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Levels and location are crucial in determining the effect of ROS on lifespan

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Reactive oxygen species (ROS) cause molecular damage that accumulates with age and have been proposed to be one of the primary causes of aging. However, recent work indicates that ROS have beneficial roles in an organism and that the relationship between ROS and aging is complex. We have shown that increasing ROS levels or oxidative damage does not necessarily lead to decreased lifespan. We have also shown that in some cases increasing ROS can promote longevity. Further investigation of the factors that determine the effect of ROS on lifespan demonstrate that both the levels and location of ROS are important in predicting the impact of ROS on longevity. Increasing superoxide levels in the cytoplasm results in decreased lifespan, while increasing superoxide levels in the mitochondria leads to increased lifespan. Within the mitochondria, mild elevation of superoxide levels promote longevity, while high levels of superoxide are toxic. Thus, a new paradigm is emerging in which ROS are neither good nor bad but levels and location makes it so.

Keywords: aging, *C. elegans*, genetics, lifespan, mitochondria, oxidative stress, reactive oxygen species, superoxide dismutase, superoxide

Abbreviations: FRTA, free radical theory of aging; PQ, paraquat; ROS, reactive oxygen species; SOD, superoxide dismutase.

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The free radical theory of aging (FRTA) was first proposed by Denham Harman in 1956.[1] This theory suggests that aging results from the accumulation of oxidative damage caused by reactive oxygen species (ROS) that are generated by normal metabolism. Since that point in time, the FRTA has become one of the most widely accepted and widely tested theories of aging and has contributed to the belief that antioxidants are beneficial for human health. However, recent work has cast doubt of the FRTA and suggests that the theory is in need of revision.

Multiple groups have tested the FRTA in worms by focusing on the antioxidant enzyme superoxide dismutase (SOD). SOD is the only eukaryotic enzyme capable of detoxifying superoxide and acts by converting superoxide to hydrogen peroxide, which is subsequently converted to water. In *C. elegans* there are 5 *sod* genes: *sod-1, sod-2* and *sod-4* are the primary cytoplasmic, mitochondrial and extracellular *sod* genes respectively, corresponding to *SOD1, SOD2* and *SOD3* in humans. Worms also have 2 inducible *sod* genes that are absent in humans: *sod-3* in the mitochondria and *sod-5* in the cytoplasm. These genes are normally expressed at low levels but can be induced under stress. To test the FRTA, we and others measured the lifespan of worms with deletions in each of the 5 individual *sod* genes.[2-4] In contrast to the predictions of the FRTA and despite the fact that at least some of these mutations increased both oxidative damage and sensitivity to oxidative stress, deletion of individual *sod* genes was found to have little or no detrimental effect on longevity. In fact, we subsequently showed that worms with deletions in all 5 *sod* genes, which have no SOD activity, still have a normal lifespan.[5] This provided strong evidence that increasing ROS levels does not necessarily decrease lifespan.

One of the most interesting and surprising findings from these experiments was the fact that deletion of *sod-2*, the primary mitochondrial *sod* gene, resulted in significantly increased lifespan.[4] Based on

this observation, we and others tested to see whether this finding could be reproduced using the ROS-generating compound paraquat, which is thought is thought to specifically increase superoxide levels in the mitochondria through redox cycling. Using a dilution series of paraquat concentrations, it was found that low levels of paraquat cause a dose-dependent increase in lifespan, while high concentrations of paraquat are toxic.[5-8] This indicates that the levels of superoxide are crucial in determining the effect of ROS on longevity (**Fig. 1**). Similarly, it has been shown that other chemicals that increase ROS levels can also increase longevity including arsenite,[9] juglone,[10] rotenone,[11] 2-deoxy-D-glucose,[12] lonidamine,[13] and D-glucosamine.[14] Additional support for the ability of ROS to promote longevity comes from observations of multiple long-lived mutants (*clk-1, isp-1, nuo-6* and *daf-2*), which have been shown to have elevated levels of ROS.[6,15,16] In each case, limiting the increase in ROS levels through treatment with an antioxidant significantly decreased their lifespan, but not the lifespan of WT worms, suggesting that the elevation in ROS levels is required for their longevity. Importantly, the ability to elevated ROS levels to increase lifespan is not specific to worms but appears to be conserved across species.[14,17,18,19]

To gain further insight into the relationship between ROS and aging, we examined how subcellular localization of ROS influenced the effect of ROS on longevity.[16] To do this we used a genetic approach based on the fact that superoxide is unable to cross biological membranes.[20] Preventing the expression of SOD in a particular compartment of the cell (cytoplasm, mitochondria or extracellular) should result in a localized, compartment-specific increase in the levels of superoxide. To increase our ability to observe a difference, we performed these experiments in a *clk-1* background (*clk-1* is a mitochondrial mutant with increased levels of ROS and increased longevity.[21,22]) We found that increasing superoxide levels in the mitochondria resulted in markedly increased lifespan. In contrast, increasing superoxide levels in the cytoplasm had the opposite effect of decreasing lifespan. Importantly, when lifespan was decreased because of too much cytoplasmic superoxide, it was still possible to increase lifespan by increasing superoxide levels in the mitochondria. Thus, cytoplasmic and mitochondrial superoxide have opposing effects on lifespan and each impacts longevity independently of the other compartment (**Fig. 2**). It is also interesting to note that increasing cytoplasmic or mitochondrial superoxide both result in increased sensitivity to oxidative stress despite having opposite effects on lifespan. This indicates that sensitivity to oxidative stress can be experimentally dissociated from longevity.

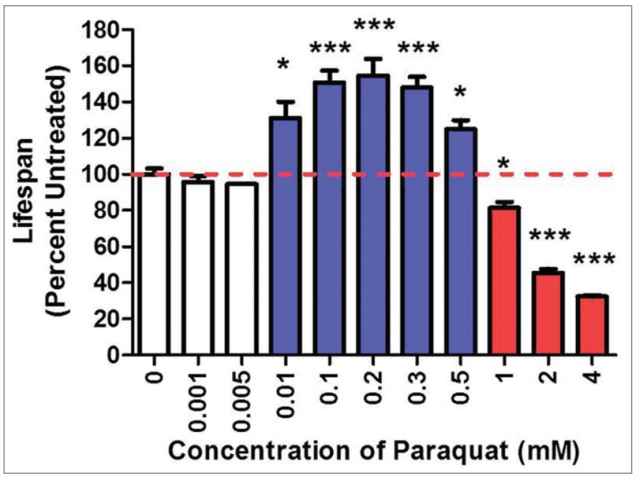

Figure 1. Levels of ROS are crucial in determining the effect of ROS on lifespan. To examine the effect of levels of superoxide on lifespan, worms were transferred to plates containing increasing concentrations of paraquat (PQ) at day1 of adulthood and their lifespan was measured. PQ generates superoxide in the mitochondria through redox cycling. At very low concentrations of PQ ($\leq$ 0.005 mM), there is no effect on lifespan. Low concentrations of PQ cause a dose-dependent increase in lifespan that is maximal between 0.1 and 0.2 mM in WT worms. Higher concentrations of PQ ($\geq$1 mM) cause decreased longevitiy. We hypothesize that mild elevations in mitochondrial superoxide levels increase lifespan by engaging pro-survival signaling, while higher levels of superoxide are toxic by causing high levels of oxidative damage. Error bars indicate SEM. *p $<$ 0.05, **p $<$ 0.01, ***p $<$ 0.001. Data is adapted from Van Raamsdonk and Hekimi, 2012.

At present one of the major obstacles to further defining the relationship between superoxide and longevity is the difficulty in specifically measuring superoxide levels due to their transient and reactive nature.[23] Ideally this would be done in a subcellular compartment-specific manner in live worms. One promising approach will be to use genetically-encoded biosensors that could be targeted to different tissues using tissue-specific promoters and to subcellular compartments with targeting sequences. At present, genetically-encoded probes have been developed for the detection of hydrogen peroxide;[24] however, probes for superoxide are still a work in progress.[25-27] Until such tools become available, detection of ROS will be limited to whole worm measurements using ROS-sensitive dyes, and indirect measures of ROS such as sensitivity to oxidative stress and oxidative damage.

In summary, the view that ROS are damage-causing molecules that contribute to aging is being replaced by a new paradigm defined by a complex relationship between ROS and longevity. While high levels of ROS are toxic, accumulating evidence indicates that low levels of ROS can be beneficial. ROS can act in intracellular signaling pathways, at least some of which influence longevity. Our recent work demonstrates that both the levels and location of ROS are important in determining the effect of ROS on lifespan.[5,16] While the FRTA might apply to cytoplasmic ROS, it cannot account for the observation that elevated mitochondrial superoxide increases lifespan. Combined, this work suggests that for antioxidants to have a beneficial effect on human health, it may be necessary to target them to specific parts of cell, possibly to specific tissues in the body, and to only use antioxidants in

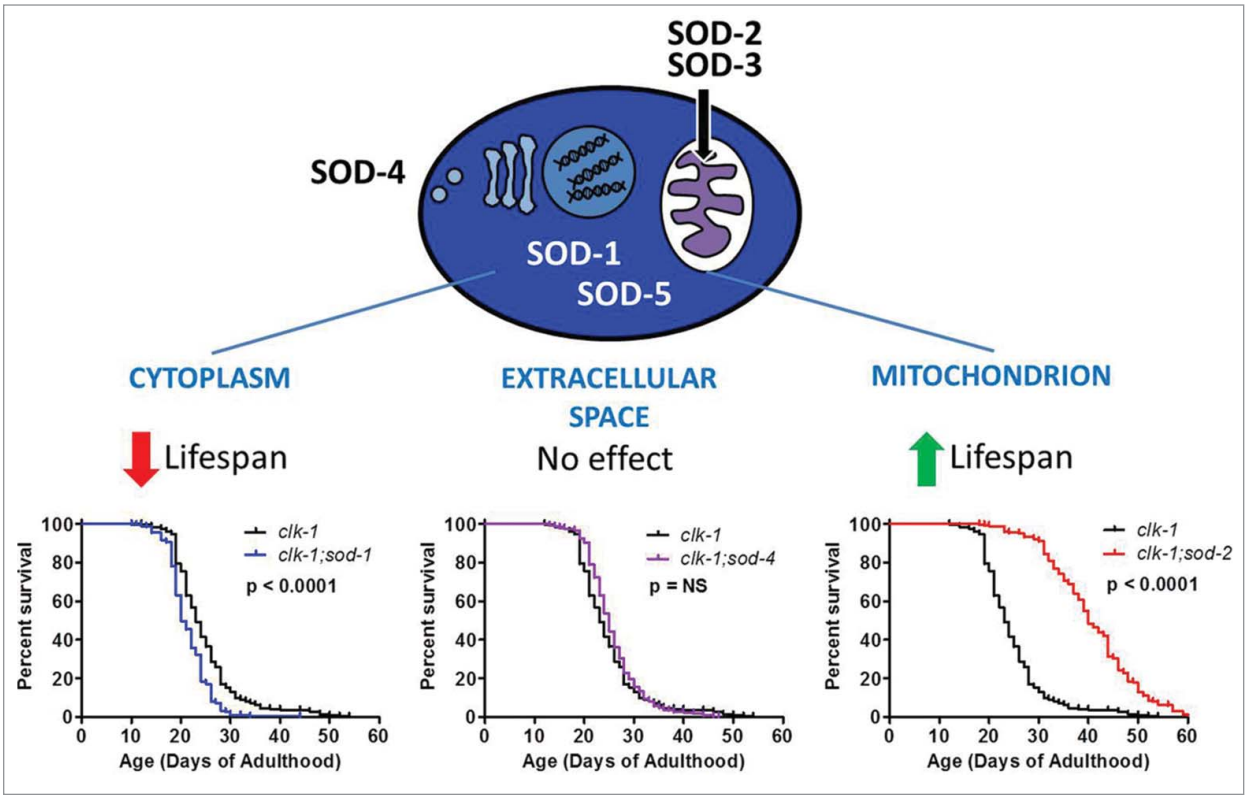

Figure 2. Location of ROS is crucial in determining the effect of ROS on lifespan. To examine the effect of subcellular localization of superoxide on lifespan, we tested the lifespan of different *sod* deletion mutants in a *clk-1* background, which is sensitive to ROS. SOD-1 and SOD-5 are present in the cytoplasm, SOD-2 and SOD-3 are present in the mitochondrial matrix, while SOD-4 is secreted extracellularly. Increasing cytoplasmic superoxide through deletion of *sod-1* resulted in decreased lifespan, while increasing mitochondrial superoxide through deletion of *sod-2* increased longevity. There was no change in lifespan when the extracellular *sod* gene was deleted. Survival curves are modified from Schaar et al., 2015.

instances where ROS levels are elevated above the levels required for optimal longevity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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