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# Minocycline, but not ascorbic acid, increases motor activity and extends the life span of *Drosophila melanogaster*.

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**Keywords:** minocycline, ascorbic acid, motor activity, life span, *Drosophila melanogaster*.

**Abstract.** In the present study we compared the effects of minocycline and ascorbic acid in the life span, motor activity and lipid peroxidation of *Drosophila melanogaster,* in an effort to find a substance capable of providing protection against oxidative stress in aging. In the flies treated with minocycline a very significant increase in the life span (101 ± 1.33 days) was observed when compared to those treated with ascorbic acid and controls (42.3% and 38.4%, respectively). The motor activity of minocycline treated flies also increased significantly with respect to control and ascorbic acid fed flies, from the 3rd to the 9th week of treatment. With regard to lipid peroxidation, it was found that the levels of malondialdehyde (MDA) in flies treated with minocycline showed no statistical differences to the control on the first day of treatment, but a significantly lower content on the day of 50% survival. In contrast, in flies treated with ascorbic acid significantly elevated levels of MDA compared to control and minocycline treated flies were detected throughout. These results suggest a protective effect of minocycline against oxidative stress and aging in *D. melanogaster*. An inhibitory effect on reactive oxygen species production may be an important contributing factor.

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**La minociclina, pero no el ácido ascórbico, incrementa la actividad motora y extiende el período de vida de *Drosophila melanogaster*.**

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**Palabras clave:** minociclina, ácido ascórbico, actividad motora, sobrevida, *Drosophila melanogaster*.

**Resumen.** En el presente estudio se compararon los efectos del ácido ascórbico y la minociclina en la duración del periodo de vida, la actividad motora y la peroxidación lipídica de *Drosophila melanogaster* en un esfuerzo por encontrar una sustancia capaz de proporcionar protección contra el estrés oxidativo en el envejecimiento. En las moscas tratadas con minociclina se observó un aumento significativo en la duración de la vida (101 ± 1,33 días) en comparación con los tratados con ácido ascórbico y los controles (42,3% y 38,4%, respectivamente). La actividad motora de las moscas tratadas con minociclina aumentó significativamente cuando se comparó con las tratadas con ácido ascórbico y el control, desde la 3ra hasta la 9na semana de tratamiento. Con respecto a la peroxidación lipídica, se encontró que los niveles de malondialdehído (MDA) en moscas tratadas con minociclina no mostraron diferencias estadísticas con relación al control en el primer día de tratamiento; sin embargo, se detecto una disminución significativa de la concentración de MDA cuando se alcanzó el 50% de sobrevida. En contraste, en moscas tratadas con ácido ascórbico observamos que los niveles de MDA estaban significativamente elevados, cuando se compararon con las moscas tratadas con minociclina y el control a lo largo de todo el tratamiento. Estos resultados sugieren un efecto protector de la minociclina frente al estrés oxidativo y el envejecimiento en *D. melanogaster,* aunque un efecto inhibidor sobre la producción de especies reactivas del oxígeno puede ser un factor contribuyente importante.

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## INTRODUCTION

The balance between the production of free radicals and the antioxidant defenses in the body has important health implications. If free radicals generation is elevated or antioxidant defenses are diminished, a condition of "oxidative stress" develops which may cause chronic damage. Free radicals have been implicated in several health problems. Cancer, atherosclerosis, stroke, myocardial infarction, senile cataracts, acute respiratory distress syndrome and rheumatoid arthritis are just a few examples. Numerous studies have shown the protective effects of antioxidant nutrients on these health problems, which may in turn be beneficial for prolonging life span (1-4).

Superoxide is among the most abundant reactive oxygen species (ROS) produced by the mitochondria, and is involved in cellular signaling pathways. Superoxide and other ROS can damage cellular macromolecules and measured levels of oxi-

dative damage products are positively correlated with aging (5). It seems that induced stress increases aging, because the deleterious effects of free radicals are progressive and accumulative (6, 7).

Minocycline is a semisynthetic second-generation tetracycline. Its antioxidant efficacy was demonstrated using the zymosan-stimulated polymorphonuclear leukocytes (PMNL) and the cell-free, xanthine-xanthine oxydase system. At concentrations comparable to therapeutic blood levels, minocycline HCL inhibited ROS ($O^{2-}$, $H_2O_2$ and OH•) production by PMNL (8). Minocycline was found to be able to block NO-induced neurotoxicity in cerebellar granule neurons treated with NO by inhibiting NO-induced phosphorylation of p38 MAP kinase. These finding may explain the neuroprotective mechanism of minocycline in models of global and focal cerebral ischemia, the R6/2 model of Huntington´s disease, as well as glutamate-induced neurotoxicity in mixed neuronal/glial cultures (9).

Shimazawa *et al*. in 2005, studied cultured retinal ganglion cells (RGC-5, a rat ganglion cell line transformed using E1A virus) and/or the in vivo retinal damage induced by N-methyl-D-aspartate (NMDA) intravitreal injection in mice. In addition, they examined minocycline's putative mechanisms of action against oxidative stress. These authors found that minocycline has neuroprotective effects against *in vitro* and *in vivo* retinal damage, and that an inhibitory effect on ROS production may contribute to the underlying mechanisms (10).

Ascorbic acid (AA) is a powerful water-soluble antioxidant which protects low-density lipoproteins from oxidation and has been shown to reduce the deleterious effects of oxidants in the central nervous system. Sales Santos *et al*. in 2009, studied the neuroprotective effects of AA in rats, against the observed oxidative stress during seizures induced by pilocarpine. This antioxidant treatment significantly reduced the lipid peroxidation level and nitrite content as well as increased the superoxide dismutase and catalase activities in the hippocampus of adult rats after seizures induced by pilocarpine (11)

In the present study we compared the efficacies of minocycline and ascorbic acid in the life span, motor activity and lipid peroxidation in *Drosophila melanogaster*, in an effort to find a substance capable of providing protection against the aging associated oxidative stress.

## MATERIALS AND METHODS

### *Drosophila melanogaster* stocks

Male wild-type flies of *D. melanogaster* (Oregon wild strain) were used. Flies were reared in a light/dark (LD) cycle of 12h:12h at a temperature of 25°C. The standard corn meal contained: 0.3 g of agar-agar, 5 g of corn flour, 1.5 g of yeast, 1.25 mL of 100% ethanol, 5 mL of a brown sugar solution (100 g of sugar in 100 mL distilled water), 0.65 g of methyl p-hydroxybenzoate (Sigma Chemistry Co. MO. USA), and 43.75 mL of water (12).

### Longevity

Two days old males were transferred to glass vials (Pyrex culture 9.6 × 100 mm) containing 1 mL of the test food. The flies were held in groups of five per vial. Fresh solutions of minocycline (Sigma Chemical Co. USA) and ascorbic acid (Sigma Chemical Co. USA) were prepared daily at a concentration of 0.05 mM and 0.36 mM, respectively, in standard corn meal, following the concentrations standardized by dose response curves (13). In each vial 1 mL of the control food or of the minocycline or ascorbic acid containing food was added. The vials were closed with cotton stoppers. Every

day at 10 a.m., the dead flies were counted and survivors were transferred to freshly prepared food. Three replicates of each treatment and control were done using 200 flies in each group. The studies were carried out between January and December of 2010.

**Motor activity**

The number of movements of individual *Drosophila melanogaster* from the treatments and control groups were determined using the DAM2 Drosophila Activity Monitor (Trikinetic) (14).

**Malondialdehyde (MDA) determination**

The MDA concentration was evaluated by measuring the TBARS according to the TBA test described by Ohkawa *et al*. (15), with modifications (14).

**Soluble protein concentrations**

The Bicinchoninic Acid Protein Assay Kit was used to determine soluble protein concentrations within whole body homogenates of treated and control *Drosophila melanogaster*, following the methodology described by (14).

**Statistical analysis**

Data are expressed as mean % SEM and were analyzed by means of the Analysis of Variance and the Bonferroni´s multiple comparison tests where appropriate. The significance between specific means was determined by Student´s t test. Differences were considered statistically significant when $p<0.05$.

## RESULTS

The life span of minocycline treated male *Drosophila melanogaster* was 101 ± 1.33 days. The treatment with minocycline resulted in a highly significant ($p<0.01$) increased longevity (38.4%) when compared to control (73 ± 0.33 days) and 42.3% when compared to ascorbic acid (71 ± 1.15 days) fed flies. The median survival with minocycline treatment (80 ± 0.33 days) also increased significantly ($p<0.0001$) when compared to control (49 ± 0.33 days) (Fig. 1). The median survival with ascorbic acid (51 ± 0.00 days) showed no statistically significant differences when compared to control (49 ± 0.33 days) (Fig. 2). Only minocycline significantly extended the longevity, and it did this by extending the health span (delaying the onset of senescence) when compared to control.

The highest motor activity was detected between 15:00 p.m. and 0:00 a.m. when the means of the number of movements per week in the animals treated with minocycline, ascorbic acid and control were compared.

During weeks 1, 2, and 8 no statistically significant differences between groups were detected. With the exception of these three weeks, minocycline treated flies showed a highly significant increase in motor activity during the 9 weeks of observation as compared to controls (Fig. 3).

On the 3$^{rd}$ week significant differences ($p<0.05$) were found when comparing the mean number of movements in *Drosophila melanogaster* treated with minocycline (18.8 ± 1.65) and control flies (15.01 ± 1.05).

In the 4$^{th}$ week a significant increase ($p<0.01$) was observed when the mean number of movements of *Drosophila melanogaster* treated with ascorbic acid (10.56 ± 0.425) was compared to control (8.25 ± 0.23). Highly significant increases ($p<0.01$) were also detected in the mean number of movements of flies treated with minocycline (15.84 ± 0.44) as compared to control.

During the 5$^{th}$ week a significant increase ($p<0.01$) in the number of movements of *Drosophila melanogaster* fed with

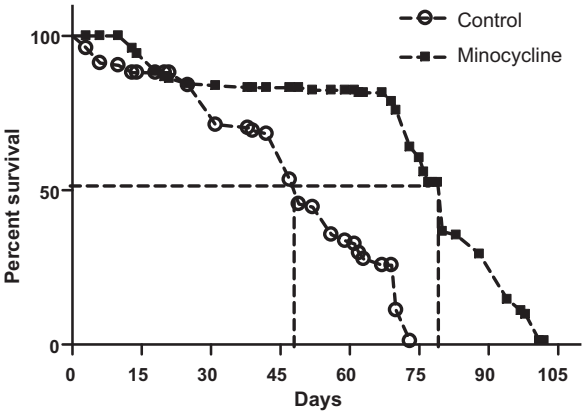


Fig. 1. Median Survival for *Drosophila melanogaster* raised in control medium and in medium containing minocycline. Flies were housed in groups of 5 per tube with 12:12 h light/dark cycle at 25 ± 1°C and transferred to fresh food three times/week. Three replicate trials were performed with male flies (Method of Kaplan and Meier).

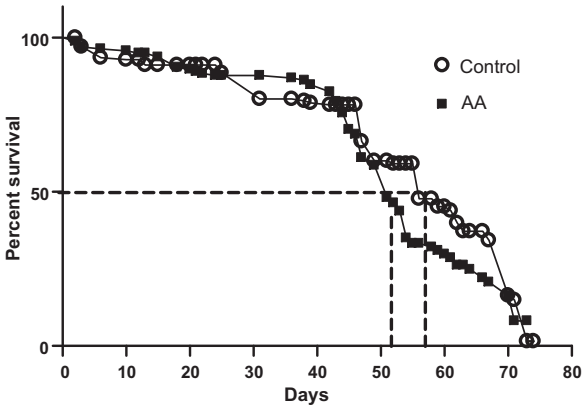


Fig. 2. Median Survival for *Drosophila melanogaster* raised in control medium and in medium containing ascorbid acid (AA). Flies were housed in groups of 5 per tube with 12:12 h light/dark cycle at 25 ± 1°C and transferred to fresh food three times/week. Three replicate trials were performed with male flies (Method of Kaplan and Meier).

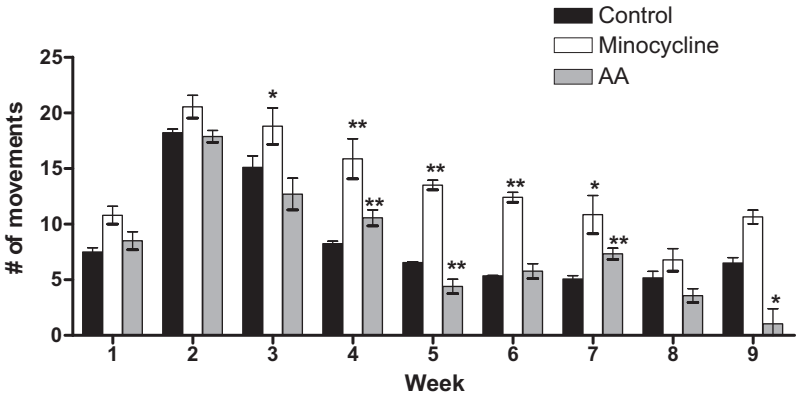


Fig. 3. Mean total motor activity in control and flies treated with minocycline and ascorbic acid. The period measurement was of 9h (between 15:00-00:00 hours at intervals of 15 min daily). The values represent the mean number of movements during each week. Flies were housed in groups of 5 per tubes with 12:12 h light/dark cycle at 25 ± 1°C and transferred to fresh food three times/week. Three replicate trials were performed with male flies. * $p< 0.05$, ** $p< 0.01$ versus control. In the 1st, 2nd and 8th week there were no statistically significant differences between groups.

minocycline (13.51 ± 0.44) was noted when compared to control (6.54 ± 0.84). The treatment with ascorbic acid decreased ($p<0.01$) the number of movements (4.4 ± 0.12) when compared to control and minocycline treated flies.

At week 6th, a highly significant increase was observed ($p<0.01$) in the mean number of movements of flies treated with minocycline (12.41 ± 0.46) when compared to control (5.35 ± 0.029) and ascorbic acid treated flies (5.77 ± 0.67). No dif-

ference was detected between control and ascorbic acid fed flies.

On the 7th week a significant increase ($p<0.05$) was detected in the mean number of movements (7.13 ± 0.14) of ascorbic acid treated flies as compared to control flies (5.09 ± 0.31). The minocycline treated flies significantly increase the number of movements (10.85 ± 1.73) ($p<0.05$) when compared to control.

During the 9th week a significant decrease was detected ($p<0.05$) in the mean number of movements of flies treated with ascorbic acid (1.05 ± 0.52), the control (6.5 ± 0.49) and minocycline fed flies (10.64 ± 0.61).

With regard to lipid peroxidation, whole body homogenates of 10 flies treated with ascorbic acid in day one showed an increase ($p<0.01$) in MDA concentrations (29.99 ± 1.07 nmoles MDA/mg protein) with respect to control (15.33 ± 0.32) and minocycline treated flies (14.99 ± 0.76). During the period of 50% survival the flies treated with ascorbic acid also exhibited a significant increase in MDA concentrations (31.74 ± 0.87) with respect to control (15.68 ± 0.49) ($p<0.05$) and minocycline (13.79 ± 2.11) ($p<0.01$) fed flies. A decrease ($p<0.01$) in MDA concentrations (13.79 ± 2.11) with respect to control (15.68 ± 0.49) was detected with minocycline (Fig. 4).

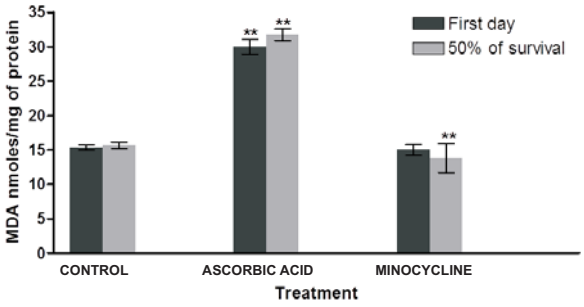


Fig. 4. Levels of MDA in *Drosophila melanogaster* in control medium and in medium containingminocycline or ascorbid acid. Three replicate trials were performed with male flies. ** $p< 0.01$ versus control.

## DISCUSSION

The life expectancy of *Drosophila melanogaster* is relative to its basal metabolic rate (16) and can be altered by diet, temperature, light and exposure to antioxidants. The effect of free radicals in the *Drosophila melanogaster* life span has been amply studied (17-19). The flies used in our work were maintained in daily 12 h light:12 h dark cycles. Under these conditions, activity levels peak near ¨dawn¨ and ¨dusk¨. Similar pattern has been reported in wild-type *Drosophila melanogaster* (20). Treatment with the two antioxidants did not alter this pattern of activity; only the size of the peak in the evening was modified in the presence of minocycline and AA treated groups. It has been previously shown that neither at the individual nor at the population level could a significant correlation between spontaneous locomotor activity and life span be found (17). However, the impact of interventions on health span is commonly evaluated by means of motor performance test such as spontaneous motor activity and the geotaxis test. The increase of 38.4% in the life span of flies fed with minocycline when compared to controls and 42.3% with respect to flies treated with ascorbic acid was higher than that observed in *Drosophila melanogaster* treated with melatonin in a previous study from our lab (12). These results are also in agreement with previous reports evaluating the effects of antioxidants on lifespan and health span of *Drosophila*. For example, Ortega-Arellano *et al*. (21) reported that polyphenols exposure prolog life span and restore locomotor activity in *Drosophila melanogaster* chronically exposed to Paraquat. Interestingly, Oxenkrug *et al*. (22) have recently shown that minocycline

treatment increases the lifespan and motor activity of *Drosophila melanogaster*. Our results confirm and complement the results of these authors. However, it is important to note that in the Oxenkrug *et al*. study a dose of 0.87 mM was used while we evaluated a dose approximately 15 times lower (0.05 mM).

In their study, Oxenkrug *et al.* propose that mynocycline effect on lifespan could be mediated by the modulation of the tryptophan-Kynurenin metabolic pathway. However, many of the protective effects of minocycline have been explained by its antioxidant capacity by interfering with the production of ROS through four different mechanisms of action: 1) its ability to trap free radicals comparable to alpha-tocopherol (23). 2) A direct action on the enzyme complexes that lead to the generation of ROS. 3) A direct effect on the expression of genes involved in the production of ROS (p. ej: COX-2 and iNOS). 4) Its ability to block activation of microglia, since the latter, once activated, is one of the main sources of production of ROS and NO, $H_2O_2$ and superoxide anion (3).

Morimoto *et al*. (24) have demonstrated the neuroprotective effects of minocycline in neurologic disease models featuring cell death. This action was evaluated both *in vitro* and *in vivo*. For the *in vivo* study, focal cerebral ischemia was induced by permanent middle cerebral artery occlusion in mice. Minocycline at 90 mg/kg administered intraperitoneally 60 min before or 30 min after (but not 4 h after) the occlusion reduced infarction, brain swelling, and neurologic deficits 24 h after the occlusion. For the *in vitro* studies, they used cortical-neuron cultures from rat fetuses in which neurotoxicity was induced by 24-h exposure to 500 $\mu$M glutamate Furthermore, the effects of minocycline on oxidative stress [such as lipid peroxidation in mouse forebrain homogenates and free radical-scavenging activity against diphenyl-p-picrylhydrazyl (DPPH)] were evaluated to clarify the underlying mechanism. Minocycline significantly inhibited glutamate-induced cell death at 2 $\mu$M and lipid peroxidation and free radical scavenging at 0.2 and 2 $\mu$M, respectively. It looks as if the direct antioxidant effect of minocycline is a major contributing factor for the increase in life span of *D. melanogaster* shown in our work.

Bonilla *et al*. (14) found that minocycline increased the life span and motor activity and decreased MDA formation of manganese treated *D. melanogaster*.

Lipid peroxidation is usually considered as an estimator of oxidative stress. The decrease in lipid peroxidation by minocycline reported in the present work lends support to the antioxidant activity of this compound. We can not rule out that other mechanisms are implicated in this enhancement of lifespan by minocycline. On the contrary, it is possible that the antioxidant effect and kynurenine pathway inhibition cooperate in minocycline effect. Future studies should address this issue.

The evidence of the lipid peroxidation-inhibiting effects of vitamins E and C, *in vitro*, is plentiful (25-30). Vitamin C has been shown to have a similar effect in a few (31, 32) but not in all (33) studies. Mice pretreated with vitamin C 24h before nicotine exposures were strongly protected against nicotine-induced DNA damage (34). Vitergan Zinc Plus (mixture of vitamins C, E, beta-carotene and minerals copper, selenium, and zinc), protects against the genotoxic effects of the chemotherapeutic free-radical generator Doxorubicin (35). Also reported what vitamins C and K3 have significant antiproliferative and apoptotic effects when used in combination. This combination enhances the efficacy of gemcitabine against bladder cancer in vivo (36).

Vitamin C can also act as a pro-oxidant under certain conditions (37, 38). In fact, although ascorbate normally acts as a powerful scavenger of reactive species, in the presence of iron (or copper) ions it can become pro-oxidant, stimulating damage to biomolecules by promoting the formation of OH• (39). In our work, the highly significant increase in MDA levels produced by ascorbic acid seems to indicate that at the dose administered to the flies (0.36 mM) it behaves as a pro-oxidant. In fact, a recent report it was shown that the repeated ijection of ascorbic acid induces dopaminergic neurotoxicity through generation of oxidative stress, and that this toxicity was related to the decline of glutathione in both the substantia nigra pars compacta and striatum (40).

In conclusion, *D. melanogaster* treated with minocycline exhibited an increased life span and motor activity when compared to controls and flies treated with ascorbic acid. MDA levels in flies fed with minocycline showed no statistical differences with the controls on day one, but significantly lower levels of MDA on the day of 50% survival. In ascorbic acid treated *D. melanogaster* elevated levels of MDA compared to controls and minocycline fed flies were detected. The significance and mechanisms of these protective effects of minocycline against oxidative stress and aging remain to be demonstrated, although an inhibitory effect on ROS production may be an important contributing factor.

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