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The point about oxidative stress in molluscs

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Abstract

In the normal metabolism of the aerobic cell, oxygen is used for various biochemical reactions. Because of its two lone electrons of parallel spins, the molecular oxygen is stable. However, oxygen generates Reactive Oxygenated Species or ROS by successive transfer of electrons. The ROS have a strong reactivity and can potentially interact with all other cellular components (lipids, proteins, DNA). They are at the origin of oxidations in chain by creating radicals. The cell has antioxidant systems which limit the effects of the ROS. These systems are composed of enzymes such as glutathione reductase, glutathione peroxidase, etc., and molecules of nonenzymatic nature like the reduced glutathione or vitamins. The production and the destruction of the radicals of oxygen coexist in a weak balance. If this balance is broken in favour of the ROS, an oxidative stress is generated. Xenobiotics could influence this balance by catalysing production of ROS.

Key words: oxidative stress; molluscs; ROS; antioxidant; xenobiotics

List of abbreviations:

CAT: catalase,

DT-d: DT-diaphorase,

GPx: glutathione peroxydase,

GRd: glutathione reductase,

GSH: reduced glutathione,

GST: glutathione S-transferase

MDA: malondialdehyde

PAH: polyaromatic hydrocarbons

H₂O₂: hydrogen peroxide,

MDA: lipoperoxydation,

PCB: polychlorinated biphenyls

PCO: peroxidation of proteins

SeGPx: glutathione peroxydase seleno-dependent,

SOD: superoxide dismutase,

Introduction

Face to chemical stress, each type of organisms, and for any species has a capacity of adaptation, based on regulating processes. These processes make it possible to maintain physiological homeostasis and the integrity of the individual, structural or functional deteriorations remainder entirely reversible or reparable. Passed the threshold of toxicity, the more marked irreversible attacks lead to a pathological state which results in a significant deterioration of the individual performances and later lead to death of the organism. Now, many xenobiotics are recognized like exerting their harmfulness by catalysing production of oxygenated radicals (Winston and Di Giulio, 1991). So the impact study of the toxic effects of the contaminants rejected into the environment requires a preliminary knowledge of the normal physiological mechanisms of adaptation (ecophysiology) and the comprehension of deteriorations of these processes induced by the contaminants (ecotoxicology).

Oxygen holds a capital place in the diversification of the species and their occupation of a majority of ecosystems. Being at the base many biochemical processes of the metabolism of the aerobic organisms, oxygen are an essential molecule. However, its oxidizing capacities make of it a potentially aggressive element for the majority of the bio-molecules. In order to limit its harmful effect, the antioxidant mechanisms were set up which make it possible the organism to maintain the rate of radicals on a low basal level. In the event of oxidative stress, the antioxidant systems can be exceeded, then causing the oxidation of different molecules and leading to cellular dysfunctions.

In a context of a multiple contamination in particular in water ecosystem, the study of oxidative stress is very used in biomonitoring. The molluscs are especially used in this type of study on account of their characteristics. We will try to resume the data about oxidative stress in these organisms.

Nature and origin of the Reactive Oxygenated Species (ROS)

The free radicals are atoms or molecules unstable presenting one or more lone electrons. To reach a better level of stability, they will yield or tear off electrons from molecules met. ROS create new radical species thus, causing oxidations in chain. All the bio-molecules of the cell (nucleic acids, lipids, proteins, polysaccharides) are potential substrates of ROS.

A significant criterion in the characterization of the radicals is their diffusion capacity which reflects the level of stability of the ROS. A little reactive form tends to act far from its site of production and thus has a significant diffusion. On the contrary, a very reactive species acts very quickly and its diffusion is so limited.

Molecular oxygen O_2 can be regarded as a radical species since it has two lone electrons; however, this molecule has a significant stability, the simultaneous addition of two electrons being difficult. In order to carry out this reaction, enzymes will create intermediates which constitute the ROS in particular during respiration and photosynthesis.

The superoxide anion radical $O_2^{\cdot -}$ is produced during endergonic reduction of molecular oxygen by capture of an electron. This reaction can be spontaneous in aerobic medium. $O_2^{\cdot -}$ is then generated primarily in membranes because of the high solubility of oxygen in hydrophobic medium (Gutteridge and Halliwell, 1993). However, it is produced during various reactions. Metals of transition such iron and enzymes are implied in its formation. The flavoenzymes and the xanthine oxidase activated by ischemia produce it (Fukai *et al.*, 2002). In addition, the phagocytic cells generate some for the degradation of the immune complexes by the means of four enzymes: NADPH oxidase, superoxide dismutase, nitric oxide synthase and myeloperoxidase (Babior, 1978a, 1978b). The superoxide anion radical is characterized by a low reactivity. Moreover, it does not have the capacity to pass the membranes; it remains limited to the compartment where it was produced. But it is at the origin of the oxidation of lipids. In fact, the deterioration of the membrane structures is carried out by nucleophilic attack between fatty acids and glycerol of phospholipids. $O_2^{\cdot -}$ can act at the same time like an oxidant and a reducer. In the presence of some metals (manganese or vanadium), it catalyses reactions of oxidation in chain for example oxidation of many molecules of NAD(P)H (Liochev and Fridovich, 1989). On the contrary, within the framework of metals of transition (iron or copper) present at the active sites of enzymes, it presents a reducing behaviour (Liochev and Fridovich, 1989).

Hydroxyl radical $OH\cdot$ can be produced during the thermal reactions or under the effect of ionizing radiations. It can also be generated during a reaction implying iron and which is translated by the Fenton's reaction. The hydroxyl radical can be also produced by homolytic fission of the H_2O_2 . This reaction of Haber-Weiss is catalysed by metals of transition. The first stage is the reduction of the superoxide anion radical

and the second corresponds to the reaction of Fenton. The Haber-Weiss reaction can be inhibited by chelating of metals, in particular the desferrioxamine in the case of iron. The hydroxyl radical is very reactive and thus its diffusion is limited. It will interfere with the first molecules met, generally on the level of the site of production. It acts, either by addition or by wrenching of hydrogen from the target molecule, or by transfer of electrons. It is at the origin of the lipid peroxidation.

The H_2O_2 is produced by the dismutation of the superoxide anion radical. This anion leads spontaneously to H_2O_2 under the conditions of physiological pH. This reaction can be accelerated by action of superoxide dismutases (Fridovich, 1975). The H_2O_2 is moderately reactive but its diffusion is high, having the capacity to cross the membranes. Its intracellular concentration is very weak between 0.001 and 0.1 μM (Sies, 1991). However, in mitochondria and peroxysomes, these concentrations can reach higher levels (Boveries *et al.*, 1972). The H_2O_2 holds a significant place among the ROS because it plays the role of intermediate in the production of other reactive radicals. By the means of metals of transition (copper, iron), it gives rise to the hydroxyl radical. Moreover, the H_2O_2 is an intracellular signalling factor (Sundaresan *et al.*, 1995).

The nitric oxide $NO\cdot$ is not always regarded as a ROS. Indeed, it plays at the same time a role in the destruction and the production of radicals. Moreover, it is not very reactive with the cellular components and reacts with radicals generating of less reactive species. It is thus able to inhibit lipid peroxidation (Rubbo *et al.*, 2000). Conversely, the nitric oxide combined with the superoxide anion radical involves the formation of peroxynitrite, highly toxic radical (Beckman and Koppenol, 1996). The NO is produced from endogenous or exogenous NO donors or thanks to a reaction of oxidation of the arginine in the presence of oxygen and NADPH. This reaction is produced in particular on the level of the phagocytic cells (Marletta, 1994). The nitric oxide and the radicals which result from this are gathered under the term of Reactive Nitrogenized Species RNS.

The hydroperoxyl ($ROO\cdot$) and alcoxyl ($RO\cdot$) radicals rise from the peroxidation of the lipids. These radicals allow the propagation gradually of the lipid peroxidation. After degradation, they lead to aldehyde formation organized in three major groups: 2-acetaldehydes (e.g. 2-hexenal), 4-hydroxy-2-acetaldehydes (e.g. 4-hydroxynonenal) and ketoaldehydes (e.g. malondialdehyde) (Uchida, 2003).

Origin and role of ROS

The major role of the endogenous production of ROS is an activity of regulation. Indeed, the radicals can interact directly with the molecules containing sulfhydryl groups and thus change their conformation. This type of regulation can in particular affect molecules implied in the mechanisms of signal transduction like protein kinase C (Dalton *et al.*, 1999). They allow the regulation of many other molecules (Babior *et al.*, 1997). Studies showed that the H₂O₂ can replace insulin in its role of growth promoter (Allen and Tresini, 2000). Thereafter, other experiments showed the stimulation of the production of H₂O₂ by insulin and nerve growth factor (Mukherjee *et al.*, 1978; Mukherjee and Mukherjee, 1982). The nitric oxide plays itself a role in the vasodilatation and the neurotransmission by activation of enzymes (Allen and Tresini, 2000). The radicals also play the roles of control of various factors of transcription (Sen and Packer, 1996).

A significant source of free oxygenated radicals comes from the redox cycles and of the oxidation catalysed by cytochrome P450 monooxygenases. These enzymes allow the addition of a functional group to the exogenous compounds. The redox cycles pass by reactions of oxidation, reduction and hydrolysis, each mediated by transfers of electrons. At the time of each reaction, ROS are formed.

Many exogenous compounds can stimulate the production of ROS (Tables 1-3). Several modes of action were described.

Many xenobiotics catalyse the microsomal transfer coming from the NAD(P)H towards oxygen of electrons and then involving ROS formation. It is the case of the nitroaromatic compounds (e.g. nitrofurantoin), quinones (e.g. menadione), and derived from the bipyridium (e.g. paraquat). Various studies showed a production NAD(P)H-dependent of ROS stimulated by contaminants (Lemaire *et al.*, 1994; Peters *et al.*, 1996; Lemaire and Livingstone, 1997; Livingstone *et al.*, 2000).

A number of compounds will involve the formation of active species of oxygen after metabolism during which they become themselves of the radicals as quinones. In this case, xenobiotic is first reduced by a NADPH-dependent reductase during the reaction on phase I producing a radical. This last can then transfer an electron to oxygen. A superoxide anion radical is generated. In each cycle, two potentially harmful compounds can thus be produced (Winston and Di Giulio, 1991; Goepfert *et al.*, 1995).

A deficiency in metal can also lead to an oxidative stress. Indeed, several metals are integrated into proteins; it is the case of the copper in Cu/Zn-SOD (L'Abbe and Fischer, 1984; Taylor *et al.*, 1988). In the rat, a feeding without coppers leads to a rise of the quantity of oxidized proteins and to a fall of the activity of Cu/Zn-SOD in erythrocytes (Sukalski *et al.*, 1997). However, in excess, copper can increase the rate of malondialdehyde, marker of the lipid peroxidation, and induce a reduction in the rate of glutathione. Indeed, copper is suitable for catalyse the production of hydroxyl radicals via the reaction of Haber-Weiss (Kadiiska *et al.*, 1993; Bremmer, 1998).

The ionizing and ultraviolet rays are also of significant sources of radicals of oxygen by break of the molecules.

Origin of ROS in molluscs

In ecotoxicology, many compounds such as PAH, PCB and metals were implicated in the induction of the production of radicals at the laboratory like *in situ* (Tables 1-3). However, it has a lack of data about the exact mechanism of action of these compounds. The measurement of the oxidative stress is generally carried out by the follow-up of the modifications of the activity levels of enzymes (CAT, SOD, etc.) and of the rates of the molecules (GSH, etc.) implied in the antioxidant defence in two main tissues, digestive gland and gills. These studies involved a hierarchical organization of the cellular answers and more specifically of the antioxidant enzymes. Thus, CAT is regarded as an enzyme presenting a clear and early response to contamination (Wenning *et al.*, 1988). The induction of the GPx is generally noted in a concomitant way to that of the CAT and sometimes to that of SOD (Rodriguez-Ariza *et al.*, 1993). In molluscs, as in the mussel *Mytilus galloprovincialis*, SOD seems to be a stable enzyme, seldom presenting variations of activity (Livingstone *et al.*, 1995). The various studies can however show contradictory results. Thus, the SOD is sometimes described like presenting a modification of activity in a concomitant way at the CAT as in *Geukensia demissa* after 12 h of exposure to the paraquat (Wenning *et al.*, 1988). Géret *et al.* (2002) describe a reduction in the levels of seleno-dependent and total GPx activities in *Ruditapes decussatus* exposed to copper (0.5; 2.5 and 25 $\mu\text{g.L}^{-1}$) after one day of exposure. At the reverse, the carp *Cyprinus carpio morpha* presents an increase in the GPx activity after 1 day of exposure to copper (5, 10, 25 and 50 $\mu\text{g.L}^{-1}$) (Radi and Matkovics, 1988).

Other molecules implied in antioxidant defences are measured, most current being GSH. The reduction in the rate of reduced GSH was observed at bivalves *M. galloprovincialis* and *Unio tumidus* in correlation with the presence of PAH and PCB in the medium (Regoli and Principato, 1995; Doyotte *et al.*, 1997; Cossu *et al.*, 1997, 2000). The modification of the rate of reduced GSH as well as balance between the rates of reduced and oxidized glutathione (GSH/GSSG) can be correlated with the variation of GRd activity. Patel *et al.* (1990) observed a reduction in the GRd activity and an increase in the rate of oxidized glutathione during the exposure of bivalves (*Anadara granosa*) to mercury. In this context, the GRd activity also constitutes an interesting enzyme in the study of the oxidative stress. The reduction in the rate of reduced GSH was connected to the induction of the lipid peroxidation in particular in the mussel exposed to metal contaminants (Viarengo *et al.*, 1988). Nevertheless, the lipid peroxidation is generally correlated with the reduction in the whole of the antioxidative enzymes (Doyotte *et al.*, 1997; Cossu *et al.*, 1997, 2000; Géret *et al.*, 2002). The environmental parameters are also suitable for induce a variation of pro-oxidant/antioxidant balance. Many studies showed seasonal variations of the antioxidant activities at marine species (Viarengo *et al.*, 1991; Orbea *et al.*, 2002). These variations are due to the fluctuations of temperature, salinity, the oxygen rate and the quantity of food available (Viarengo *et al.*, 1991). Changes of seasonal nature were also studied in order to understand their implication in the answers of the antioxidant systems to the contaminants. This kind of studies allows establishing the link between ecophysiological parameters and ecotoxicological reactions (Niyogi *et al.*, 2001a, 2001b).

Effects of ROS and diseases

ROS can act on the whole of the cellular components. The variations of level of these radicals thus have significant effects on cellular functions. The ROS influence in particular the thiol groups of proteins, leading to the formation of intra- or inter-molecular disulphide bridges.

The most studied action of ROS is the lipid peroxidation. This reaction is mainly carried out by the hydroxyl radical (Stegeman *et al.*, 1992a; Steinberg, 1997). This process corresponds to reactions in chain. After rearrangement and addition of oxygen, peroxy (ROO·) and alkoxy (RO·) radicals are generated. Oxidation is propagated thereafter with other unsaturated lipids and can even reach proteins. The

oxidation of phospholipids membranes involves disturbances of these structures. As a first consequence, we can observe a reduction in fluidity of the membranes and the inactivation of the receptors and enzymes located at their level (Snell and Mullock, 1987). In a second time, this oxidation and particularly oxidized products increase the permeability of the membranes, in particular with the calcium ions leading to cellular death (Gutteridge and Halliwell, 1990). On the level of the mitochondria like lysosomes, the lipid peroxidation results in the lysis of these organelles and the release of enzymes. These enzymes then catalyse the decomposition of proteins, nucleic acids and cellular polysaccharides (Horton and Fairhurst, 1987; Snell and Mullock, 1987; Pre, 1991). As example, the oxidation of the polyunsaturated lipids can induce the appearance of cardiovascular diseases (Wattanapitayakul and Bauer, 2001).

In a general way, during this reaction, various compounds are produced such malondialdehyde (MDA) and 4-hydroxynonenal (HNE), both able to bind to proteins and to form adducts. Indeed, these compounds react in a spontaneous way with cysteines of proteins and with glutathione. The 4-hydroxynonenal can inhibit the synthesis of the nucleic acids and proteins, and block the cellular proliferation (Benedetti *et al.*, 1982, 1986; Esterbauer and Cheeseman, 1990; Esterbauer, 1993).

Another action of the ROS relates to proteins. The oxidation of proteins derives from direct action of ROS or indirect interaction with the alcoxyl (RO·) or peroxy (ROO·) radicals generated at the time of the lipid peroxidation. The amino acids most sensitive are those including sulfhydryl groups such methionine and tryptophan. This oxidation can involve of: (i) change of protein conformation by modification of some amino acids; (ii) generate bridges between proteins and proteins and lipids; (iii) cuts (Levine *et al.*, 1994). The structural modifications induce functional changes in particular cellular metabolism (Shacter *et al.*, 1994). Indeed, the oxidation of proteins can result in a disturbance of ionic transport, enzymatic activities and calcium homeostasis. The damaged proteins are then more sensitive to the proteases action, and thus destroyed more quickly, this being able to induce tissue degradation (Rice-Evans *et al.*, 1991).

The nucleic acids are also targets for the free oxygenated radicals. The damage is not specific: simple or double cuts, formation of abasic sites, covalent bonds between DNA or DNA and proteins, and modifications of the bases, the most reached being the deoxyguanosine oxidized in 8-hydroxy-2'-deoxyguanosine (8-

OHdG) (Meneghini, 1988; Dizdaroglu, 1991; Spencer *et al.*, 1996). The DNA damages are mainly caused by the hydroxyl radical (OH·). Superoxide anion radical can cause also cuts of DNA and lesions of the bases. If guanine is the majority target, each base can undergo these attacks (Halliwell and Dizdaroglu, 1992).

Finally, the glucid oxidations in presence of metals involve protein cuts. This reaction is initiated by the hydroxyl radical, which tears off a hydrogen atom to the one of carbons close to the glycoprotein. Other radicals are produced such as the peroxy radical.

The cytotoxicity of the radicals of oxygen takes part in the development of much pathology. The oxidative stress is thus implied in the disease of Alzheimer (Bowling and Beal, 1995; Ihara *et al.*, 1997). The damage caused by the radicals of oxygen among parkinsonian patients was shown and would be related to a deficiency of the system of defence in brain, in particular in SODs activity (Radunovic *et al.*, 1997).

Conversely, an overproduction of radicals of oxygen is implicated in the development of some diseases. At the time of the respiratory syndrome of distress, an infiltration of fluid is observed in the air cells resulting from damage of the endothelium of the capillaries. At the people reached of this syndrome, the lungs contain a significant number of neutrophils (Weiland *et al.*, 1986). The production of ROS is also implied in rheumatoid arthritis. The therapies against this pathology include antioxidant components (Reglinski *et al.*, 1997). Balance between pro-oxidants and antioxidants systems would be also implied in the phenomenon of cellular ageing (Sagar *et al.*, 1992). The oxidative stress would increase during cellular differentiation and ageing (Sohal *et al.*, 1990). However, the implication of the radicals in cellular ageing is not cleared up. Indeed, the studies do not show all the same variations according to the age (e.g. Mizuno and Ohta, 1986; Sohal *et al.*, 1990; Hussain *et al.*, 1995; Sahoo and Chainy, 1997; Kim *et al.*, 2002).

Effects in molluscs

At the marine molluscs, the physiological and morphological modifications in response to the chemical stress are not much studied. Indeed, the appearance of pathologies is generally synonymous with irreversible damage. The major observations related to hepatic pathologies such as the increase in the occurrence of parasitic infections, ignition and necrosis in the fish (Vethaak, 1992). However, in molluscs, the majority of the studies evaluate the impact of pollution by the

appearance of neoplasia (Malins *et al.*, 1988; Kinae *et al.*, 1990). In molluscs, the observation of pathologies does not correspond to a major axis of ecotoxicological studies. Nevertheless, *in situ* works bring back the observation of a blood neoplasm, haematopoietic neoplasm, disseminated neoplasia, hemic neoplasia, leukaemia or proliferate cellular disorder (Krishnakumar *et al.*, 1999). This syndrome was observed overall on 15 species of bivalves including 4 of oysters, 6 of clams and 5 species of mussels (re-examined of Elston *et al.*, 1992). This disease is characterized by the proliferation of circulating haemocytes. They present a significant core of lobed form which compared fills the major part of the cell to the cytoplasm. Moreover, one or more micronuclei are observed as well as a high frequency of mitoses (Farley, 1969; Mix, 1983). The origins of this neoplasia remain discussed. Indeed, some authors advance a potential implication of carcinogenic compounds (Lowe and Moore, 1978; Farley *et al.*, 1991). Other studies connect on the contrary, the appearance of this pathology to a retrovirus or to a genetic disposal of the individuals (Couch and Harshbarger, 1985; Elston *et al.*, 1988). Nevertheless, Lowe and Moore (1978) showed the appearance of neoplasms in the mussel *Mytilus edulis* subjected to a pollution of domestic and industrial nature including hydrocarbons. Other authors blame these same xenobiotics in the appearance of these tumours in different bivalves, in particular of oysters, subjected to the pollution generated by the shipwreck of Amoco Cadiz in France (Balouet *et al.*, 1986; Barry and Yevich, 1975; Yevich and Parszcz, 1977). Conversely, Mix and Schaffer (1983) do not note any incidence of PAH on the frequency of appearance of neoplasia in *M. edulis*. In addition, Farley *et al.* (1991) showed a linear correlation between the appearance of neoplasms and the tissue concentration in chlordane. The exposures in laboratory lead in the same way to contradictory results, with either inductions of tumours or no incidence following treatments with the PAH or other xenobiotics (Khudoley and Syrenko, 1978; Rasmussen *et al.*, 1983a, b, 1985; Winstead and Couch, 1988; Krishnakumar *et al.*, 1999). However, the implication of the oxidative stress is however not proven in a sure way (Elston *et al.*, 1988; Moore *et al.*, 1991; Krishnakumar *et al.*, 1994, 1999). Moreover, in *Mytilus edulis* and *Mya arenaria*, an increase in the frequency of appearance of the neoplasms is observed during the coldest months of the year. These observations can be related to the reduction in the activities of the antioxidant enzymes at low temperatures (Elston *et al.*, 1992).

Defences of the organisms

The production and the action of the ROS must be controlled in order to limit the cellular damage. This limitation is carried out initially by sequestration, even the destruction, of the systems pro-oxidants such as the complexation of free metals by metallothioneines. The antioxidant systems also include enzymes whose activity involves the destruction of the reactive oxygenated species. These enzymes can act by the means of metals of transition. A last means of fight against the oxidative stress is the stop of oxidations in chain of the cellular components. The antioxidant capacities are variable from one species to another. Moreover, it is allowed that these activities vary according to the seasons. Lastly, another adaptation of the organisms to the increase in the production of ROS is the induction of the synthesis of antioxidant molecules.

Two categories of antioxidant systems are generally defined: antioxidant enzymes and molecules without enzymatic activity.

Three major enzymes act jointly for the destruction of the ROS in the cell: SODs, CAT and GPxs. The GRd can be added to these enzymes even if it does not present a direct role in the catabolism of the oxygenated radicals.

SODs (SOD; EC. 1.15.1.1) will allow thereafter the destruction of the superoxide anion radical by dismutation out of H_2O_2 dealt by CAT. The two enzymes, SOD and CAT, have the same principal localization in the cell, the peroxysomes. Isoenzymes of the SOD are found in the various compartments of the cell, but their active site has a tertiary structure overall good preserved, made of a hydrophobic well where the superoxide anion radical fits. The reaction of dismutation is catalysed by a metal from which nature makes it possible to distinguish three types of isoenzymes. During the reaction, the metal ion captures an electron of the superoxide anion radical. SODs seem to be very significant enzymes because of their ubiquity and of their localization at the same time intra- and extra-cellular (Stegeman *et al.*, 1992b). Cu/Zn-SOD (35 kDa) was identified for the first time in 1968, in bovines erythrocytes by McCord and Fridovitch (McCord and Fridovich, 1969). In addition to its localization in the cytoplasm, its presence was also shown on the external face of the endothelial cells and in the blood plasma. Later on, it was also detected in the peroxysomes, the lysosomes and the core of the eukaryotes cells (Beyer *et al.*, 1991). This isoenzyme is made up of two identical sub-units from approximately 15 000 Da each one, to which two metal atoms are added: copper and zinc. The function of destruction of the

superoxide anion is provided by copper whereas zinc would have only one structural role. Cu/Zn-SOD was described at the vertebrate ones, the aquatic and terrestrial invertebrates, like at the plants on the chloroplastic and cytosolic level. More recently, an extra-cellular form noted EC-SOD, was characterized at the vertebrate ones, then at the invertebrates, and more precisely the nematode *Caenorhabditis elegans* (Hjalmarsson *et al.*, 1987; Wilson *et al.*, 1994; Folz *et al.*, 1997). Indeed, this extracellular copper/zinc-SOD was detected in the fluids circulating like plasma, the lymph and the synovial liquid (Marklund, 1982; Fridovich, 1995). However, it would be mainly related to the proteoglycans of the cellular membrane and only less than 1% would be present in circulating form (Karlsson and Marklund, 1987; Karlsson *et al.*, 1988; Adachi *et al.*, 1995).

The mitochondrial matrix contains Mn-SOD in eukaryotes and bacteria and Fe-SOD in plants and bacteria. These two isoenzymes are also present in lysosomes, peroxisomes and nuclear compartment. Fe-SOD is localised in the chloroplasts of plants and constitutes for those the most significant form. These two shapes of SODs present analogies of structure; however, it is allowed that the Mn-SOD is inducible by the superoxide anion radical, whereas it would not be the case of Fe-SOD. Various substances are able to inhibit the SOD with for some, specificity with respect to an isoenzyme. Thus the Cu/Zn form is inhibited by cyanide (Weisiger and Fridovich, 1973) and Mn-SOD by a treatment to sodium dodecyl sulfate. The H₂O₂ inactivates Fe-SOD (Hodgson and Fridovich, 1975). However, Yim *et al.* (1990) bring back an inhibition at the same time of Cu/Zn-SOD and Fe-SOD by H₂O₂. All these enzymes are also inhibited by elimination of their metal of transition, by chelators. However, this inhibition is reversible. Lastly, the activity of Mn-SOD is inhibited with pH 9 whereas that of Cu/Zn-SOD is not influenced by the pH. Deficiency in SODs or their inhibition increases the sensitivity of the organisms to oxidants. In this general context, it was shown in particular that the mitochondrial Mn-SOD is essential for the life. Indeed, Mn-SOD deficiency was implicated in the appearance of serious pathologies, the production of superoxide anion becoming very significant. Thus, the knockout mice for Mn-SOD die after the birth or suffer from neuro-degenerative diseases (Melov *et al.*, 1998). The form of Mn-SOD, contrary to that of Cu/Zn-SOD, would be controlled by the superoxide anion and in a general way by the radicals of oxygen (Liu *et al.*, 2000). In the bacteria, this induction brings into play a locus *soxR* which controls the transcription of nine genes implied in the synthesis of enzymes for

the production of NADPH, the repair of the DNA, the protein synthesis and the membrane permeability (Harris, 1992). The form of Mn-SOD is thus inducible by cytokines in various cellular types and by ionizing radiations (Masuda *et al.*, 1988). Studies showed that the tumoral necrosis factor TNF- α could induce the expression of the manganese-SOD (Wong *et al.*, 1989, 1995). In the same way, Otieno *et al.* (2000) showed the transcriptional regulation of Mn-SOD by the chemoprotective 3H-1,2-dithiol-3-thiol. Conversely, the rates of mRNA of Cu/Zn-SOD do not vary following this treatment. In fact, Cu/Zn-SOD cytosolic appears less significant in the limitation of the oxidative stress. Indeed, the transgenic animals not expressing this enzyme present a normal phenotype (Ohlemiller *et al.*, 1999).

CAT (CAT; EC. 1.11.1.6) is present primarily at peroxisomal level. This inducible enzyme allows the destruction of H₂O₂ out of water and oxygen. It is a hemoprotein including an iron atom per unit, the number of units varying according to species. It catalyses a two stages reaction corresponding to a catalasic activity. However, the CAT can also present a peroxidasic activity (Leguille-Cossu, 1996). The CAT has other functions during the normal function of the cell. Thus, this enzyme catalyses the detoxication of substrates such alcohols and phenols in connection with the reaction of reduction of hydrogen peroxide (Akyilmaz and Dinckaya, 2003). However, generally, this enzyme is regarded as being able to catalyse only the destruction of hydrogen peroxide (Stegeman *et al.*, 1992b). It was shown in the rat, the possibility of a transcriptional induction of CAT under treatment by a chemoprotective, 3H-1,2-dithiole-3-thione (Otieno *et al.*, 2000). Its localization makes it possible this enzyme to carry on an activity complementary to the GPx. In the bacteria, its activity is induced by H₂O₂ on the level of the locus oxyR (Harris, 1992).

One second way of destruction of H₂O₂ utilizes the GPxs (GPx; EC.1.11.1.9). The enzymatic activity is coupled with the oxidation of the GSH and generates alcohols. The GPxs are also able to reduce other peroxides. These enzymes are localised in the cytoplasm and the mitochondrial matrix of the cells. They include two categories: the SeGPx and a Se-independent form, which corresponds in fact to a glutathione S-transferase with a peroxidasic activity. This last form is a dimer of 50 kDa localised mainly in the microsomes and catalyses only the reduction of organic peroxides. The SeGPx is a tetrameric metalloenzyme (80 kDa) of which each sub-unit comprises a selenium atom in the form of a selenocysteine residue and incorporated in the active site thanks to a selenocysteine-specific RNA (Spallholz and

Boylan, 1991). A selenium deficiency thus will involve an inhibition of this enzyme. There are other inhibitors of GPx whose metals by a thiolooprive action (cadmium and lead) and in a general way the detergents (triton, etc.). The catalytic mechanism proposed for the reduction of peroxides by GPx passes by the oxidation of the active site in selenic acid (SeOH). The SeOH is transformed by adjunction of a molecule of reduced GSH. The addition of one second molecule of GSH regenerates the active site of the enzyme and the oxidized glutathione (GSSG). Moreover, Ursini *et al.* (1982) described another form of Se-dependent GPx in the liver on pig, the phospholipid hydroperoxide peroxidase (PLGPx; EC. 1.11.1.12). This enzyme is a monomer (22 kDa) and is implied in the protection of the liposomes and membranes against the oxidative damage. Contrary to the preceding enzyme, its selenium requirement is less strict (Spallholz and Boylan, 1991).

The GRd (GRd; EC. 1.8.1.7) is not always recognized as an antioxidant enzyme. It can nevertheless be included in this category because it makes it possible to reduce the oxidized glutathione (GSSG) according to a NADPH-dependent process, and it is thus at the base of the regeneration of reduced GSH necessary to the operation of the GPxs and of many other enzymes of the cell. Balance between GSSG and GSH is capital in the maintenance of cellular homeostasis (Winston and Di Giulio, 1991).

In the cell, all these enzymes will intervene in concert, each one according to a specific cellular under-localization, in order to control the quantity of free radicals.

Other enzymes are regarded as having an antioxidant action. It is the case of DT-d and the glutathione S-transferase. Indeed, the glutathione S-transferase presents a peroxidasic activity with respect to organic peroxides and belongs to the group of the Se-independent GPx. DT-d, also indicated as NAD(P)H oxidoreductase 1 (NQO1, EC. 1.6.99.2) catalyses the reduction of quinones by addition of two electrons, thus generating hydroquinones which are more easily excreted after conjugation with sulfates groups or glucuronide (Cadenas, 1995). This enzyme thus makes it possible to produce a quinoline stable form without passage by radicals intermediates. In this direction, DT-d can be regarded as an antioxidant enzyme. However, hydroquinones are also able to generate radical species of oxygen, or to react directly with the DNA in reactions of alkylation (Cadenas, 1995). In this last case, DT-d constitutes an enzyme of bioactivation. More recently, a new family of antioxidant enzymes, the peroxiredoxines, was described in some procaryotic organisms and mammals (Chae

et al., 1994). These proteins have homologies of sequence with the thioredoxine peroxidase of yeast and they have a peroxidasic function. Six groups were defined according to their sequences and their immunological properties (Kang *et al.*, 1998; Chae *et al.*, 1999; Seo *et al.*, 2000). In human, they are expressed in the brain, each of the six groups presenting of the particular localizations (Kang *et al.*, 1998; Chae *et al.*, 1999; Seo *et al.*, 2000). Their differential expression was connected to neurodegenerative disorders such as the disease of Alzheimer (Krapfenbauer *et al.*, 2003).

Antioxidants of nonenzymatic nature exist too. An antioxidant capacity is conferred on the GSH (L- γ -glutamyl-L-cysteinyl glycin) by the presence of the thiol group carried by the cysteinic residue. The GSH is a tripeptide of glutamate (L-Glu), cysteine (L-Cys) and glycine (Gly), the glutamate and cysteine being connected by γ -peptide connection. It is synthesized by the consecutive action of two enzymes, γ -glutamylcysteine synthetase and the GSH synthetase. In the cells, the GSH is present mainly in its reduced form GSH and represents the most significant thiol in eukaryotes cells (0,2 to 10 mM). An increase in the proportion of oxidized form (GSSG) translates an oxidative stress. The GSH exerts many functions in the cell. It intervenes in the processes of reduction such as the synthesis and the degradation of proteins, the formation of deoxyribonucleotides, the regulation of the enzymes and the protection of the cells against the free radicals of oxygen. The GSH also plays the role of co-enzyme for various reactions and it is combined with compounds either endogenous (oestrogens, prostaglandins and leucotrienes) or exogenous (drugs and xenobiotics), thus taking part in their metabolism. The GSH thus indirectly supports the detoxication of the radical compounds by its function of co-substrate of the antioxidant enzymes such as the GPxs. Moreover, it is the co-substrate of a significant enzyme in the process of detoxication: the GST. The GSH is thus regarded as a central element of antioxidant defences. Indeed, a depletion in GSH induces an increase in the sensitivity of the organisms to xenobiotics or overall, with generating processes of radicals (Jones *et al.*, 1995; Conners, 1998).

Other compounds known as low-weight molecular have an antioxidant role. Thus, the lipoic acid in reduced form can reduce the GSH and the peroxy radicals. It also has a chelating capacity of metals, quenching of free radicals (Kagan *et al.*, 1992) and of regeneration of others antioxidants like the ascorbic acid and the vitamin E (Packer *et al.*, 1995). Other protective elements are brought by the food: vitamin E,

vitamin C and pigments such carotenoids. These antioxidant systems make it possible to stop the chain reactions, in particular those of the lipid peroxidation. Indeed, these substances are localised on the level of the membranes and destroy the free radicals by collecting the lone electron. Other natural compounds also have an antioxidant character: urea, thiourea, mannitol and dimethyl sulfoxide. In substitution for iron, zinc exerts also an antioxidant action. Metals constitute however a particular case because they can at the same time generate radicals and destroy them.

Antioxidants in molluscs

The antioxidant systems known in the mammals are found in the marine organisms. All in all, the antioxidant activities are lower at the aquatic species compared to those of the mammals. In particular, the Mn-SOD is little expressed in tissues of *M. edulis*, Cu/Zn-SOD being the main form (Livingstone et al., 1992; Manduzio et al., 2003). Furthermore, the expression of the Cu/Zn-SOD is modulated by xenobiotics. Manduzio et al. (2003) described the induction of expression of a Cu/Zn-SOD isoform in mussel *M. edulis* exposed to contaminants in field and laboratory. However, the bivalve molluscs have levels of activity in digestive gland of the same order as those measured in the liver of fish. At these organisms, the antioxidant activities however are influenced by various factors: (i) an anaerobic respiration gives rise to a reduction in the enzymatic activities and lipid peroxidation, levels returning to normal when oxygenation is restored (Viarengo et al., 1989); (ii) the laying involves an increase in the antioxidant activities in March-April, followed by a progressive reduction at spring whereas the availability in food and the temperature increase (Solé et al., 1995); (iii) the age sensitizes with the oxidizing effects by reduction of the antioxidant capacities what results in an increase in the rates of lipid peroxidation (Viarengo et al., 1991). In the same way, the seasonal fall in antioxidant enzymes is concomitant to an increase in the rate of lipid peroxidation. However, this decrease could be compensating by an augmentation of GST activity in gills of mussels (Power and Sheehan, 1996; Sheehan and Power, 1999; Manduzio et al., 2004). This observation is all the more pronounced since the water is polluted as described in the harbour of Le Havre, which is characterized by a general contamination by various compounds such as PAHs, PCBs and heavy metals (Manduzio et al., 2004).

Conclusion

In spite of many studies about oxidative stress in molluscs, there still exists many questions. This could be explaining sometimes contradictory data. It would be interesting to study thoroughly physiological natural factors which could induce modification between pro-oxidant and antioxidant systems. In particular, among these factors, the phenomena of hypoxia/anoxia could have an important impact. Moreover, the disappearance of environment being able to be considered as free from pollution and so being able to constitute a reference is limiting. It is all the more significant to develop rapidly reliable tools of diagnostic of environmental safety that the levels of pollution increase and new xenobiotics are synthesized each year.

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