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Age-related lipid peroxidation in the digestive gland of mussels: The role of the antioxidant defence systems

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Summary. The main cellular defence systems against free radical-mediated oxidative stress are significantly reduced in the digestive gland of aged (> 10 years old) compared to younger (2–4 years old) mussels (*Mytilus edulis* L.). Moreover, the concentration of lipid peroxidation products (malondialdehyde) is increased in the same age group with respect to younger animals. The obtained data indicate that an impairment of the antioxidant defence systems would render the older animals more susceptible to peroxidative stress, thus supporting the general significance of the free radical theory of aging.
Key words. Aging; mussels; free radicals; antioxidant systems; lipid peroxidation.

Among the various concepts of biological aging[1], the free radical theory[2] has aroused particular interest because of its general significance and applicability. According to Harman, the structural and metabolic changes which occur in aging cells are mainly related to free radical reactions and, among these, to the peroxidation of membrane lipids.

Free radicals generally arise from: i) exposure of cells to ionizing radiation; ii) non-enzymatic reactions of oxygen with metal cations and organic compounds; iii) enzymatic reactions involved in the electron transport of the respiratory chain, in phagocytosis and in the activity of the cyt-P450 system. These reactions, in which oxygen is the main source of free radicals, seem to be responsible for the lipid peroxidation as well as for aging processes.

A related concept is that the intracellular redox state becomes more pro-oxidizing, and that aging organisms are more susceptible to oxidative stress. Indeed, recent studies indicate that cellular aging may be related to a decreased capacity to inactivate free radicals, due to a reduced activity of the antioxidant systems involved in protection against peroxidative damage[3–6].

Even though this subject has been studied in different mammalian tissues, information on marine invertebrates, including mussels, is still lacking. These intertidal, sessile, filter-feeding organisms are adapted to, and therefore tolerant of, variations of a wide range of environmental parameters (such as oxygen, temperature, food availability, etc.)[7]. In particular, during tidal exposure they are periodically subjected to either hypoxia or anoxia followed by aerobic recovery. Such conditions are thought to give rise to an enhanced free oxygen radical production in tissues, due to the simultaneously enhanced flux of reducing equivalents and oxygen[8,9]. Therefore, because of the characteristics of their metabolism, mussels seem particularly suited for studies on the role of free radicals in age-related lipid peroxidation. Reduced coenzyme-dependent oxyradical production has been observed in digestive gland microsomes and cytosol[10] of mussels, and it is stimulated by redox cycling quinone[11] and nitroaromatic compounds[12].

Data are presented here on the antioxidant systems and lipid peroxidation in the digestive gland of mussels (*Mytilus edulis* L.) of three different age groups (I age = 2–4 years; II age = 6–8 years; III age = > 10 years). The average life span of this species is about 12 years[7].

In this work, the concentration of both aqueous-phase (GSH) and lipid-soluble (vitamin E, carotenoids) antioxidant compounds, as well as the activity of antioxidant enzymes such as superoxide dismutase (SOD – EC 1.15.1.1.), catalase (EC 1.11.1.6.) and GSH peroxidase (EC 1.11.1.9.) were evaluated. As is already known, naturally-occurring antioxidants can act as reductants or as free radical scavengers, while antioxidant enzymes play a fundamental role in scavenging superoxide anion radicals (superoxide dismutase) and in the metabolism of H_2O_2 and lipid hydroperoxides (catalase, GSH peroxidase)[13–15]. Finally, the determination of the tissue levels of malondialdehyde (MDA) was utilized as an indicator of the lipid peroxidation process.

Methods

Animals and treatments: Mussels (*Mytilus edulis*) of three different age groups (I = 2–4 years; II = 6–8 years; III = > 10 years, corresponding to the size classes 2.9–4.2 cm, 5.3–6.3 cm, > 7 cm, respectively) were collected from the River Lynher near Plymouth (UK). The tissues (digestive gland) were dissected out and damp-dried to minimize differences in water content.

Reduced glutathione: The tissues were homogenized in 10 vol of 5% TCA (trichloroacetic acid) and then centrifuged at 30.000 × g for 30 min. The supernatants were diluted with 5 mM EDTA/0.1 M Na-phosphate buffer, pH 8. The GSH concentrations were evaluated utilizing the fluorometric assay described by Hissin and Hilf[16].

Lipid peroxidation: Lipid peroxidation was evaluated by determining the concentration of malondialdehyde (MDA) by HPLC on a Waters carbohydrate analysis column (n.84038), utilizing the method described by Esterbauer et al.[17] with slight modification[18].

Vitamin E: The vitamin E content was evaluated by HPLC on a Supelcosil PLC-SI (Supelco; n. 5-9180) column as described by Burton et al.[19].

Carotenoids: Carotenoids were extracted from the digestive gland as described by Campbell[20]. The total carotenoid concentration was evaluated spectrophotometrically, comparing the absorbance at 451 nm of the petroleum ether extracts with that of a standard solution of β-carotene in petroleum ether.

DNA: DNA concentration was evaluated by Burton's method (1956)[21].

Antioxidant enzyme activities: The tissues were homogenized in 5 vol of 20 mM Tris buffer, 0.5 M sucrose, 0.15 M NaCl, pH 7.6 and centrifuged at 500 × g for 15 min at 4 °C. The supernatants were then centrifuged at 12.000 × g for 30 min at 4 °C. The 12.000 × g supernatants were subsequently chromatographed on a Sephadex G-25 column (Pharmacia PD-10 columns) and utilized for the evaluation of the enzyme activities.

Superoxide dismutase (SOD): SOD activity as evaluated with the xanthine oxidase-cytochrome c method as described by McCord and Fridovich[22]. The cytochrome c reduction by superoxide anion radicals generated by the xanthine-xanthine oxidase system was monitored spectrophotometrically, at the wavelength of 550 nm. One unit of SOD is defined as the amount of enzyme giving a 50% inhibition of the cytochrome c reduction at pH 7.8 and 25 °C.

Catalase: Catalase activity was determined on both the 12.000 × g supernatant and the pellet (this latter containing mitochondria and peroxisomes), following the decomposition of H_2O_2 at pH 7, 25 °C, at 240 nm wavelength[23].

GSH peroxidase: GSH peroxidase activity was evaluated as described by Günzler and Flohé[23], utilizing both H_2O_2 and cumene hydroperoxide as substrates. The GSSG formed in the GSH peroxidase reaction is continuously and instantaneously reduced by an excess of GSH reductase, to provide a constant GSH level in the reaction mixture in the presence of NADPH. The NADPH oxidation rate is followed at 340 nm at 25 °C. The assay conditions were chosen to optimize the ratio between the enzymatic and the non-enzymatic oxidation of NADPH. The activity with H_2O_2 as substrate represents the selenium-dependent peroxidase, whereas the activity with cumene hydroperoxide represents the sum of this and the Se-independent (GSH transferase) GSH peroxidase activity.

Materials: All reagents were of analytical or HPLC grade. Enzymes and standards were from Sigma Chemical Company (St. Louis, USA).

Results and discussion

Table 1 shows the GSH content in the digestive gland of mussels of different ages. The results indicate that the level of this tripeptide, which is regarded as one of the main soluble antioxidants in the cells, tends to decrease with aging. In particular, the GSH content is significantly lower (about 30–40%) in the > 10-year-old animals than in the first age group.

Similar results were obtained for the levels of lipid soluble antioxidants (vitamin E, carotenoids). The concentration of vitamin E, the main membrane lipid-soluble antioxidant, was lower (−47%) in the digestive gland of older animals when compared to the first age group (table 2). Moreover, the total carotenoid content (table 3) was significantly decreased in both the second and the third age groups with respect to the younger animals (about −45% and −55%, respectively). The observed differences in the total carotenoid content may be related to lower feeding rates in mussels of different ages. However, it must be mentioned that the levels of these antioxidant compounds in mussel tissues have been demonstrated to fluctuate widely during the year[20, 24].

Data concerning the evaluation of the antioxidant enzyme activities (table 4) showed a significant decrease in catalase activity in the digestive gland of the second and third age groups with respect to the younger animals (−34% and −25%, respectively). Also, the GSH perox-

Table 1. Glutathione content in the digestive gland of mussels of different ages.

	GSH (μg/g tissue)	%	(μg/mg DNA)	%
2–4 years	452.02 ± 31.5	100	165.91 ± 6.08	100
6–8 years	365.10 ± 27.69 n.s.	81	145.97 ± 29.15 n.s.	87
> 10 years	313.07 ± 16.04*	69	99.93 ± 11.41*	60

Data are expressed as μg/g wet wt and μg/mg DNA ± SD; * = $p < 0.01$; n.s. = not significant (Mann-Whitney's U-test).

Table 2. Vitamin E content in the digestive gland of mussels of different ages.

	Vitamin E (μg/g tissue)	%	(μg/mg DNA)	%
2–4 years	15.11 ± 0.36	100	5.66 ± 0.16	100
6–8 years	11.74 ± 1.87 n.s.	68	4.57 ± 1.02 n.s.	80
> 10 years	9.52 ± 0.70*	53	3.02 ± 0.40**	53

Data are expressed as μg/g wet wt and μg/mg DNA ± SD; * = $p < 0.05$; ** = $p < 0.01$; n.s. = not significant (Mann-Whitney's U-test).

Table 3. Total carotenoid content in the digestive gland of mussels of different ages.

	Carotenoids (μg/g tissue)	%	(μg/mg DNA)	%
2–4 years	227.11 ± 17.44	100	85.16 ± 7.19	100
6–8 years	126.23 ± 22.63*	54	49.65 ± 7.92*	58
> 10 years	113.14 ± 8.23*	50	36.11 ± 5.41*	42

Data are expressed as μg/g wet wt and μg/mg DNA ± SD; * = $p < 0.01$; n.s. = not significant (Mann-Whitney's U-test).

Table 4. Antioxidant enzyme activities in the digestive gland of mussels of different ages.

	SOD U/g wet wt	GSH-peroxidase (total activity/g wet wt)	Catalase	%
2–4 years	593.01 ± 85.59	660.77 ± 149.56[a] 121.42 ± 33.72[b]	1437.96 ± 78.76	100
6–8 years	583.71 ± 224.67 n.s.	545.08 ± 137.59[a] n.s. 114.84 ± 79.39[b] n.s.	952.21 ± 130.24*	66
> 10 years	568.75 ± 217.94 n.s.	465.10 ± 124.56[a] n.s. 112.95 ± 40.44[b] n.s.	1085.17 ± 129.96*	75

Data are expressed per g wet weight ± SD; * = $p < 0.01$ (Mann-Whitney's U-test); n.s. = not significant. One unit of SOD (superoxide dismutase) is defined as the amount of enzyme giving a 50% inhibition of the cytochrome c reduction (see Methods).
Total catalase activity, determined in both the 12.000 × g supernatant and pellet (the latter containing mitochondria and peroxisomes), is expressed as μmoles H_2O_2 consumed/min/g tissue. GSH-peroxidase activity is expressed as nmoles NADPH consumed/min/g tissue utilizing as a substrate (a) cumene hydroperoxide; (b) hydrogen peroxide. Statistical differences are referred to values obtained from the first age group (2–4-year-old mussels).

Table 5. Malondialdehyde (MDA) content in the digestive gland of mussels of different ages.

	MDA (nmoles/g tissue)	%	(nmoles/mg DNA)	%
2–4 years	75.82 ± 15.81	100	28.49 ± 6.49	100
6–8 years	90.32 ± 9.96 n.s.	119	35.84 ± 9.24 n.s.	126
> 10 years	131.95 ± 31.19*	174	40.60 ± 4.13*	142

Data are expressed as nmoles/g wet wt and nmoles/mg DNA ± SD. * = $p < 0.01$; n.s. = not significant (Mann-Whitney's U-test).

idase activity, when evaluated utilizing cumene hydroperoxide as a substrate, appeared to decrease with age, but the observed changes were not statistically significant; SOD activity was similar in the different age groups.

Finally, table 5 shows data on lipid peroxidation, as judged by determining the levels of malondialdehyde (MDA) in the digestive gland of mussels of different ages (MDA is usually considered to be one of the main products of the peroxidation of membrane lipids [13]). The results demonstrate that the MDA content is significantly higher in age group III (> 10 years), than in younger (2–4 years) animals. A slight, but not statistically significant increase of the MDA content was also observed in the age group II mussels compared with the younger ones.

Our results, which represent the first data on the aging process in the tissues of marine invertebrates, indicate that, in the digestive gland of mussels, the levels of the antioxidant defence systems show a general tendency to decrease with age. Particularly, a significant decrease of both soluble (GSH) and membrane (vit. E, carotenoids) antioxidant compounds, as well as of the activity of catalase, the enzyme involved in the detoxication of H_2O_2, is observed in the > 10-year-old mussels compared to the first age group.

With regard to this, it must be taken into account that bigger (older) animals, having a lower oxygen consumption per g wet weight, would presumably have a lower rate of oxyradical production, so that the lower levels of the antioxidant defence systems may still be adequate for scavenging the free radicals that arise. However, it may

also be the case that a reduction of the antioxidant defence systems would render the older animals more susceptible to free radical-related oxidative stress and, consequently, to cellular peroxidative damage, as indicated by the net increase in the content of toxic aldehydes (MDA) observed as a function of age.

These results are consistent with the general definition of aging as the progressive accumulation of changes which are responsible for the decreased ability of the organism to maintain the homeostatic equilibrium and to adapt to various environmental stimuli[25]. In this view, the increased susceptibility to peroxidative stress observed in aging mussels would affect their capacity to respond to the natural fluctuations of oxygen levels. Moreover, since mussels are able to accumulate in their tissues high levels of environmental contaminants[26], such as metal cations or organic compounds, which are known to stimulate the production of free radicals[10-12,27], the age-related decrease of the antioxidant defence systems would impair the natural protection against the potential toxicity of pollutants.

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Biophoton emission from *Daphnia magna*: A possible factor in the self-regulation of swarming

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Summary. The formation of swarms by planktonic organisms was first described almost 100 years ago, but the mechanisms governing the development of patterns in population size and density are still not understood. In this study, we investigated one biophysical factor that may play an important role in swarm-formation. Spontaneous ultraweak photon emission in the visible range has been well documented for living cells, tissues and individuals in the plant and animal kingdom, including humans. We demonstrate here that the intensity of light emitted by the planktonic crustacean *Daphnia magna* is a function of population density in relation to body size. The effects are discussed on the basis of the theory of Dicke[1,2], and it is suggested that biophoton emission may be a basic factor in the self-regulation of swarm density.
Key words. Swarm density; animal distance; body size; photons; self-regulation; *Daphnia magna*.

Pattern formation in time and space represents a fundamental principle in the differentiation and evolution of living systems, and probably of all matter[3,4]. Many physical, chemical and biological factors which may control this process have been studied in the broad range from the molecular to the ecological levels, and many hypotheses have been put forward. One of the phenomena which is least understood, although it has often been