Effects of ethanol intake on anti-oxidant responses and the lifespan of Caenorhabditis elegans

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1. Introduction

Liquor is a traditional beverage consumed by all ethnic groups globally and the most commonly consumed beverage. The raw material for the production of liquor is grain, which is fermented to produce alcohol. Alcoholism is a chronic disease that is considered an important public health issue by the World Health Organization.

The use of different model organisms to study the physiological effects and toxicity of alcohol has attracted the attention of many scholars. The genome of Caenorhabditis elegans contains highly conserved genes as determined by comparative genomic analysis; approximately 60% of human genes have an ortholog in C. elegans (Petersen, Dirksen, & Schulenburg, 2015). As a model organism with a short lifespan, C. elegans can be easily cultivated, handled, and observed microscopically (Goldstein, 2016). Thus, it has become the most popular model organism in scientific research. Many researchers have studied the aging mechanism of C. elegans. Tullet et al. (2017) used C. elegans to demonstrate that SKN-1 promotes longevity by a mechanism other than protection against oxidative damage. According to Upadhyay, Chompoo, Taira, Fukuta, and Tawata (2013), Alpinia zerumbet leaf extract enhanced stress resistance and prolong lifespan in C. elegans. Kumsta et al. (2014) reported that decreased expression of integrin-linked kinase increased resistance to heat stress and lifespan in C. elegans without substantially affecting cytoskeletal integrity. In recent years, researchers have found that low concentrations of alcohol have a certain effect on C. elegans longevity (Castro, Khare, Young, & Clarke, 2012; Chen, O’Halloran, & Kovacs, 2014; Roberson, Kuddo, Abebe, & Spong, 2013; Yu, 2011; Yu, Zhao, Ma, Fu, & Zhao, 2011). When a large amount of alcohol is consumed, a series of...
acute behaviors is triggered and a stress response is generally activated in *C. elegans*. Under this stressed state, the lifespan of the nematodes is shortened.

Nonetheless, how changes in antioxidant enzymes and oxygen free radicals in response to alcohol stress are correlated with shortened lifespan remains largely unknown. In this study, oxidative stress in *C. elegans* induced by different concentrations of alcohol and the relationship between alcohol exposure and movement and lifespan were investigated. We expected our data to serve as a reference for further screening of plant functional components that may reduce the toxic effects of alcohol.

### 2. Materials and methods

#### 2.1. Materials

Wild-type *C. elegans* strain N2 and *Escherichia coli* OP50 were obtained from the *Caenorhabditis* Genetics Center (CGC, University of Minnesota). Ethanol (99.98% purity), calcium chloride anhydrous (CaCl₂), sodium chloride (NaCl), sodium hydroxide (NaOH), magnesium sulfate (MgSO₄), peptone, agar, and yeast were purchased from Sinopharm Chemical Reagent Co., Ltd. 2,7-Dichlorodihydrofluorescein diacetate (H₂DCF-DA), superoxide dismutase (SOD), superoxide anion radical (O₂·⁻), and hydroxyl radical (–OH) kits were purchased from Nanjing Jiancheng Bioengineering Institute. Catalase (CAT), glutathione peroxidase (GSH-PX), apoptosis detection, and total antioxidant capacity kits were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. 5-Fluoro-2′-deoxyuridine (FUDR) was purchased from Sigma-Aldrich.

#### 2.2. Methods

##### 2.2.1. Culture of *C. elegans*

Synchronous basal and liquid cultures of *C. elegans* were grown according to Stiernagle (2006).

##### 2.2.2. Alcohol exposure

The alcohol exposure experiment was performed in liquid culture. According to the results of previous experiments (unpublished), alcohol induces a high mortality rate in nematodes when used at concentrations >5%, whereas it does not induce significant mortality at concentrations ≤1%. As this concentration range was not suitable for the purpose of this study, we selected final alcohol concentrations of 1–5%. *C. elegans* nematodes synchronized and grown to the larval L4 stage were sterilized by exposure to FUDR at a final concentration of 0.12 mM for 12 h. Then, the animals were divided into six treatment groups: a control group (without alcohol) and 1%, 2%, 3%, 4%, and 5% (v/v) ethanol-treated groups. An equal volume of the different alcohol solutions was added to the respective cultures, whereas an equal volume of sterilized water was added to the control group, with three repetitions for each group. Nematodes (2,000 ± 100/mL) were exposed to the respective treatments for 24 h at 20°C under gentle shaking. Each culture was sealed with sealing film to avoid error caused by the volatilization of ethanol. After the treatments, a small portion of the culture was used to observe movement of the nematodes. The remaining culture was centrifuged at 1,150 × g for 2 min at 4°C, and the nematodes were collected for the determination of other indicators.

##### 2.2.3. Behavioral analysis

After treatments, ten nematodes from each group were transferred to fresh nematode growth medium (NGM) plates and incubated at 20°C for 5 min. Head swings and pharyngeal swallows in every nematode were observed and recorded at intervals of 30 s under a stereo microscope equipped with video-recording software. Averages were calculated. In addition, the motion trajectory of each nematode was recorded using the stereomicroscope, and the tracks of all groups were analyzed.

##### 2.2.4. Determination of CED-3 and CED-9

The apoptosis protein CED-3 and anti-apoptosis protein CED-9 in the homogenate of alcohol-treated *C. elegans* were quantified using the respective apoptosis detection kits according to the manufacturer’s instructions. The standard curves revealed the relationship between absorbance and the CED-3 concentration between 0 and 20 ng/mL (Y = 12.29X−1.7291, R² = 0.9952) and absorbance and the CED-9 concentration between 0 and 12 ng/mL (Y = 6.609X−0.8801, R² = 0.9975). The amounts of CED-3 and CED-9 in each sample were calculated according to the respective standard curves. Cell apoptosis in *C. elegans* was detected by acridine orange (AO) staining according to the method of Plemel et al. (2017).

##### 2.2.5. Antioxidant enzyme assay

H₂DCF-DA was used as a fluorescent probe to detect reactive oxygen species (ROS) in *C. elegans*. The contents of GSH-PX, SOD, CAT, O₂·⁻, and –OH and total antioxidant capacity of *C. elegans* were determined using assay kits according to the manufacturers’ instructions.

##### 2.2.6. Lifespan assessment

The liquid culture method reported by Solis and Petrascheck (2011) was used to determine the lifespan of nematodes. Briefly, late L4 larvae were divided into six treatment groups (A–F). The larvae were seeded in 96-well microtiter plates at 20 larvae/well in 120 μL of suspension and were cultured. For each treatment, there were eight experimental repetitions. The plates were sealed using adhesive tape to avoid contamination and evaporation. The plates were shaken on a microtiter plate shaker for 2 min and then incubated at 20°C. The tape was removed every 4 days under a sterile environment to allow fresh oxygen to enter the culture wells. Nematode survival was evaluated every 2 days by observing movement. Strong light, especially blue light, triggers movement in nematodes and was therefore used to determine the viability of the animals. Data were recorded until all the nematodes in each well had died. Kaplan–Meier analysis was used to analyze survival after treatments.

##### 2.2.7. Statistical analysis

The data are expressed as the mean ± SD. Means were compared using one-way ANOVA. *P* < 0.05 was considered significant, and *P* < 0.01 was considered extremely significant. All analyses were performed using SPSS 18.0 software.

### 3. Results

#### 3.1. Alcohol at high concentration reduces nematode movement

Compared with that in the control group, the pharynx pumping rate was significantly increased in the 1% alcohol
group \( (P < 0.01) \), not significantly different in the 2% and 3% alcohol groups, and significantly decreased in the 4% and 5% alcohol groups (Figure 1). In the experimental groups, pharynx pumping decreased with increasing alcohol concentration. Furthermore, high levels of alcohol (4–5%) significantly reduced the frequency of nematode head bending. Nematodes cultured with relatively low concentrations of alcohol (1–3%) showed no significant difference in the frequency of head bending. Therefore, the frequency of head bending decreases with increasing concentration of alcohol.

The effect of alcohol at different concentrations on the motion trajectory of nematodes is illustrated in Figure 2. Table 1 shows the wavelength of the motion trajectory of the nematodes \( (n = 15) \). High concentrations of alcohol \( (\geq 3\%) \) significantly impacted the trajectory of nematodes, whereas low concentrations did not. In the presence of 3% alcohol, the wavelength was significantly shorter than in the absence of alcohol \( (P < 0.01) \) and the degree of body bending was increased. At 4% concentration, the nematodes adopted abnormal body postures, and the trajectory became ring-shaped with a decreased crawl speed. At the highest alcohol concentration of 5%, the nematodes were mostly stationary, stiff, and cowered, and the trajectory was abnormal, with no statistically observable wavelength. Based on these results, nematodes are resistant to the biological toxicity of alcohol to a certain extent, but not at high concentrations.

### 3.2. Effect of alcohol on apoptosis in C. elegans

CED-3 and CED-9 protein levels in C. elegans after exposure to alcohol at different concentrations are shown in Figure 3. All alcohol-treated groups demonstrated increased CED-3 and CED-9 expression compared with the control group. The groups exposed to lower alcohol concentrations exhibited the highest expression, which tapered off with increasing levels of alcohol. CED-9 and CED-3 protein levels in C. elegans treated with 2% alcohol were 1.19 and 1.20 times \( (P < 0.01) \) those in the control group, respectively. As seen in Figure 3, the ratio of CED-9 to CED-3 in each test group was either increased or decreased, indicating a considerable effect from alcohol. The control and test group nematodes exhibited uniform dark green or yellow-green fluorescence. No stain-enhanced pyknosis, blob shape, or cylindrical apoptotic nuclear morphology was observed (Figure 4), indicating that alcohol did not cause significant cell apoptosis.

### 3.3. Effect of alcohol on antioxidant properties in C. elegans

#### 3.3.1. Effect of alcohol on ROS

The ROS content in C. elegans was estimated based on DCF fluorescence intensity and is shown relative to the content in the control group, which was set as 100%, in Figure 5a. The alcohol-treated groups had lower ROS contents than the control group (except 3% group) \( (P < 0.01) \). ROS levels in nematodes tended to increase under low alcohol concentration \( (<3\%) \), but with an increase in alcohol concentration \( (>3\%) \), ROS levels tended to decrease. The 1% alcohol treatment group exhibited a 46.16% decrease in ROS content compared with the control group. In the 3% alcohol treatment group, ROS production and clearance in the

### Table 1. Trajectory wavelength and swing of nematodes exposed to different concentrations of alcohol.

| Index         | Control \((n = 15)\) | 1%    | 2%    | 3%   | 4%   | 5%   |
|---------------|----------------------|-------|-------|------|------|------|
| Wavelength \((\mu m)\) | 206.40 ± 10.06 | 202.00 ± 1.798 | 203.60 ± 7.73 | 177.20 ± 6.374** | 148.40 ± 8.182** | –    |

\* \( P < 0.05 \), \** \( P < 0.01 \).
nematodes were in dynamic balance, without a significant difference from the control group. At 5% alcohol, the content of ROS was 47.40% lower than that in the control group.

3.3.2. Effects of alcohol on antioxidant enzyme activities
SOD activity increased with increasing alcohol concentration up to 3% but tended to decrease at higher concentrations (>3%) (Figure 6a). The results demonstrated that a relatively low dose of alcohol increased the total SOD activity, whereas at high concentration, alcohol reduced the total SOD activity in the nematodes. SOD is an important antioxidant enzyme that scavenges superoxide anion radicals to produce hydrogen peroxide or oxygen. The stress reaction induced by alcohol at low concentration can promote total SOD activity and O$_2^{-}$ scavenging over time in nematodes. The high-concentration alcohol-treated

![Figure 2. Effects of different concentrations of alcohol on C. elegans trajectories.](image)

Figure 2. Efectos de diferentes concentraciones de alcohol en las trayectorias de C. elegans.

Locomotion trajectories of (A) control nematodes and (B–F) nematodes treated with 1%, 2%, 3%, 4%, and 5% alcohol, respectively. *P < 0.05, **P < 0.01.

Trayectorias de locomoción de (A) nematodos de control y (B – F) nematodos tratados con 1%, 2%, 3%, 4% y 5% de alcohol, respectivamente. * P < 0.05, ** P < 0.01.

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![Figure 3. Effects of alcohol on CED-3 and CED-9 protein expression in C. elegans.](image)

Figure 3. Efectos del alcohol en la expresión de las proteínas CED-3 y CED-9 en C. elegans.

*P < 0.05, **P < 0.01 compared with the control group; ΔP<0.05, ΔΔP<0.01 compared with the control group.

* P < 0.05, ** P < 0.01 en comparación con el grupo de control; ΔP <0.05, ΔΔP <0.01 en comparación con el grupo de control.
groups may have exhibited decreased mitochondrial $O_2^-$ production owing to the slowing down of the biological rhythm. This in turn slowed SOD activity, which was nonetheless significantly higher than that in the control group. CAT and GSH-PX eliminate the main oxidation products produced in vivo and prevent them from producing more strongly oxidized hydroxyl radicals ($^\cdot$OH). GSH-PX and CAT activities were significantly increased in the test group nematodes (Figure 6a), indicating that a stress response was initiated in response to alcohol. In the 2% alcohol group, CAT and GSH-PX activities were increased by up to 1.71 and 1.63 times those in the control ($P < 0.01$), respectively.

The total antioxidant capacity is the sum of anti-oxidative molecules and enzyme levels in a system and serves as an index to measure the effect of lipid peroxidation. It comprehensively reflects the ability of an organism to scavenge ROS. As seen in Figure 6b, the total antioxidant capacity of the test groups rose significantly, which coincided with the increased activities of the three antioxidant enzymes. At alcohol concentrations <2%, the total antioxidant capacity of nematodes increased with increasing alcohol concentration. In groups exposed to alcohol at concentrations >2%, the total antioxidant capacity decreased with increasing alcohol concentration.

### 3.3.3. Effect of alcohol on free radical inhibition

$O_2^-$ and $^\cdot$OH are the most important causes of lipid peroxidative nucleic acid breakage, which at excessive levels can accelerate body aging. Therefore, the ability to inhibit the production of $O_2^-$ and $^\cdot$OH is an important indicator of the resistance to damage caused by intermediate oxidation by-

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**Figure 4.** Effects of alcohol on apoptosis in nematodes.

**Figura 4.** Efectos del alcohol sobre la apoptosis en nematodos.

Bright-field images of (A1) control nematodes and (B1–F1) nematodes exposed to 1%, 2%, 3%, 4%, and 5% alcohol, respectively. A2–F2 represent the corresponding dark-field images.

Imágenes de campo brillante de nematodos de control (A1) y nematodos (B1–F1) expuestos a alcohol al 1%, 2%, 3%, 4% y 5%, respectivamente. A2 – F2 representan las imágenes de campo oscuro correspondientes.
products. Figure 5b demonstrates that the inhibition of $O_2^{-}$ production was significantly higher in the alcohol test groups than in the control group and increased with (1%-4%) increasing alcohol concentration. When the alcohol concentration reached 5%, the resistance to $O_2^{-}$ weakened, but it was still significantly higher than in the control group. The data demonstrated that alcohol at low concentrations can stimulate the nematode body to suppress $O_2^{-}$ production. However, at a certain concentration, alcohol-mediated inhibition of $O_2^{-}$ production decreased. Alcohol inhibited the reduction in $\cdot$OH in a dose-dependent manner. In particular, $\cdot$OH inhibition in the 5% alcohol group was decreased by 49.93% ($P < 0.01$) compared with that in the control group.

3.4. C. elegans lifespan

The effect of alcohol on lifespan is visualized in the survival curves of C. elegans (Figure 7). Interestingly, exposure to 1% and 2% alcohol significantly extended lifespan ($P < 0.01$, Table 2). The median lifespan in the 2% alcohol group was 1.19 times that in the control group. This effect was diminished and alcohol became clearly harmful at higher concentrations. At >3% alcohol, the average lifespan of the nematodes decreased significantly. The median and average lifespan were 0.56 times those in the control group. At 4–5% alcohol, a significantly shortened lifespan was observed ($P < 0.01$).

4. Discussion

C. elegans is covered with a cuticle that is impermeable to many pharmacological agents. However, the species does not have organs or tissues corresponding to the respiratory system, suggesting it uses other methods to absorb external substances. To verify that alcohol can enter nematodes only through feeding channels, researchers previously exposed C. elegans to 400 mM (2.4%)-500 mM (3%) alcoholic environments, and the results showed that the internal alcohol concentrations of C. elegans were 22 ± 0.8 mM and 29 ± 0.5 mM, respectively – considerably lower than those of the environment. The findings revealed that alcohol internalization through the epidermis is unlikely in nematodes. The internal alcohol concentration of 22 ± 0.8 mM in C. elegans is equivalent to 0.1% alcohol in human blood, corresponding to the common legal driving limit of 21.7 mM. The internal alcohol concentration of 29 ± 0.5 mM in nematodes is comparable to ethanol levels associated with intoxication in humans (Davies et al., 2003). Based on the results of this study and other studies in China and abroad, we determined the concentrations of ethanol used in our experiments.

The results revealed that alcohol significantly affected nematode movement; locomotion was enhanced by exposure to a low concentration of alcohol (1%) but slowed down under high concentrations of alcohol, resulting in incoordination and even apparent paralysis and limited eating. Recently, Davies, Blackwell, Raabe, and Bettinger (2015) studied the effects of acute long-term alcohol exposure on C. elegans locomotive behavior. They reported that nematodes exposed to a high concentration of alcohol (400 mM) demonstrated a strong initial decrease in crawling but later partially resumed normal locomotive speed despite the continued presence of alcohol. At high concentration, alcohol has an anesthetic effect and slows the movement of organisms (Huang, Li, & Wang, 2009). This is probably because alcohol activates the BK channel of SLO-1 protein subunit (slo-1 encodes the pore-forming subunit of a large-conductance $\text{Ca}^{2+}$-activated K$^+$ channel) and subsequently limits excitatory neurotransmitter release in C. elegans. This results in the slowing down of locomotion and incoordination (Crowder, 2004). Mitchell et al. (2007) showed that pharynx pumping and eating slowed down in C. elegans exposed to alcohol, in a concentration-dependent manner (Mitchell et al., 2007). No significant apoptotic effect of alcohol was identified in nematodes in this study. Although the expression of CED-9 and CED-3 was promoted, it was still relatively balanced.

The inhibition of free radical production is usually associated with antioxidant indicators representing the antioxidant capacity of the body (Weidner, Król, Karamać, & Amarowicz, 2018). The ROS content in the 1% alcohol group was significantly lower than that in the control group. It is possible that a low concentration, alcohol causes oxidative stress, disrupting the original metabolic balance in the body. This is turn enhances the ROS scavenging capacity or may even eliminate some of the ROS produced in the body. In the 5% alcohol group, nematodes also showed low
ROS levels. This could be explained by the effect of body paralysis induced by 5% alcohol, leading to decreased ROS production and restriction of normal alcohol metabolism due to the low metabolic rate. Thus, the alcohol could not be normally metabolized and less free radicals were produced. Currently, it is believed that oxidation in the body is a major factor affecting human lifespan. Among other factors, improving antioxidant activity and reducing ROS production may prolong life (Gems & Doonan, 2009; Yee, Yang, & Hekimi, 2014). A certain concentration of alcohol can induce ROS stress tolerance, promoting cellular resistance to stress and providing a protective effect against aging (Cypser, Tedesco, & Johnson, 2006). Henderson and Johnson (2001) showed that DAF-16 protein can localize to the nucleus to improve cell protection and repair ability and enhance stress resistance. Alcohol, like any stressor, can inhibit the insulin/insulin-like signaling (IIS) pathway. Alcohol also increases the gene and protein expression of SOD-3, which transforms cell-damaging substances such as ROS into safer compounds, effectively eliminating ROS damage (Patananan, Budenholzer, Eskin, Torres, & Clarke, 2015).

*Figure 6. Effects of alcohol on major antioxidant enzymes in C. elegans.*

*Figure 6. Efectos del alcohol sobre las principales enzimas antioxidantes en C. elegans.*

**P < 0.01 compared with the control group; ΔP < 0.05, ΔΔP < 0.01 compared with the control group; #P < 0.05, ##P < 0.01 compared with the control group.

** P < 0.01 en comparación con el grupo control; ΔP <0.05, ΔΔP <0.01 en comparación con el grupo de control; #P < 0.05, ## P < 0.01 en comparación con el grupo de control.

C. elegans became more active with improved antioxidant ability – including SOD, CAT, GSH-PX, and total antioxidant capacity – under low concentrations of ethanol. This is possibly owing to the alcohol stimulation, which passively improves the body’s antioxidant defense function. When the alcohol concentration is high enough to significantly inhibit C. elegans locomotion, the antioxidant capacity decreases. Likely, when the alcohol concentration is too high, it seriously affects normal body functions, reducing the self-repair ability and increasing oxidative stress. This effect might also be due to the paralyzing effect of high concentrations of alcohol on the body, slowing basal metabolism, and thus blocking alcohol metabolism. This in turn would result in the
production of more harmful intermediate products, such as acetaldehyde, which directly damages cells. Moreover, alcohol is known to inhibit normal signalling in the nervous system (Zhu, Zhang, & Li, 2014). In the current study, alcohol at low and high concentrations had differential effects on the total antioxidant capacity in nematodes, and therefore, the lifespan of nematodes was explored under the various concentrations. The results of the lifespan assay were largely consistent with those of the antioxidant enzyme and locomotion tests. A low concentration of alcohol prolonged the lifespan of nematodes, whereas a high concentration shortened the lifespan. Accordingly, a previous study reported that at low concentration, alcohol prolonged nematode lifespan through the regulation of conserved gene signaling pathways, gene expression of age-1 and pdk-1 in the IIS pathway was decreased, and akt-1 gene expression was suppressed through negative feedback. Consequently, gene expression of let-363, involved in the mTOR pathway, was decreased, which had an inhibitory effect on downstream proteins in the IIS pathway. Thus, alcohol at low concentration can regulate genes related to aging and in turn, prolong lifespan (Wang, Wang, Jin, Guo, & Zhang, 2017). Ren et al. (2017) studied effects of the strong oxidizing agent paraquat on nematode lifespan. They found that nematode lifespan was slightly extended at an appropriate concentration of paraquat, and the mechanism may involve activation of a protective response and upregulation of antioxidant-related gene expression under long-term exposure. Chen et al. (2014) proposed a similar mechanism.

In this study, the ability of the nematodes to inhibit $O_2^-$ increased after alcohol intake, whereas the ability to inhibit $\cdot OH$ decreased. We speculate that alcohol intake induced oxidative stress and enhanced free radical production, which in turn activated a compensatory defense response, including enhanced antioxidant capacity of the body, involving SOD, CAT, and GSH-PX, and thus, the ability to inhibit $O_2^-$ production was improved. With the increase in anti-oxidant activities in vivo, the overall levels of free radicals were lower than those in the control group, which explains why we observed no obvious apoptosis in the nematodes. $\cdot OH$ can be generated from $O_2^-$ and $H_2O_2$. Thus, as the ability to inhibit $O_2^-$ was enhanced and $O_2^-$ decreased, downstream $\cdot OH$ production decreased, and accordingly the ability of the body to inhibit $\cdot OH$ decreased. Numerous studies have reported a complex interaction between endogenous oxidants, antioxidants, and life span (Remacle & Renard, 1996; Guan Xin-Lei, 2015; Moriwaki et al, 2013). There may be various reasons for the differential $O_2^-$ and $\cdot OH$ inhibitory capacities in C. elegans upon alcohol exposure, which require further study.

5. Conclusion

The current study demonstrated that exposure to alcohol at various concentrations affects oxidative stress, movement, and lifespan in C. elegans. Upon exposure to alcohol at low concentrations, nematodes became more active, resistance to oxidative stress was increased, oxidation resistance and free radical scavenging ability were improved, and the overall level of free radicals was reduced, eventually slowing down aging. However, nematodes could not fully resist oxidative stress and biological toxicity induced by alcohol at high concentration, and therefore, the lifespan was shortened under such exposure. The results of this study may serve as a reference for the screening of drugs that reduce the side effects of ethanol, which will be the next focus of our research group.

Table 2. Lifespan of nematodes exposed to different concentrations of alcohol.

| Group          | Average lifespan (d) | 95% Confidence interval | Maximum lifespan (d) | Median (d) | 95% Confidence interval |
|---------------|----------------------|-------------------------|----------------------|------------|-------------------------|
| Control (n = 98) | 18.80 ± 0.58         | 19.94 - 17.65           | 30.00                | 18.00 ± 0.789 | 19.53 - 16.45           |
| 1% (n = 110)  | 21.31 ± 0.60**       | 24.30 - 22.12           | 34.00                | 24.00 ± 0.599** | 25.17 - 22.83           |
| 2% (n = 103)  | 22.40 ± 0.96**       | 24.30 - 20.50           | 38.00                | 24.00 ± 1.445** | 26.83 - 21.17           |
| 3% (n = 98)   | 10.53 ± 0.54**       | 11.60 - 9.46            | 26.00                | 10.00 ± 0.658** | 11.29 - 8.71            |
| 4% (n = 101)  | 8.69 ± 0.23**        | 9.15 - 8.23             | 12.00                | 8.00 ± 0.270** | 8.53 - 7.47             |
| 5% (n = 104)  | 8.08 ± 0.13**        | 8.33 - 7.82             | 12.00                | 8.00 ± 0.102** | 8.20 - 7.80             |

*P < 0.05, **P < 0.01
Disclosure statement
No potential conflict of interest was reported by the authors.

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