproteins and proteins. Polyunsaturated fatty acids are particularly sensitive targets for oxidative damage, due to the presence of double-bonds. However, monounsaturated fatty acids, such as oleic acid, appear protective against membrane-related ROS damage.

The mechanisms of defense either prevent ROS generation or scavenge these toxic compounds. The scavenging pathway includes (i) oxidoreduction of several "protecting" compounds such as vitamins E, C and glutathione and (ii) enzymatic catalysis (glutathione peroxidase, catalase). It is very important to understand this two steps mechanism. First, a "lightening conductor"-like process allowing the avoidance of the harmful consequence of oxidizing a biological compound thanks to a quicker oxidation of a given metabolite alternately oxidized and reduced. Secondly, a succession of enzymatic processes (glutathione peroxidase (GPX) and reductase (GPR) or catalase) allowing a final reduction leads to an actual scavenging process (Figure 2). Indeed, if

several natural compounds act as "antioxidant", this is mostly due to their property of changing from oxidized to reduced status representing a first line of defense, this action must be followed by the endogenous antioxidant pathway represented by the key antioxidant enzymes GPX and catalase.

Numerous evidences involve oxidative stress in several pathological disorders and it has been recognized as a factor of morbidity in acutely ill patients. Indeed, oxidative stress alters cellular homeostasis by ischemia/reperfusion or by activation of ROS production from immune cells. In such acutely ill patients, antioxidant capacity is often depressed after a few days due to a high rate of consumption and/or of losses in urine of several key components, in fluid drainage or through the burned skin. However, some of these patients are also frequently previously depleted, mostly because of preexisting chronic or sub-acute disease, thus favoring the risk of oxidative damage. If several experimental and clinical studies have already pointed out the potential benefits of antioxidant therapy, it was quite disappointing in others and the question of antioxidant supplement is not completely solved. Hence, further studies are needed to assess the clinical conditions associated with an unequivocal beneficial effect, but it is highly probable that a better approach of oxidative stress management in acutely ill patients will permit to improve their outcome.

Assessing the efficacy of a given antioxidant component needs to consider not only its mechanisms of action (known or supposed) but also the resulting effect in vivo, beyond its action. As an example, vitamin supplements are given to correct a potential vitamin deficiency and/or to increase the vitamin storage in the body. This could be assessed in principle,

although storage compartments are not easily accessible and often only plasma or red cell are considered, but this does not imply an actual efficacy on the whole pathway in the clinical situation. Even worse, opposite effects have been reported. Indeed, considering the complexity of the entire antioxidant pathway, an activation of ROS scavenging rate would require a coordinated effect of many different steps. This opens the question of the consequence beyond the target of any intervention.

When SOD-gliadin, as natural oral antioxidant, is used in the prevention of oxidative stress, different levels of determination must be considered in the attempt of assessing its efficacy, each of them being a complement of the others.

SOD is a fragile enzyme and therefore determination of its enzymatic activity is a prerequisite, if considering its action is obtained through its activity. This can be performed by a classic spectrophotometric enzymatic assay (Xanthine-/XOD-NBT), which can be performed on each batch of the raw material (Extramel®). This first assessment of enzymatic activity is then used as reference for assessing this enzymatic activity in the final product (SOD-gliadin). Assessment of the activity in the final product is routinely achieved by gel-electrophoresis. Superoxide dismutase (SOD) is electrophoresed on polyacrylamide gel. SOD is then localized by soaking the gel in nitro-blue tetrazolium (NBT), followed by an immersion in a solution containing TEMED and RIBOFLAVIN. Gel is then illuminated to induce superoxide radical ( $O_2^{\cdot-}$ ) generation thanks to the photo-oxidation of riboflavin in the presence of Temed and Oxygen. While illuminated, the gel becomes uniformly blue except at the spots where SOD is present. SOD activity is appreciated by the achromatic zone present on the gel. A commercial kit is now available permitting spectrophotometric

determination (SOD ASSAY KIT-WST). It provides similar results and the equivalence between the two spectrophotometric methods have been demonstrated (Zhou & Prognon). Therefore, it is possible to assess the enzymatic activity of the final product, *i.e.* after manufacturing, by either method.

2) Of course it is not possible to assess this activity in the digestive tract: *i.e.* after its absorption. Therefore, an unknown remains concerning the form and the intimate mechanism in the gut and assessing its activity *in vivo* relies on the determination of some appreciable consequences. Strikingly contrasting with vitamins and some other metabolites supplemented in the food, it is of course not possible to assume here that SOD, an exogenous and heterologous protein, given orally, may replenish one or more of the various endogenous pools of this enzyme. Hence, assessing endogenous SOD-activity(ies) with oral treatment with SOD-gliadin reflect no less, but no more, than other indicators of the global activity of key-enzymes and intermediates involved in the ROS-scavenging pathway. Any change in one or more of the steps and/or intermediates of this pathway indicates a global response of the pathway to a signaling event consecutive to the oral supplement. A very similar mechanism follows the physiological adaptation to an increased oxidative pressure: hyperoxia, tobacco, alcohol, etc. have been shown to activate the scavenging pathway as a response to the ROS aggression. As stated above, activation of the antioxidant (scavenging) pathway indicates an exposure to an enhanced risk. This has a very important consequence that is in principle the effect of oral administration of SOD-gliadin is very similar to that of an exposure to an increased risk of oxidative stress, only the signal responsible for the

response is different! The parameters of the antioxidant pathway, which are accessible and susceptible to a modulation, involve enzymatic activities such as: Mn-SOD, Cu,Zn-SOD, GPX, GPR, catalase and mostly reduced and oxidized glutathione. The enzymes are endogenous proteins under transcriptional regulation. The “pro *versus* antioxidant equilibrium” is the matter of a delicate balance since besides their potential harmful effects, ROS are intracellular signaling molecules and an adequate level of intracellular “oxidative stress” should be maintained. Accordingly, the level of the antioxidant enzyme activity is finely tuned to the actual exposure to oxidative stress: “too much or too low are both unsuitable”.

Again it is important to stress the difficulty in interpreting the results of these enzymatic determinations. Chronic depletion of antioxidant elements provided by lack in the diet (vitamins, minerals and few other compounds) might lead to a depression of the global transcription leading to low enzyme activities, but this situation might also indicate a very moderate exposure to oxidative stress. The difference between the two situations depends on the levels of the dietary “antioxidant elements” low with chronic depletion and high the case of an equilibrated diet with low exposure to oxidative stress.

Conversely high level of defense potentially indicates an adapted response to oxidative stress exposure, the question being to know if the initial oxidative stress were harmful or simply a temporary signal. This could be determined by measuring the damage of endogenous compounds by oxidative stress. As it was stated above, the very short half-life of ROS renders these molecules not very accessible for a routine use in clinical practice although several analytical methods useful for research

purposes (RPE). Hence the choice in practice is to assess the result of oxidative stress by assessing damaged molecules: MDA, TBARS, di-ene conjugated metabolites (pentane or ethane) and lipids (isoprostanes). Each of these compounds has specific advantages and drawbacks, isoprostanes being often proposed as a good marker. Again, interpreting these data is delicate! Indeed, an increase in these oxidized metabolites indicates clearly an oxidative stress, it does not inform really if the phenomenon is acute (signal leading to an adapted response) or prolonged and potentially harmful.

Among the endogenous molecules susceptible to peroxidation, the DNA is very prominent. Assessment of DNA damage can be performed as described in the comet assay (electrophoresis of DNA). This test is a good marker of oxidative stress at the nucleus level and antioxidant capacity, the lesions depending on both. Interestingly the presence of DNA breaks also indicates the capacity of the endogenous repairing machinery. Hence it is a global assessment of the situation and a good marker when clearly abnormal. The value of this test has been amplified by exposing cells (lymphocytes) to a calibrated exogenous oxidative stress *ex vivo*, allowing determining the capacity of these cells to buffer the exogenous challenge. Hence it is possible to determine the basal situation and the capability of response to acute and severe challenge.

The final level for assessing the effect of an antioxidant therapy on oxidative stress is to measure directly the pathological effects and their consequences. With SOD-gliadin, several abnormal events, in both animal and human works, have been shown to respond to the oral supplement. Such effect was demonstrated with the consequence of exposure to hyperbaric

oxygen, where the damaged DNA was significantly less after SOD-gliadin as compared to controls, validating then the whole concept from oral vegetal extract to cellular antioxidant defense. A significant effect has also shown in several animal models (mices, rats, pigs) with an effect on tumor development, insulin sensibility and oxidative stress related to sepsis. Finally, it was recently shown in a prospective study that this supplement was also responsible for an improvement of lipid profile and atheromatous lesions after long-term supplementation.

As conclusion, it is important to underline the fact that a simple appreciation of oxidative stress is not pertinent since a similar result on several parameters might be due to different if not opposed situation. The best criteria, and the most difficult to evidenced, is an effect in vivo on a function clearly related to oxidative stress.

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Figure 1

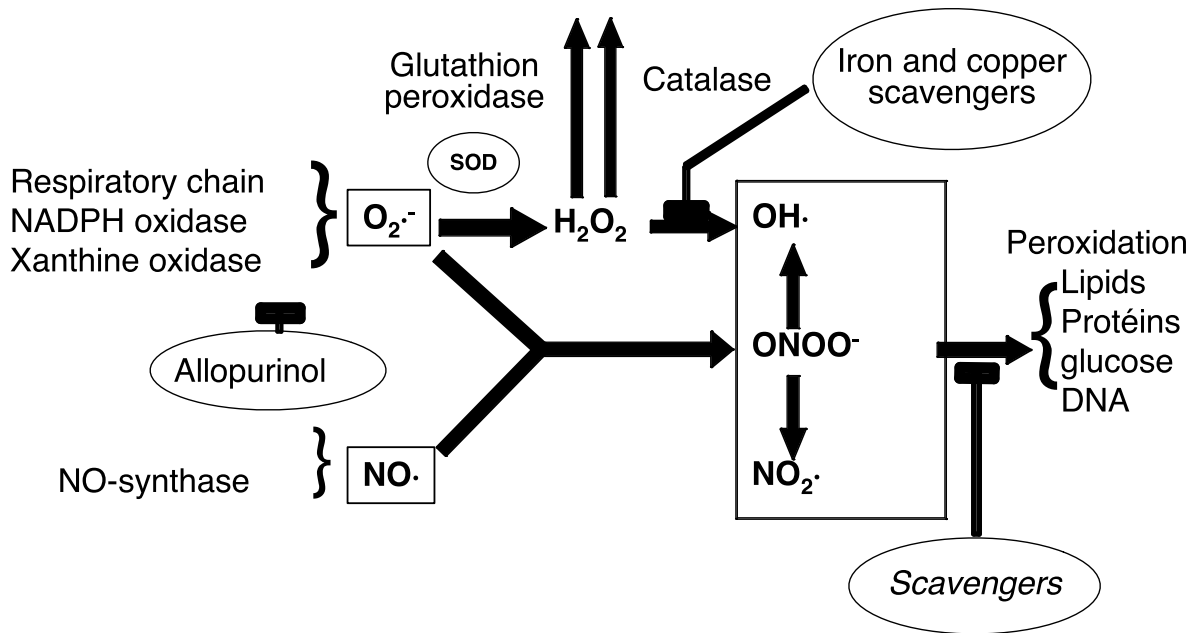


Figure 2

Hydrogen Peroxide and ROS scavenging pathway.

