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ANTI-AGING EFFECT OF RIBOFLAVIN VIA ENDOGENOUS ANTIOXIDANT IN FRUIT FLY DROSOPHILA MELANOGASTER

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Abstract: *Objectives:* This study investigated the effect of riboflavin on aging in Drosophila melanogaster (fruit fly). *Design:* Experimental study. *Setting:* Naval Medical Research Institute. *Participants:* Fruit fly Drosophila melanogaster. *Intervention:* After lifelong supplement of riboflavin, the lifespan and the reproduction of fruit flies were observed. Hydrogen peroxide (H_2O_2) was used to mimic oxidative stress damage to fruit flies and the survival time was recorded. *Measurements:* The activity of copper-zinc-containing superoxide dismutase (SOD1), manganese containing SOD (SOD2) and catalase (CAT) and lipofuscin (LF) content were determined. *Results:* Riboflavin significantly prolonged the lifespan (Log rank $\chi 2=16.677$, $P<0.001$) and increased the reproductive capacity ($P<0.01$ for day 15; $P<0.05$ for day 30) of fruit flies by lifelong supplement. The survival time of fruit flies damaged by H_2O_2 was significantly prolonged (Log rank $\chi 2=15.886$, $P<0.001$), the activity of SOD1 ($P<0.01$) and CAT ($P<0.01$) was enhanced, and the accumulation of LF ($P<0.01$) was inhibited by riboflavin supplement. *Conclusion:* Riboflavin prolonged the lifespan and increased the reproduction of fruit flies through anti-oxidative stress pathway involving enhancing the activity of SOD1 and CAT and inhibiting LF accumulation. Riboflavin deserves more attention for slowing human aging.

Key words: Riboflavin, aging, lifespan, reproduction, oxidative stress.

Introduction

Riboflavin, one of the human essential vitamins, is the central component of the cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), and as such required for a variety of flavoprotein enzyme reactions (1). It is essential for cellular functions, growth and development. There are reports from as early as the 1940s of various congenital malformations associated with riboflavin deficiency (2). Diverse skeletal and soft tissue abnormalities are well described in the offspring of rats and mice fed riboflavin-deficient diets (3). Furthermore, riboflavin deficiency has been recently related to variety of disease, and riboflavin supplement was reported to protect against these disease, such as neurodegeneration (4), cancer (5), stroke (6) and cardiovascular diseases (7).

More than 70% of people over 65 years suffer from at least two chronic diseases such as heart disease, stroke, cancer, arthritis and diabetes (8). Though riboflavin deficiency has been observed in elderly people with high proportion (9-11), little attention regarding riboflavin deficiency is paid. Recent interest in the putative role of riboflavin in protecting against various diseases inspires us to wonder whether it will be beneficial to supplement riboflavin lifelong, and further slow the process of aging.

The purpose of the present study was to assess the anti-aging effect of lifelong supplement of riboflavin in Drosophila melanogaster (D. melanogaster, fruit fly). Meanwhile, the mechanism through anti-oxidative stress pathway was further studied.

Materials and Methods

Material

Riboflavin (R9504) and antiseptic (1% Ethyl-4-hydroxybenzoate in 75% alcohol) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Glucose (63005518), agar (10000582), hydrogen peroxide (H_2O_2) (Lot: 20120601), Chloroform (40064966) and methanol (40064292) were purchased from Sinopharm Chemical Regent Co., Ltd (Shanghai, China); yeast was purchased from Angel Yeast Co., Ltd (Yichang, China); Superoxide dismutase (SOD) activity assay kit (S0103) was purchased from Beyotime Biotechnology Co., Ltd (Haimen, China). Catalase (CAT) activity assay kit (A007-1) was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Quinine sulfate (LQ3281) was purchased from Hefei Bomei Biotechnology Co., Ltd. (Hefei, China).

Fruit fly strain and culture conditions

Wild type Oregon K line of the D. melanogaster fruit fly was kindly provided by Professor Ze-Sheng Zhang of Tianjin University of Science and Technology, Tianjin, China. The fruit flies were housed in 50ml plastic vials containing 5ml culture medium, and the vials were kept at 25±1°C, 60±5% humidity on a 12h:12h light/dark cycle. The fruit flies were transferred to fresh culture medium twice a week. The basal culture medium consisted of 72g cornmeal, 72g glucose, 10g yeast, 6g agar, 40ml antiseptic and water to prepare 500ml of medium. The mixture was cooked and poured into vial (5ml in each).

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Table 1
Lifespan parameters in fruit flies following lifelong supplement of riboflavin

Group	Median lifespan (day)	Mean lifespan (day)	Chang from Control (%)	Log rank	Maximum lifespan (day)
Control	48.0±1.4	45.4±1.2	-	-	75.2±7.9
Riboflavin	51.0±1.7	51.8±1.4	14.1	$\chi 2$=16.677, P<0.001	86.6±6.0[a]

Maximum lifespan was calculated by the average lifespan of the longest surviving 10% of fruit fly population in each group; Data are presented as mean±S.D. (n=200). a. P<0.001 vs the control group.

Lifespan assay

A total of 400 male fruit flies (eclosion within 8 h) were randomly divided into 2 groups: the control group and the riboflavin group. The fruit flies in the riboflavin group were fed with culture medium supplemented with riboflavin at the final concentration of 120μg/ml. The fruit flies in the control group were fed with basal culture medium. The number of dead fruit flies were recorded every 3 days until all died. The survival time was observed. The lifespan curve was drawn, and the median and mean lifespans were calculated. Maximum lifespan was calculated by the average lifespan of the longest surviving 10% of the fruit fly population.

Reproduction assay

Reproduction was chosen as another index of aging to assess the anti-aging effect of riboflavin. With different sexes separated strictly, both male and female fruit flies (eclosion within 8 h) were divided into groups and housed as described above. On days 15 and 30, one couple of fruit flies from the same group was merged into one vial containing corresponding culture medium. After consecutively housing for another 7 days, the parent fruit flies were expelled. Since the first fruit fly of the filial generation eclosion, the amount of fruit flies eclosion in the following 7 days was counted and the sex ratios were calculated. Ten parents of fruit flies were performed for each group.

Survival time (H_2O_2 exposure) assay

Oxygen-containing free radicals are considered to be involved in the mechanisms of aging (12). H_2O_2 is usually used to mimic oxidative stress (13). The present experiment was designed to examine the protective effect of riboflavin against acute oxidative stress induced by H_2O_2 in fruit flies. Male fruit flies (eclosion within 8 h) were divided into groups and housed as described above. On day 30, the fruit flies were first starved for 2 h, and then transferred into new vials containing a filter paper saturated with 9% H_2O_2 in a 6% glucose solution. The numbers of dead fruit flies were recorded every 4 h until all fruit flies died.

Enzymes assay

To eluciate the mechanisms responsible for the lifespan prolonging effect of riboflavin on fruit flies, we examined the effect of riboflavin on the activity of the antioxidant enzymes, SOD and CAT and the lipofuscin (LF) content. Male fruit flies (eclosion within 8 h) were divided into groups and housed as described above. On day 0, 25 and 45, the fruit flies were collected for the determination. Copper-zinc-containing SOD (SOD1) activity, manganese containing SOD (SOD2) activity and CAT activity were detected according to the manufacturer's instructions. The LF concentration was determined as previously described (14). Briefly, the fruit fly homogenate was extracted with chloroform:methanol (2:1, v/v) and centrifuged at 3000×g for 10min. The absorbance (excitation, 330nm and emission, 410nm) was measured using a Hitachi Fluorospectrophotometer-850 (Tokyo, Japan). Standardization was carried out by a freshly prepared solution of 0.1μg/ml quinine sulfate as 10U. The LF concentration was calculated from the fluorescence of 0.1μg/ml quinine sulfate and expressed as U/mg body weight.

Statistical analysis

Data are expressed as mean±S.D. Comparisons among groups were made by analysis of variance (ANOVA) followed by Dunnett's-t test. The Log-rank test was used to evaluate the equality of survival curves. P<0.05 was considered to indicate statistically significant difference.

Results

Riboflavin prolonged the lifespan of fruit flies

As shown in Fig. 1, lifelong supplement of riboflavin at the concentration of 120μg/ml significantly prolonged the lifespan of fruit flies. The median and the mean lifespan parameters shown in Table 1 confirmed the result. The riboflavin supplement prolonged the mean lifespan of fruit flies by 14.1% (Table 1).

Riboflavin strengthened the reproduction of fruit flies

No matter the fruit fly parents obtained from day 15 or 30, the amount of the total first filial generation (P<0.01 for day 15; P<0.05 for day 30) and the male (P<0.05 for day 15; P<0.05 for day 30) were significantly increased by riboflavin supplement, respectively (Fig. 2). Furthermore, the male proportion in the offspring was up-regulated by riboflavin supplement (P<0.05) (Table 2). No effect of riboflavin supplement was observed on the amount of the female offspring (Fig. 2).

Figure 1

Lifelong supplement of riboflavin prolonged the lifespan of fruit flies. Fruit flies received lifelong supplement of riboflavin at the concentration of 120μg/ml. Fruit flies in the control group were fed with basal culture medium. The numbers of dead fruit flies were recorded every 3 days until all died. Data were analyzed by the Log-rank test (n=200)

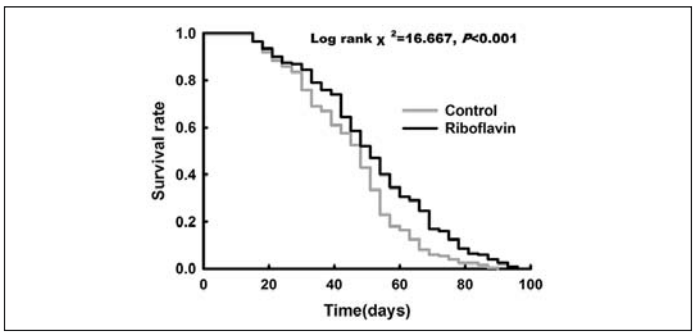
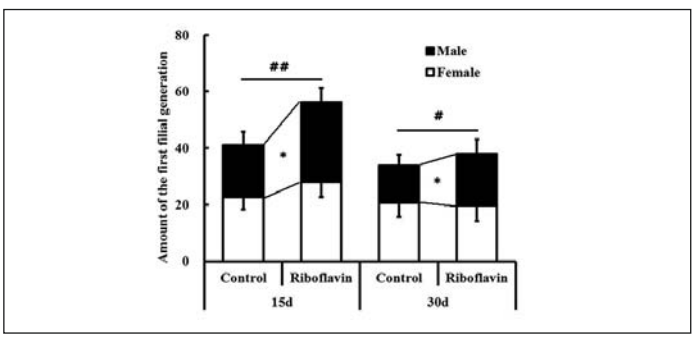


Figure 2

Riboflavin strengthened the reproduction of fruit flies. With different sexes separated strictly, fruit flies (eclosion within 8 h) were grouped and housed. On days 15 and 30, one couple of fruit flies were merged for 7 consecutive days. The amount of the first filial generation eclosion in the following 7 days was counted. Data are presented as mean±S.D. (n=10 couples), and were analyzed by ANOVA followed by Dunnett's-t test. *P<0.05 compared with the amount of male fruit flies in the control group at the same time point; #P<0.05, ##P<0.01 compared with the total amount of fruit flies in the control group on the same time point

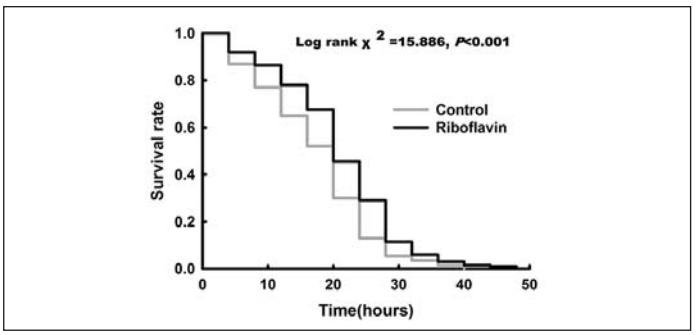


Riboflavin prolonged the survival time of fruit flies exposed to H_2O_2

The results showed that riboflavin supplement at the concentration of 120μg/ml significantly prolonged the survival time of the fruit flies exposed to H_2O_2 (Fig. 3). Riboflavin supplement significantly prolonged the mean survival time of the fruit flies by 20% (Table 3). These results suggested lifelong supplement of riboflavin promoted the ability of anti-oxidative stress in fruit flies.

Figure 3

Riboflavin inhibited damage of fruit flies exposed to H_2O_2. Fruit flies (eclosion within 8 h) were grouped and housed. On day 30, after starving for 2 h, fruit flies were transferred to new vials containing a filter paper saturated with 9% H_2O_2 in a 6% glucose solution. The numbers of dead fruit flies were recorded every 4 h until all died. Data were analyzed by the Log-rank test (n=200)



Riboflavin enhanced the activity of some antioxidant enzymes

The activity of total SOD was found significantly decreased with age, and riboflavin supplement significantly inhibited the decreasing compared with the control group on both days 25 and 45 (Fig. 4A). The activity of SOD1 was enhanced by riboflavin supplement on day 45; though this trend was also observed on day 25, no statistical difference was found (Fig. 4B). No effect of riboflavin on the activity of SOD2 was found (Fig. 4C). Compared with the control group on day 45, riboflavin supplement significantly enhanced the activity of CAT (Fig. 4D). LF accumulation increased with age, and riboflavin supplement significantly inhibited this accumulation on day 45; though this trend was also observed on day 25, no statistical difference was found (Fig. 4E).

Discussion

Aging is a natural bio function degeneration process of organism. Slowing the process of human aging has been studied for centuries. As an essential nutriment for human, riboflavin has been related to aging. Riboflavin deficiency was reported in older individuals both in vivo (15) and in vitro (16). And the cause was reported not due to the intestinal transport of riboflavin (17). While, to the best of our knowledge, this is the first time the effect of riboflavin supplement on physiological aging was observed.

As we mentioned previously (18), the various advantages made D. melanogaster suitable for using as an organism model in the study of physiological processes affecting lifespan. In the present study, we chose D. melanogaster as an in vivo model to assess the effect of riboflavin supplement on aging. The result showed that riboflavin supplement significantly prolonged the lifespan of fruit flies.

To confirm the anti-aging effect of riboflavin, reproduction

Table 2
Reproduction parameters in fruit flies following riboflavin supplement

Group		The amount of the first final generation			Raising rate vs Control (%)			Ratio of the amount of female to male
		♀	♂	Total	♀	♂	Total	
Control	15d	22.7±4.4	18.4±4.6	41.1±6.9	-	-	-	1.23±0.21
	30d	20.7±5.1	13.1±3.8	33.9±8.4	-	-	-	1.58±0.34
Riboflavin	15d	28.1±5.4	28.1±5.0[a]	56.3±11.0[b]	23.8	52.7	37.0	1.00±0.18[a]
	30d	19.6±5.3	18.4±5.1[a]	38.8±8.8[a]	-5.5	40.4	14.4	1.06±0.22[a]

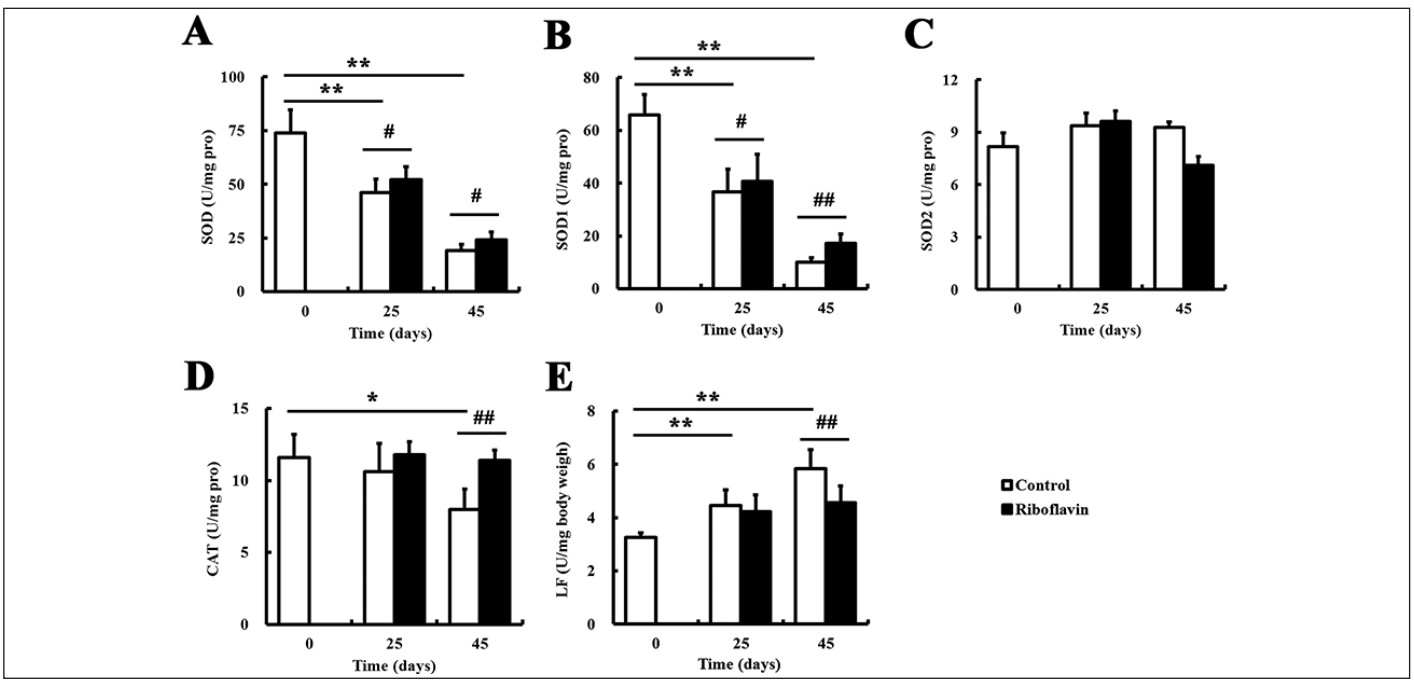
Data are presented as mean±S.D. (n=10 couples); a. P<0.05, b. P<0.01 vs the control group at the same time point.

Table 3
Survival time parameters in fruit flies exposed to H_2O_2 following riboflavin supplement

Group (mg/ml)	Median survival time (hour)	Mean survival time (hour)	Chang from Model (%)	Log rank (vs Model)
Model	20.0±0.7	17.4±0.6	-	-
Riboflavin	20.0±0.7	20.9±0.6	20	$\chi 2$=15.886, P<0.001

Data are presented as mean±S.D. (n=200).

Figure 4
Effect of riboflavin on the antioxidant enzymes in fruit flies. Fruit flies (eclosion within 8 h) were supplement with 120μg/ml riboflavin. Fruit flies were grouped and housed. On days 0, 25 and 45, fruit flies were collected for the detection of the activity of the following antioxidant enzymes. Data are presented as mean±S.D. Data were analyzed by ANOVA followed by Dunnett's-t test. n=6,*P<0.05, **P<0.01 compared with day 0; #P<0.05, ##P<0.01 compared with the control group on the same time point. (A) Riboflavin enhanced the activity of total SOD of fruit flies on both days 25 and 45. (B) Riboflavin enhanced the activity of SOD1 of fruit flies on day 45. (C) Riboflavin had no influence on the activity of SOD2 in fruit flies. (D) Riboflavin enhanced the activity of CAT in fruit flies on day 45. (E) LF accumulation increased with age, and riboflavin supplement significantly inhibited this accumulation on day 45



was chose as another index to assess the anti-aging effect of riboflavin supplement, as it is consensus that reproduction is negatively correlated with aging (19-20). Riboflavin deficiency was reported to result in adverse reproductive outcomes (21), and increase the incidence of congenital abnormalities during pregnancy (22-23). We showed that riboflavin supplement to parents significantly increased the amount of the offspring of fruit flies. This result further verified the anti-aging effect of riboflavin supplement. These closely implied that riboflavin had the probability to be exploited for anti-aging application. Interestingly, except for the total amount of offspring, riboflavin supplement increased the male proportion of the offspring of fruit flies. No more information about this effect could be obtained till now.

The anti-aging effect of riboflavin prompted us to ask what will be the mechanism. Since accumulated literatures focus on oxidative stress as a criminal of aging and degenerative diseases along with aging (24-25), and previous reports showed riboflavin might have the effect of anti-oxidative stress (26-27), we investigated the anti-oxidative stress effect of riboflavin in fruit flies to further understand the mechanism. H_2O_2 induced acute oxidative stress damaged model was used to assess the anti-oxidant stress effect of riboflavin in this study. The longer survival time of fruit flies with riboflavin supplement suggested the promoted ability of anti-oxidative stress by riboflavin.

Oxidative stress is the consequence of increased production of reactive oxygen species (28), and causes progressive structural and functional alterations of cellular organelles. Antioxidants inhibit oxidative stress, and thus inhibit the process of aging (29). Endogenous enzymes superoxide dismutase (SOD) and catalase (CAT) are involved in the antioxidant defense network of human (30). LF accumulation has been related to age-dependent mortality (31), and thus is deemed to be a hallmark of aging (32). Our result, the enhanced activity of SOD1 and CAT and the inhibited accumulation of LF by riboflavin supplement, suggested that the anti-aging effect of riboflavin was associated, at least in part, with the enhanced activity of endogenous antioxidant enzymes in fruit flies.

Considering nontoxicity of riboflavin in vivo has been reported even at high dose, it is possible to intake riboflavin for long term to slow the process of human aging. No evidence for riboflavin toxicity after excessive intakes has been observed in human, as the low solubility of riboflavin keeps it from being absorbed in dangerous amounts within the digestive tract (33). Even 400mg of riboflavin intake per day for three months, no short-term side effects were reported (34). The excessive riboflavin excretes through excrement and urine.

In conclusion, riboflavin supplement prolonged the lifespan and increased the reproductive capacity of fruit flies through anti-oxidative stress involving enhancing the activity of SOD1 and CAT and inhibiting the accumulation of LF. If the relationship between riboflavin and anti-aging could be confirmed and the detailed mechanism could be clarified in more future studies, it will provide a novel release strategy for slowing human aging. Furthermore, the study reminds us not to neglect riboflavin deficiency in human aging and pregnant.

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Conflict of interest: The authors declare that there is no conflict of interest.

Ethical Standards: The experiments in this study comply with the current laws of the country in which they were performed.

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