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# 꼬마선충에서 메밀 추출물에 의한 산화성 스트레스 저항성 증가 및 수명 연장 효과

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# Buckwheat Extract Increases Resistance to Oxidative Stress and Lifespan in *Caenorhabditis elegans*

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**ABSTRACT : Buckwheat (*Fagopyrum esculentum*) has been known for having strong anti-oxidant, anti-mutagenic, and anti-carcinogenic activities. The free radical theory of aging, also known as the oxidative stress theory of aging, claims that cellular oxidative damage accumulated with time is a major causal factor of aging. In the present study, we investigated the effect of buckwheat extracts on resistance to oxidative stress and aging using *Caenorhabditis elegans* as a model system. Survival under an oxidative-stress condition induced by paraquat increased markedly following 500 ㎎/L buckwheat extracts treatment, suggesting lower cellular oxidative damage by buckwheat extracts. A lifespan assay also revealed that treatment of buckwheat extracts significantly extended both the mean and maximum lifespan in *C. elegans*. Interestingly, this lifespan-extension by buckwheat extracts was not accompanied by reduced fertility. These findings suggest that buckwheat extracts can confer longevity phenotype to *C. elegans* through its strong anti-oxidant activity and support the aging theory which emphasizes a pivotal role of oxidative stress during aging.**

***Key Words*** **:** Buckwheat, Oxidative Stress, Lifespan, Fertility, *C. elegans*

## INTRODUCTION

Buckwheat (*Fagopyrum esculentum*) is composed of 10-15% proteins, 2-3% lipids, 65-70% carbohydrates, several minerals, and vitamins (Hwang *et al*., 2006). Phenolic compounds, such as rutin, orientin, vitexin, quercetin, isovitexin, kaempferol-3-rutinoside, isoorientin, and catechins, are also contained in buckwheat (Havsteen, 1983). Among them, rutin has a strong anti-oxidant activity and results in the anti-oxidative, anti-carcinogenic, and anti-hemorrhagic properties of buckwheat (Kreft *et al*., 1994). Buckwheat meal lowers the glucose and insulin responses to a meal in healthy people, suggesting a potential beneficial role of buckwheat preventing diabetes and hyperglycemia (Koh *et al*., 2002). Extract from germinated seeds of buckwheat shows strong anti-oxidative and anti-microbial activities (Hwang *et al*., 2006). In addition, DNA oxidative damage occurred by hydroxyl radicals is significantly reduced by buckwheat honey (Zhou *et al*., 2012). Obesity-induced rats supplemented by buckwheat show increased cellular levels of antioxidant and antioxidant enzymes, including glutathione, glutathione peroxidase, and glutathione S-transferase, as well as decreased oxidative stress (Kim *et al*., 2012).

A number of hypotheses have been proposed to explain the mechanistic basis of aging. In 1956, Dr. Denham Harman first introduced the free radical theory of aging, which postulates that normal aging is due to the accumulation of random deleterious oxidative damage to tissues (Harman, 1956). Oxidative damage is mainly contributed by reactive oxygen species (ROS), which are produced as byproducts of cellular respiration. ROS can be scavenged by cellular anti-oxidants, such as vitamin E, vitamin C, and glutathione. Nutritional anti-oxidants act through different mechanisms, including neutralization of free radicals, reduction of peroxide concentrations, repair of oxidized membranes, and decreased ROS production

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(Berger, 2005). Dietary interventions of anti-oxidants have received particular attention due to their potential role in modulating oxidative stress associated with aging. Vitamin E supplementation partially suppresses age-associated gene expression profiles in the mouse heart and brain (Park *et al*., 2008). Aging rat myocardium exhibits increased oxidant production, lower ascorbic acid, and a marked increase in 8-oxo-2'-deoxy-guanosine, and all of these changes are suppressed by α-lipoic acid supplementation (Suh *et al*., 2001). Pre-treatment with coenzyme $Q_{10}$ improves the functional recovery of senescent rat hearts after aerobic stress and the heart contractile function of elderly patients after cardiac surgery (Rosenfeldt *et al*., 2002). α-lipoic acid and coenzyme $Q_{10}$ supplementation inhibit age-related alterations in the expression of genes involved in the extracellular matrix, cellular structure, and protein turnover, but have no impact on longevity or tumor patterns compared with those in control mice (Lee *et al*., 2004). Dietary supplementation with acetyl-L-carnitine in rats reverses the age-associated decline in mitochondrial function (Hagen *et al*., 1998). Global gene expression profiling has revealed several tissue-specific transcriptional biomarkers of aging, and dietary supplementation of anti-oxidants markedly retards the age-related changes in expression of these biomarkers of aging in mice (Park *et al*., 2009).

In the present study, we examined the effect of buckwheat extracts on resistance to oxidative stress to validate its antioxidant activity *in vivo*. We used paraquat (methyl viologen dichloride hydrate) as an oxidative stress inducer. Paraquat is widely used as a herbicide and can produce ROS acting as an electron acceptor (Bus and Gibson, 1984). The effect of buckwheat extracts on normal aging was also studied using *Caenorhabditis elegans* as a model system. *C. elegans* is a free living nematode widely used in various biological studies. It can be grown in the laboratory easily on agar plates containing *E. coli* as a food source and produce 300 eggs on average during their reproductive period. The life cycle and lifespan of *C. elegans* are relatively short (Wood, 1988). These make *C. elegans* as a good experimental system especially for aging researches. Here, mean and maximum lifespan of *C. elegans* and the number of progeny produced were compared between control and buckwheat extract-treated animals.

## MATERIALS AND METHODS

### 1. Sample preparation and worm culture

Water extracts of buckwheat powder were filter-sterilized using 0.2 ㎛ cellulose acetate (hydrophilic) filters (Advantec, Japan). The *C. elegans* wild-type N2 CGCb strain was used as the model system for all experiments. Worms were cultured on NGM plates (1.7% agar, 2.5 ㎎/㎖ peptone, 25 mM NaCl, 50 mM $KH_2PO_4$ (pH 6.0), 5 ㎍/㎖ cholesterol, 1 mM $CaCl_2$, and 1 mM $MgSO_4$) containing *E. coli* OP50 as the food source. All experiments were performed at 20℃.

### 2. Resistance to oxidative stress

The effect of five different concentrations of buckwheat water extracts (0, 50, 100, 500, and 1000 ㎎/L) on resistance to oxidative stress was tested *in vivo*. Age-synchronized 3-day-old young adult worms were placed on small NGM plates containing the different concentrations of buckwheat extracts. All adult worms were removed from the plate after 4 hours. The eggs were hatched, and the worms were grown to adults at 20℃. After 3 days, adult worms were transferred to fresh NGM plates containing the buckwheat extracts and 20 mM paraquat (Sigma-Aldrich, St. Louis, MO) which induces oxidative stress *in vivo*. Survival of the worms treated with paraquat was monitored three times per day until all worms were dead. A worm was scored as dead when it did not respond to mechanical stimulation. Worms were transferred to fresh NGM plates with buckwheat and paraquat every 2 days. Sixty worms were scored in each group.

### 3. Lifespan assay

Longevity assessments were performed with age-synchronized N2 hermaphrodites on NGM plates at 20℃. Five L4/young adult worms cultured on NGM plates were transferred to a fresh NGM plate and allowed to lay eggs for 4 hours. After removing five adults, the eggs were incubated at 20℃. Thirty young adults were picked 3 days after hatching and transferred to a fresh NGM plate. 12.5 ㎍/㎖ of 5-fluoro-2'-deoxyuridine (Sigma-Aldrich, St. Louis, MO) was added to the NGM plates to prevent progeny from hatching. Thereafter, worms were transferred every 2-3 days until all worms were dead. Dead worms were scored daily and removed from the plates

immediately. We compared the lifespan of worms treated with 500 ㎎/L of buckwheat extracts with that of control worms.

### 4. Determining fertility

Hermaphrodite fertility was monitored by daily transfer of ten hermaphrodites to individual fresh, spotted, NGM plates and subsequent progeny were counted 2 days later. This process was repeated until no hatched worms were found on the NGM plates. We examined the effect of a 500 ㎎/L buckwheat extracts treatment on worm fertility.

### 5. Statistical analysis

The log-rank test was employed for the statistical analysis of resistance to oxidative stress and lifespan assay. The log-rank test, also called as Mantel-Cox test, is a nonparametic test frequently used for comparing lifespan of two groups (Peto and Peto, 1972). In the statistical analysis of lifespan assay, we excluded worms lost or killed during the assay. For fertility assay, we calculated p-value using standard two-tailed student t-test.

## RESULTS AND DISCUSSION

### 1. Anti-oxidant activity of buckwheat extracts

We induced oxidative stress in young adult worms using paraquat to measure the anti-oxidant activity of buckwheat extracts. Then, survival of worms was compared between control and experimental groups treated with different concentration of buckwheat extracts. There was no difference in time required for larval development and size and growth of the worms between control and buckwheat-treated worms. The mean survival time of control worms was 42.0 h, and the buckwheat extracts significantly increased mean survival time at higher concentrations (Table 1). Mean survival times of the 500 and 1000 ㎎/L buckwheat extracts-treated worms were 68.9 and 69.1 h, respectively. Both concentrations of buckwheat extracts increased mean survival up to 64% ($p < 0.001$). A replicative experiment showed markedly increased resistance to oxidative stress by all concentrations of buckwheat extracts tested. The most effective concentration of buckwheat extracts in the replicative experiment was 500 ㎎/L as shown in Table 1. Mean survival time increased from 61.8 to 88.2 h ($p < 0.001$), and % effect was 42.8%.

**Table 1.** Increased resistance to oxidative stress following treatment with different concentrations of buckwheat extracts.

| | Conc. (㎎/L) | N | Mean survival time (h) | *p*-value** | % effect* |
|---|---|---|---|---|---|
| 1st experiment | 0 | 48 | 42.0 | | |
| | 50 | 60 | 48.8 | 0.042 | 16.4 |
| | 100 | 60 | 43.8 | 0.938 | 4.3 |
| | 500 | 60 | 68.9 | <0.001 | 64.2 |
| | 1000 | 60 | 69.1 | <0.001 | 64.6 |
| 2nd experiment | 0 | 35 | 61.8 | | |
| | 50 | 60 | 74.8 | 0.004 | 21.1 |
| | 100 | 37 | 83.9 | <0.001 | 35.9 |
| | 500 | 60 | 88.2 | <0.001 | 42.8 |
| | 1000 | 60 | 82.2 | <0.001 | 33.0 |

*% effect was calculated as [(B-C)/C] × 100, where B is the mean survival time of worms treated with each concentration of buckwheat extracts and C is the mean survival time of control worms (0 ㎎/L).
**$p$-value was determined using the log-rank test and $p$-value lower than 0.05 was considered significant.

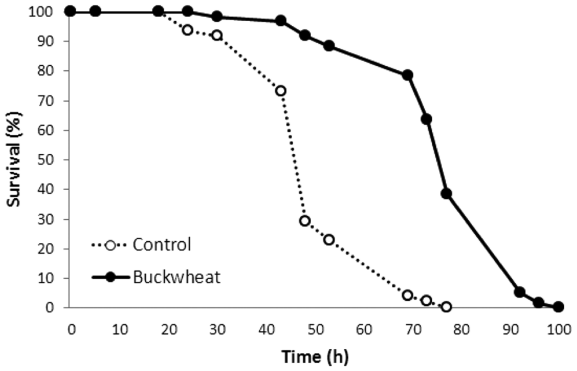


**Fig. 1. Resistance to oxidative stress induced by paraquat was compared between the control group and the buckwheat group.** The buckwheat group was treated with 500 ㎎/L of buckwheat extracts. Buckwheat significantly increased resistance to oxidative stress in *C. elegans* ($p < 0.001$).

Figure 1 shows the survival curve of the control and 500 ㎎/L buckwheat extracts-treated worms. These findings suggest that the buckwheat extracts have a strong anti-oxidant activity and can increase the survival of worms under oxidatively-stressed conditions *in vivo*. Recent study shows that buckwheat has strong antioxidant activities including hydroxyl radical and superoxide anion radical scavenging activities due to its phenolic and flavonoid contents (Sedej *et al.*, 2012). Secondary metabolites of buckwheat play a key role in the antioxidant activity of buckwheat (Kreft *et al.*, 1994). Further study focusing on the cellular pathways or organelles involved in the anti-

oxidant activity of the buckwheat extracts will be helpful to understand the underlying mechanisms of increased resistance to oxidative stress following buckwheat extracts treatment.

### 2. Longevity phenotype induced by buckwheat extracts

The free radical theory of aging suggests that oxidative stress plays a pivotal role in normal aging and determines of lifespan of an organism (Sohal and Weindruch, 1996; Beckman and Ames, 1998). Based on our finding that the buckwheat extracts reduced susceptibility to oxidative stress in *C. elegans*, we next examined the effect of the buckwheat extracts on *C. elegans* lifespan. As the 500 ㎎/L of buckwheat extracts showed the strongest anti-oxidant activity in the previous assay, we monitored the lifespan of worms treated with 500 ㎎/L of buckwheat extracts. The mean and maximum lifespans of control worms were 19.7 and 27 days, respectively. Both mean and maximum lifespan were extended significantly by treating worms with the buckwheat extracts (Table 1). The mean lifespan of worms treated with the buckwheat extracts increased to 22.6 days and their maximum lifespan was 30 days ($p < 0.001$). The percent lifespan-extending effect of buckwheat calculated using the mean lifespan was 14.4%. In the replicative experiment, mean lifespan increased from 19.3 days to 24.0 days (23.9% lifespan-extending effect) and maximum lifespan was extended from 27 days to 30 days ($p < 0.001$).

As shown in Fig. 2, the survival curve of *C. elegans* shifted to the left following treatment with the buckwheat extracts, suggesting that strong anti-oxidant activity leads to extended mean and maximum lifespan of *C. elegans*. These data support our hypothesis that increased resistance to oxidative stress provided by the buckwheat extracts can modulate the aging process and eventually confer a longevity phenotype in *C. elegans*. The lifespan-extending effect of buckwheat is reported here for the first time, and these results can be applied directly to mammalian studies and used to develop novel anti-aging nutritional supplements or natural therapeutic compounds.

**Table 2.** Effect of the buckwheat extracts on *Caenorhabditis elegans* lifespan.

| | Treatment*** | Mean lifespan (day) | Maximum lifespan (day) | *p*-value** | % effect* |
|---|---|---|---|---|---|
| 1st experiment | Control | 19.7 | 27 | | |
| | Buckwheat | 22.6 | 30 | <0.001 | 14.4 |
| 2nd experiment | Control | 19.3 | 27 | | |
| | Buckwheat | 24.0 | 30 | <0.001 | 23.9 |

*% effect was calculated as [(B-C)/C] × 100, where B is the mean lifespan of buckwheat-treated worms and C is the mean lifespan of the control.
**$p$-value was determined using the log-rank test.
***The control group was grown in NGM plates containing no buckwheat extracts and the buckwheat group was grown in NGM plates supplemented with 500 ㎎/L of buckwheat extracts.

### 3. Effect on fertility

Many *C. elegans* genetic mutants with an extended lifespan show decreased reproductive activity, such as a reduced number of progeny and a delayed reproductive period (Larsen *et al*., 1995; Gems *et al*., 1998; Hughes, *et al*., 2007). It is believed that this phenomenon might be due to a trade-off of cellular resources between aging and reproduction. Long-lived mutants seem to re-locate their cellular resources from reproduction to somatic maintenance. We were interested in whether treatment of worms with the buckwheat extracts also accompanied reduced reproduction as previously observed in several long-lived mutants. Interestingly, worm fertility was not affected by the buckwheat extracts (Fig. 3). The total number of progeny produced during the gravid period decreased slightly in worms treated with 500 ㎎/L of buckwheat extracts, but was not significantly different from that of the control. The total number of progeny in the control was $175 \pm 30.4$ (mean ± SD, $n = 9$) and that in the buckwheat-treated worms was $157 \pm 36.2$ ($n = 7$) ($p = 0.298$).

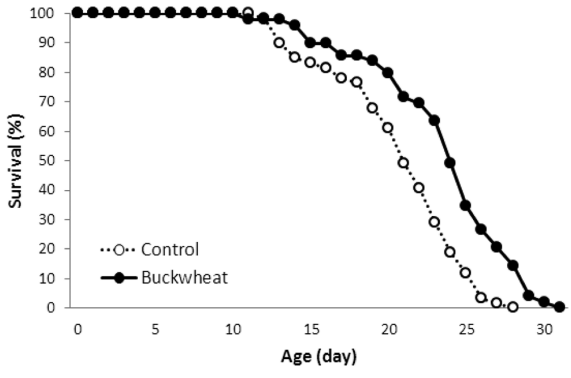


**Fig. 2. Buckwheat extended *Caenorhabditis elegans* lifespan.** 500 ㎎/L of buckwheat extracts was added to NGM plates in the buckwheat group. Buckwheat significantly increased both mean and maximum lifespan in *C. elegans* ($p < 0.001$).

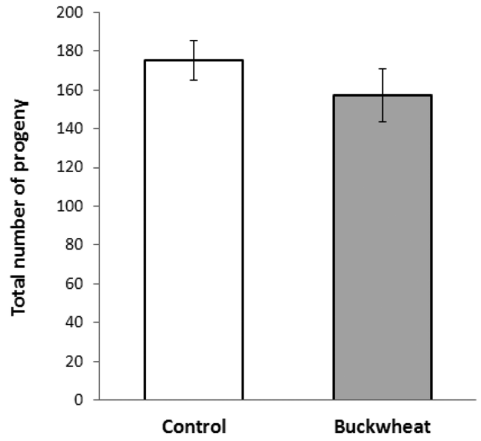


**Fig. 3. Total number of progeny produced was compared between the control and the buckwheat (500 ㎎/L) group.** Error bars indicate standard errors (SE).

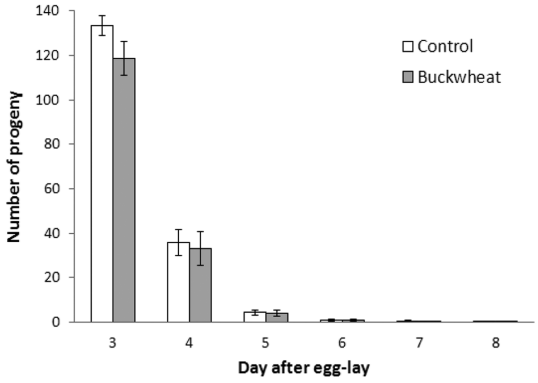


**Fig. 4. Time-course distribution of progeny production in the control and the buckwheat (500 ㎎/L) group.** Data are mean ±standard errors (SE).

The time-course distribution of progeny was also not significantly different between the control and the buckwheat-treated worms (Fig. 4). The number of progeny produced each day was similar between the two groups, and no delay in the reproductive period was observed. Taken together, we conclude that the buckwheat extracts have a strong *in vivo* anti-oxidant activity and can extend both mean and maximum lifespan in *C. elegans* without reducing fertility.

## ACKNOWLEDGEMENTS

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