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Original Article

Redox-Based Flagging of the Global Network of Oxidative Stress Greatly Promotes Longevity

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Abstract

Despite more than 50 years of investigations into the free radical theory, the direct role of oxidative stress (OS) in aging and age-related diseases remains unproven. Little progress in identifying antioxidant drugs promoting longevity has been made, likely due to selectivity toward one or few radical species, variable efficacy in vivo, inherent pro-oxidant behavior of such drugs, or lack of synergism with metabolic redox homeostasis. Silencing the wide range of reactive free radicals has a great impact on OS–linked outcomes and age-related disorders. Here we show that an innovative, redox-active, multi-radical-scavenger catalytic drug delays the age-associated decline in physiological processes and markedly prolongs the mean lifespan of the adult freshwater annelids *Aeolosoma viride* by 170%. This unprecedented extension is associated with a decreased OS status. Consistently, treatment of annelids increases their natural resistance to oxygen-derived damage without affecting mitochondrial respiration or reproductive activity. Conversely, the superoxide dismutase (SOD)-mimetic EUK 134 that we selected as a positive control led to an increase in lifespan of ~50%, the same increase previously observed in nematodes. Our results show that reduction of the global network of OS has a profound impact on aging, prompting the development of a possible redox-based therapeutic intervention to counteract the progression of aging.

Key Words: Longevity—Oxidative stress

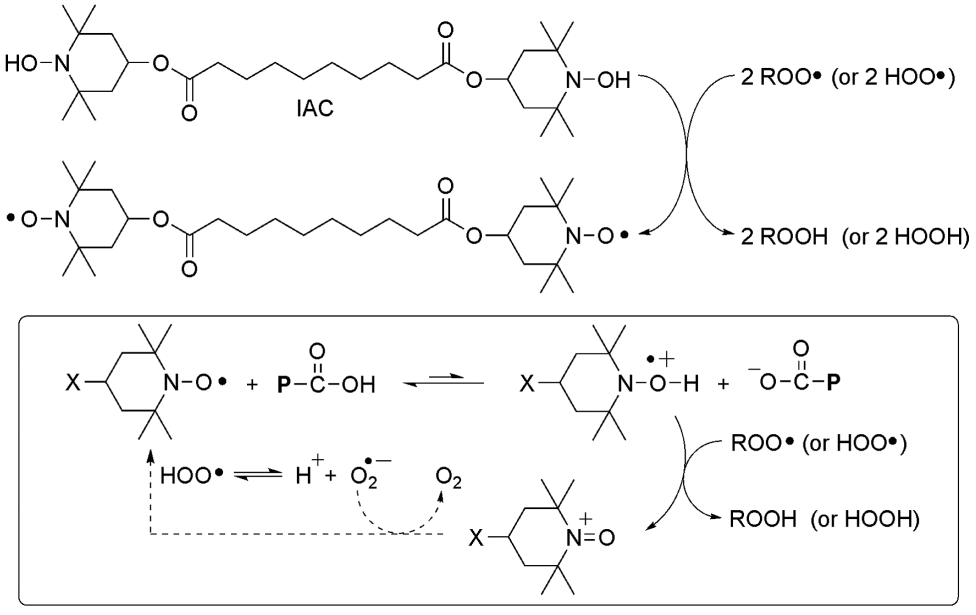
The free radical theory of aging hypothesizes that reactive nitrogen and oxygen species (ROS), which are produced in several metabolic processes, are responsible for the damage to cellular components, thereby causing a functional decline in aging (1,2). Support for this theory stems from evidence that genetic mutations and manipulations conferring resistance or limiting OS improve physical

performance or prolong lifespan in many organisms, including yeast, nematodes, fruitflies, and rodents (3–9). A considerable body of data supports some of the predictions of the free radical theory of aging also in humans (ie, oxidative damage increases with age in many human tissues). However, this theory, although widely accepted, is still debated. In fact, attempts to extend longevity with antioxidant drugs have had very limited success so far (9,10). In a few cases lifespan extension was achieved in *Caenorhabditis elegans* by treatment with neurotropic drugs but the effect was not related to direct or indirect antioxidant activity of the drug (11,12). Actually, when conventional (eg, phenolic) antioxidants have been tested, they had often produced disappointing or controversial results. For instance, resveratrol was found to improve survival of mice on a high-calorie diet (13). However, the mechanism was unrelated to its antioxidant activity [which is indeed modest (14)], but was rather found to be due to its induced calorie-restriction-like metabolism, thereby opposing the effect of the high-calorie diet. On the other hand, coenzyme Q had no effect neither on lifespan nor on antioxidant defenses of mice (15). Surprisingly, a diet lacking this antioxidant resulted in life-span extension in *C elegans* (15). The ongoing controversy is possibly related to the fact that conventional antioxidants, upon scavenging ROS, are themselves converted into pro-oxidant (semiquinone and quinone-like) by-products in the absence of suitable co-antioxidants. Hence, as previously suggested (6,15), their antioxidant role might be compromised by the pro-oxidant behavior. It is noteworthy that successful examples of lifespan extension have always resulted upon treatment with antioxidant molecules acting as mimics (10) or inducers (9) of SOD, a catalytic antioxidant that is not chemically altered by antioxidant action.

Here, we test the OS hypothesis of aging by studying the effects on life-span of an artificial hydroxylamine scavenger (IAC). IAC reacts with most—if not all—carbon, nitrogen and oxygen reactive species of biological interest (including peroxyl radicals ($ROO^{\bullet}$) and superoxide radical-anion [$O_2^{\bullet-}$ (16)] and was recently found to attenuate oxidative diseases where OS has a pathophysiological role (17–19). Unlike conventional antioxidants, IAC has an additional action: upon quenching ROS, it becomes super-activated, turning from a hydroxylamine to a nitroxide—an even more potent and catalytic antioxidant (20,21; Scheme 1).

Hence, its antioxidant behavior is modulated by redox homeostasis. We tested the effect of IAC in the *Aeolosoma viride* model system. These small limnetic freshwater annelids age rather quickly (average survival is 69 days) and share many conserved metabolic processes with nematodes and vertebrates, including aspects of the aging process (22,23). The *A viride* is optically transparent, has agamic reproduction that eliminates the variability of meiotic reproduction. In nematodes and insects, the lifetime is divided into stages interspersed with moultings, and the last one (the adult stage), which is usually short, basically involves the reproductive activity followed by death. Aeolosoma (annelids) are instead included in Lophotrochozoa that differ from the previous general plan of organization and development. In annelids, the periods of youth, maturity, and aging present a substantial continuity similar to that found even in deuterostomes, including vertebrates. This allows an expansion of the experimental model. Unlike the nematodes, the anellid tegument devoid of cuticle is the place of extensive exchanges with the medium (osmolarity, gas exchange). Population dynamics parameters are easily quantifiable and, in standardized breeding conditions, the lifespan and the number of zooids produced (reproductive activity) are repeatable, with a range of variability of 10%. The quantitative parameters that describe the reproductive activity of *A viride*, exhibit variations summarized in an initial increasing period (about 30% of the entire life) and in a later decreasing period (about 70% of the life) that corresponds to a progressive aging prior to spontaneous death (23). Thanks to these advantages, and for being genetically homogeneous, this model surpasses the gold standard *C elegans* for aging studies. The features of the *A viride* designate this non-conventional organism as a powerful system that exceeds the limits of using conventional animal models (24).

We exposed zooid populations (2 days old), which reproduce agametically by paratomic scission with pygidial budding, in cultured medium (22) containing various concentrations of IAC and



Scheme 1. Hydroxylamine scavenger (*bis*(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)-decandioate, IAC) gives fast reaction with conceivably all the oxygen centered radicals of biological interest, including superoxide, to form nitroxide, an even more potent and catalytic antioxidant.

maintained them concurrently with a control population of untreated annelids (23). Similar experiments were performed with the SOD/catalase (CAT) mimetic EUK 134, as an antioxidant positive control since it previously showed an increase of lifespan by a mean of 44% in the nematode *C elegans* (10). The annelids were transferred on alternate days to fresh medium during the entire life cycle until death. Twelve animals were individually cultured for each concentration and the lifespan of zooids was measured for each experiment.

The radical scavenger IAC had a dramatic effect on the lifespan of adult zooids, largely extending the mean lifespan from 68 to 182 days (168% increase, $p < .01$; maximum life-span 207 days; Table 1A). A dose-response analysis revealed that annelids cultured with an external concentration of 1.25 µM IAC displayed the longest prolongation of mean lifespan. Concentrations both below and slightly above this optimum concentration showed shorter extensions, whereas 10-time higher concentrations caused toxicity and drastically reduced lifespan, compared to controls. Similar results were achieved in a second independent experiment (Table 1B). The Kaplan–Meier survival curves, averaged between both experiments, are shown in Figure 1A. To the best of our knowledge, this is the longest lifespan extension recorded in a living organism exposed to a synthetic compound.

Treatment of annelids with 3.10 µM EUK 134 resulted in a lifespan increase of about 53% ($p < .05$; Table 1D). Similar results were obtained in an independent experiment (Table 1C and Figure 1B, averaged between both experiments). Our findings are therefore comparable to those reported with *C elegans* by Melov et al. (10) and confirm, in various species, the lifespan prolonging properties of the SOD/CAT mimetic EUK 134 (25). In particular, two groups of investigators (26,27) have analyzed *C elegans* exposed to EUK 134, and these studies have resulted in substantially different conclusions. Both units conclude that EUK 134 is functional as SOD mimetic in the worm and can counteract the effects of damage caused by ROS. The finding of Keaney et al. reports that this compound does not extend lifespan of animals grown in standard conditions, but only if exposed to ROS generators. The culture conditions used by Melov et al. (10) may have involved a higher level of oxidative stress that did limit *C elegans* lifespan. In two other invertebrates, the house fly Musca domestica and the fruit fly *Drosophila melanogaster*, administration of EUK 134 did not extend lifespan (28,29).

On the basis of the obtained results, the two antioxidant agents, IAC and EUK 134, probably operate with different mechanisms of action and, even if additional studies are required to demonstrate them, a possible preliminary explanation of behaviors could be ascribed to their different targets. As mentioned above, IAC is able to attenuate OS, by quenching carbon, nitrogen, and oxygen reactive species. In contrast, EUK 134 displays solely SOD and catalase enzymatic activities, scavenging superoxide and hydrogen peroxide respectively. Therefore, we propose that the achieved results may be ascribed to the different mechanisms of action. Indeed another possible explanation that the higher effectiveness of IAC compared to EUK 134 could be associated to the higher bioavailability of IAC is not supported by currently available data, which indicate a modest oral bioavailability of IAC hydrophilic form (18), thus comparable with that described for EUK 134.

In worms, probably due to the presence of a cuticle, higher antioxidant concentrations (millimolar) were necessary compared to annelids since the compounds can enter the worm only through ingestion (while annelids can exploit a passive deffusion), and this may explain the variability of lifespan extension (10,26).

To determine whether the multi-radical scavenger IAC can delay the age-related decline of physiological processes in *A viride*, we analyzed agamic reproduction of individual zooids on a daily basis and found that the concentration of IAC promoting maximum lifespan extension (1.25 µM) did not substantially alter the mean reproductive activity (Table 2A and B), whereas higher concentrations did so significantly. The same holds true for EUK 134 treatment (Table 2C and D). The treated animals also displayed

Table 1. Effect of IAC (A and B) and EUK 134 (C and D) on *Aeolosoma* Life-Span

	IAC			EUK 134		
	Treatment (µM)	Mean Life-Span (Days)	Maximum Life-Span (Days)	Treatment (µM)	Mean Life-Span (Days)	Maximum Life-Span (Days)
Experiment 1	A			C		
	—	68 ± 12	88	—	65 ± 16	85
	0.30	66 ± 19	89	1.60	85 ± 11*	97
	0.60	72 ± 19	95	3.10	94 ± 22*	122
	1.25	182 ± 21**	207	6.30	97 ± 33	139
	2.50	146 ± 31**	183	12.50	74 ± 21	105
	3.70	88 ± 28	123	25.00	52 ± 17	73
	5.00	81 ± 29	126	50.00	30 ± 3*	34
	12.50	29 ± 8**	40	100.00	18 ± 5*	26
	25.00	21 ± 3**	25			
Experiment 2	B			D		
	—	75 ± 17	95	—	64 ± 15	82
	0.30	64 ± 11	76	1.60	78 ± 1*	79
	0.60	68 ± 11	83	3.10	98 ± 23*	123
	1.25	186 ± 23**	218	6.30	91 ± 32	132
	2.50	138 ± 39**	186	12.50	81 ± 17	104
	3.70	98 ± 44	154	25.00	53 ± 10	65
	5.00	73 ± 33	105	50.00	30 ± 3*	33
	12.50	26 ± 7**	34	100.00	17 ± 5*	23
	25.00	22 ± 3**	26			

Data are means ± *SD* of 12 animals. *p* Values are compared to untreated annelids as assessed with Student's *t*-test (*$p < .05$, **$p < .01$).

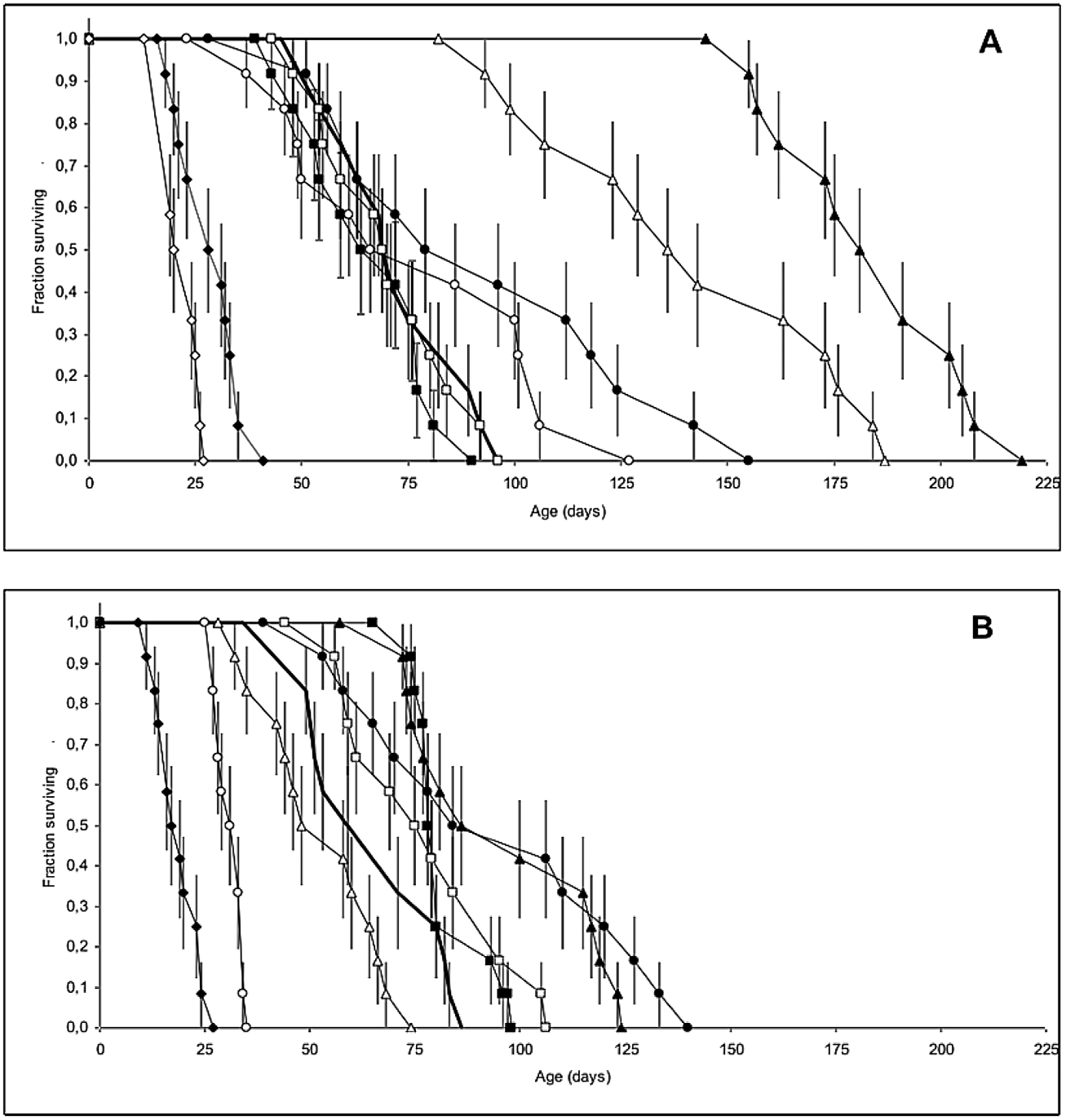


Figure 1. Adult *Aeolosoma viride* life-span extension by a diet supplemented with radical scavenger compounds. (**A**) Kaplan–Meier survival curves (±SD) of adult annelids treated with the radical scavenger IAC at various concentrations (μM). Zooids were cultured under the concentrations indicated and were scored as dead when they failed to respond to repeated touching with a platinum wire pick. ▬ Untreated; ■ 0.30 μM; □ 0.60 μM; ▲ 1.25 μM; Δ 2.50 μM; ●3.12 μM; ○ 6.25 μM; ♦ 12.50 μM; ◊ 25.00 μM. (**B**) Adult life-span survival curves of annelids fed with a diet containing the SOD/CAT mimetic EUK 134 (selected as positive control) at various concentrations (μM). ▬ Untreated; ■ 1.56 μM; ▲ 3.12 μM; ●6.25 μM; □ 12.50 μM; Δ 25.00 μM; ○ 50.00 μM; ♦ 100.00 μM. Data in A and B are the mean values between the corresponding experiments 1 and 2 reported in Table 1.

normal food ingestion and body morphology [they did not appear thin or starved (23)], suggesting that the multi-radical scavenger activity is not linked to a non-specific reduction of metabolic processes. This hypothesis is consistent with the observation that treated annelids did not appear moribund until close to the end of their prolonged life.

To further validate our hypothesis, we also measured whether exposure of annelids to IAC affects mitochondrial respiration (ie, energy supply). The growth of zooids in the presence of 1.25 μM IAC did not show any significant effect on the rate of cyanide-sensitive oxygen consumption as compared to untreated zooids (9.24 vs 8.95 pmoles $O_2 \cdot s^{-1} \cdot 10^{-2}$ animals, respectively; Figure 2A). The

Table 2. Fertility of *Aeolosoma* Treated With IAC (A and B) or EUK 134 (C and D)

	IAC		EUK 134	
	Treatment (μM)	Fzn/d	Treatment (μM)	Fzn/d
Experiment 1	A		C	
	—	0.75 ± 0.08	—	0.73 ± 0.10
	0.30	0.69 ± 0.38	1.60	0.77 ± 0.04
	0.60	0.84 ± 0.40	3.10	0.76 ± 0.09
	1.25	0.63 ± 0.11	6.30	0.67 ± 0.10
	2.50	0.38 ± 0.16**	12.50	0.54 ± 0.11*
	3.70	0.35 ± 0.07**	25.00	0.37 ± 0.09**
	5.00	0.19 ± 0.03**	50.00	0.16 ± 0.03**
	12.50	0.24 ± 0.15**	100.00	0.17 ± 0.09**
	25.00	0.25 ± 0.07**		
Experiment 2	B		D	
	—	0.77 ± 0.11	—	0.72 ± 0.11
	0.30	0.87 ± 0.34	1.60	0.76 ± 0.05
	0.60	0.89 ± 0.37	3.10	0.75 ± 0.08
	1.25	0.61 ± 0.16	6.30	0.72 ± 0.08
	2.50	0.28 ± 0.16**	12.50	0.58 ± 0.06*
	3.70	0.35 ± 0.17**	25.00	0.42 ± 0.07**
	5.00	0.18 ± 0.03**	50.00	0.16 ± 0.03**
	12.50	0.31 ± 0.14**	100.00	0.16 ± 0.09**
	25.00	0.27 ± 0.08**		

Data are means ± *SD* of the number of zooids produced daily (Fzn/d) by 12 animals over the overall fertility period (from 1 to 2 days until death). *p* Values are compared to untreated annelids as assessed with Student's *t*-test (* $p < .05$, ** $p < .01$).

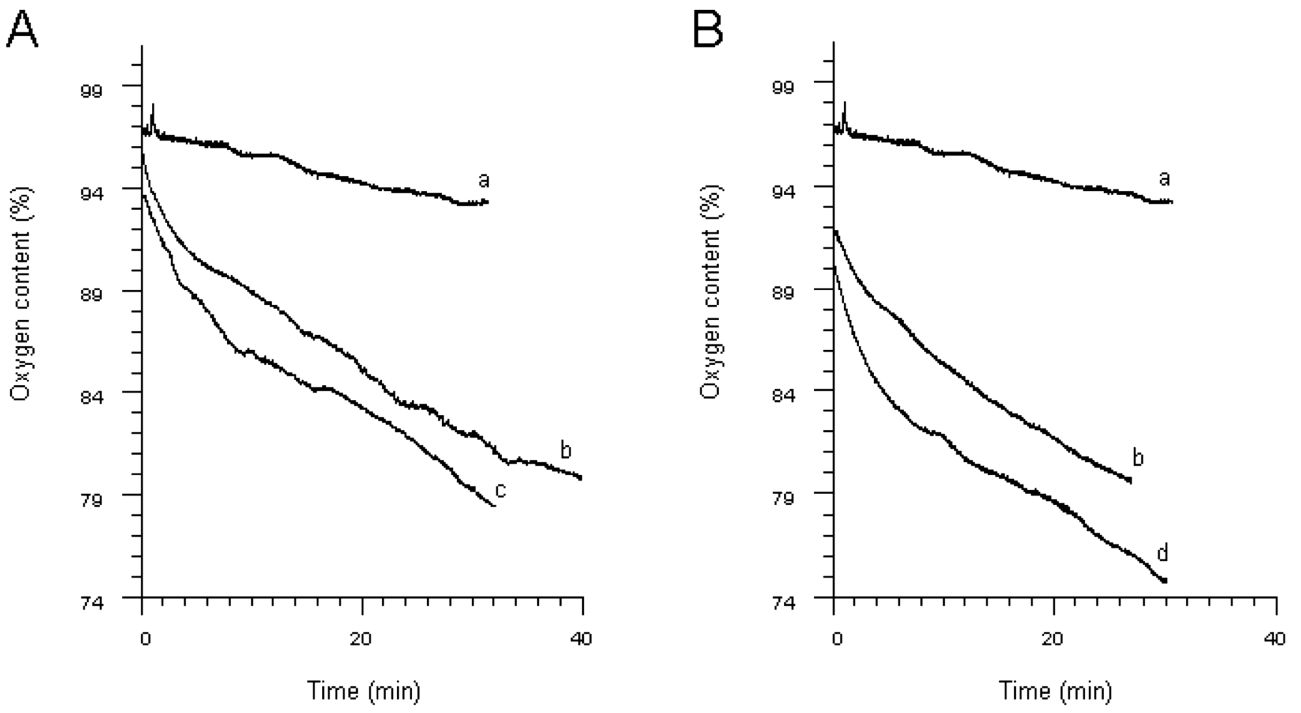


Figure 2. Respiratory rate of *Aeolosoma viride* anellids treated with different antioxidants. (**A**, **B**) The sample polarographic traces reported in the graphs indicate the oxygen consumption over time within a respirometer chamber containing, respectively, (*a*) 2 mL of water medium as blank reference, (*b*) 200 control individuals resuspended in the same volume of medium as in (*a*), (*c*) 200 individuals pre-treated with 1.25 μM hydroxylamine (IAC), and (*d*) 200 individuals pre-treated with 3.12 μM EUK 134. The oxygraphic analysis was carried out at 23°C as described in the Method Summary section. Respiration was 90%–95% cyanide-sensitive in all the animal samples, as tested by the addition of 1 mM KCN, pH 7.4 (data not shown).

marked life-span extension produced by the multi-radical scavenger in *A viride* could be due to its ability to counteract overall oxidative stress while not causing an impairment of oxidative phosphorylation, differently from caloric restriction mimetic agents (30), including some antioxidants (31). Likewise, treatment of the animals with 3.10 μM EUK 134 did not cause an alteration in the

rate of respiration (9.41 pmoles $O_2 \cdot s^{-1} \cdot 10^{-2}$ animals, in EUK-treated samples; Figure 2B).

While the above experiments clearly show that lifespan extension induced by the antioxidant IAC is not due to reduced metabolic activity, we sought for further evidence that it is instead related to its antioxidant activity. We performed whole-body electron paramagnetic resonance (EPR) spectroscopy measurements of the OS status (OSS) of *A viride* treated with 1.25 µM IAC for their entire life-span and compared the data to untreated control animals. We developed and validated this convenient approach based on the use of IAC itself as redox probe in living or excised tissues (16). Indeed, at concentration much higher than those used for the animal treatment, oxidation of IAC by metabolic reactive radical species selectively yields the corresponding mono-nitroxide, which can be detected by EPR spectroscopy and reduced back to the starting EPR-inactive hydroxylamine by ascorbate, glutathione, and a variety of antioxidant enzymes. Hence, EPR quantitation of the nitroxide provides a measure of oxidative-reductive balance within the system (16). At age intervals throughout their life-span, treated and untreated animals from two parallel populations were shortly (10 minutes) incubated with 1.0 mM IAC in the cavity of the spectrometer and the EPR spectra recorded. Untreated animals showed an OSS that significantly depended on age, following a Gaussian correlation centered at nearly half-life ($r^2 = .941$; $p < .01$; Figure 3). On the contrary, no simple dependence of OSS on age was observed in treated animals ($r^2 < .1$ for any first-order, second-order, or Gaussian correlation) and data simply appeared to oscillate around an average constant value (Figure 3). Starting from Day 21st post-IAC treatment, OSS was significantly lower ($p < .01$) in animals treated with 1.25 µM IAC than in controls until the final period of senility of control animals, when, due to metabolic decline, their OSS actually became significantly lower (at Day 100, $p < .01$) than in metabolically active treated animals. Since no control animals survived after 100 days at variance with IAC treated animals, comparison of the average OSS recorded during the first 100 days in the two populations indicated that it was lower in IAC-treated *A viride* by 36.4% ($p < .01$). Our findings provide an important support for the role of OS in aging and open the door for additional studies to verify whether the life-prolonging effects in lower organisms also occur in mammals and thus might plausibly apply to the human aging.

In conclusion, we report that the *A viride* lifespan is greatly extended by treatment of adult animals with a multi-active antioxidant like IAC, a redox-based scavenger of various free radicals and other reactive species involved in stress response pathway disregulation. We propose that IAC prolongs the zooid lifespan by reducing the integral OS network, thus strengthening the natural antioxidant defenses without affecting the mitochondrial respiration or other traits. These results ultimately designate OS as one of the major determinants driving the aging process and raise the provocative question of whether an effective antioxidant therapeutic intervention to counteract the progression of aging and age-related diseases in humans is ultimately possible.

Method Summary

Annelids

A Viride reproductive activity was recorded daily (22); survival of parental zooids and number of first filial generations/day was recorded and survival rates (S) as well as reproductive activity were calculated in terms of cumulative number of filial zooids (N) and number of offspring "born" per day [(dN/dt (23)].

Antioxidant

Treatment, renewed every 2 days, was performed either with bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)-decandioate [IAC (16)] (0.3–25 µM) or EUK134 (1.6–100 µM), the range of concentration being chosen by setting the maximal dose at 50% the minimal lethal dose and decreasing the dose by 50% at each subsequent dilution.

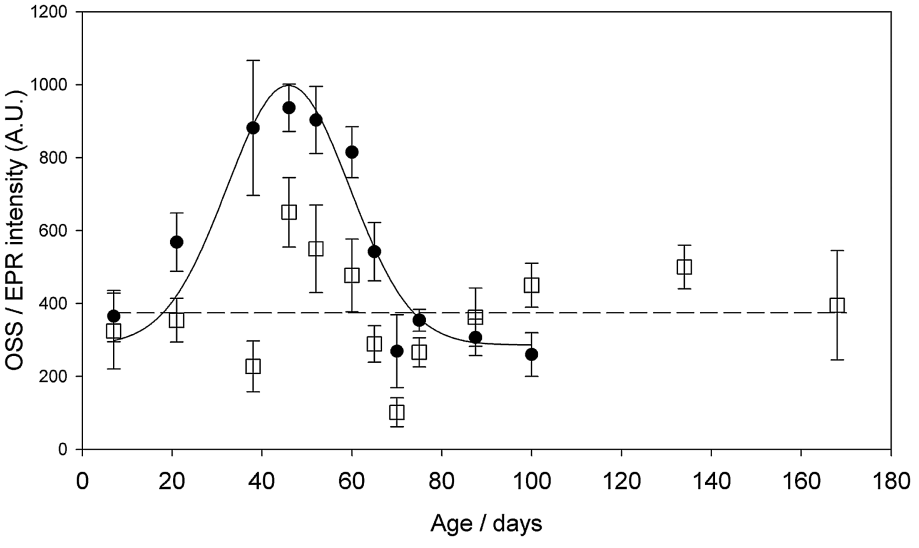


Figure 3. Oxidative stress status (OSS) of 1.25 µM IAC-treated *Aeolosoma viride* compared to untreated animals. OSS was determined by whole-body EPR spectroscopy of animals incubated at 23 ± 1°C for 10 minutes with 1 mM IAC in distilled water inside a calibrated capillary tube directly in the cavity of a Bruker Elexsys EPR spectrometer. The normalized intensity of the 1st spectral line of the corresponding mono-nitroxide was taken as measure of OSS in arbitrary units (A.U). Animals treated from Day 1st (□) had OSS oscillating around a constant value (---), at variance with control animals (●), which had OSS showing a Gaussian dependence on age (—). Treated animals had significantly ($p < .01$) lower OSS from Day 21 until Day 75, and significantly higher OSS ($p < .01$) at Day 100. On average during the first 100 days OSS in treated *Aeolosoma viride* was lower by 36.4% ($p < .01$). Error bars indicate ±2 *SD*.

Minimal lethal dose was determined in preliminary experiments as the minimal dose causing the death of three out of six worms within 3 days of exposure.

Oxygen Consumption

The oxygraphic analysis was carried out at 23 ± 1°C in a high resolution respirometer (Oroboros Oxygraph, Innsbruck, Austria) equipped with a polarographic oxygen sensor and a magnetic stirring apparatus (100 rpm); traces of oxygen consumption over time were recorded employing 200 individuals in each experiment, suspended in water medium within the respirometer chamber (2.0 mL final volume). Samples pre-treated with EUK134 or IAC doses giving the maximum life-span extention (ie, 3.10 µM EUK134 or 1.25 µM IAC) and untreated control animals as well as reference blanks were considered. The K-cyanide sensitivity of respiration was tested by addition of 1 mM KCN in buffer solution, pH 7.4 (32).

EPR Oxidative Stress Measurements

EPR measures were carried out at 23 ± 1°C with a Bruker Elexsys 500 spectrometer. At regular intervals (from Day 7 to 175 post-IAC treatment), animals from a population treated from Day 1st with 1.25 µM IAC were incubated for 10 minutes with 1.0 mM IAC in distilled water in a calibrated glass capillary tube and the EPR spectrum of the corresponding mono-nitroxide (a_N = 15.52 G; g = 2.0062) was recorded. The normalized intensity of the first spectral line was taken as measure of OSS, after correction for air oxidation of IAC (blank), in comparison with an identical control population grown in parallel. After measurement animals were discarded and removed from the population.

Statistics

Data are reported as average values and standard deviations. p Values refer to comparisons between treated and untreated animals as assessed with Student's t-test; statistical significance was set at $p < .05$ and $p < .01$.

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