

Delphinidin-3-glucoside prolongs lifespan and healthspan in *Caenorhabditis elegans* with and without environmental stress

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ABSTRACT

The beneficial effects of crude anthocyanin extracts against aging, cancer, and bacterial infections are widely documented in the literature. Delphinidin-3-glucoside (D3G) is a class of anthocyanin pigment in fruits and vegetables. Although there are *in vitro* studies on the bio-functional activities of D3G, none are *in vivo*. Thus, we examined the effects of D3G on the lifespan of *Caenorhabditis elegans* (*C. elegans*) and measured the healthspan indicators like egg-laying ability and pharyngeal pumping during its normal state and in the presence of stressors, such as heat, ultraviolet (UVA) light, and hydrogen peroxide (H₂O₂). We found out that D3G prolongs the lifespan and improves its healthspan without stress. These same effects were observed with oxidative stress but not heat and UVA stressors. D3G partially reverses the adverse effects of H₂O₂ by prolonging the lifespan and augmenting the pharyngeal pumping of treated *C. elegans*, albeit lower than untreated. Overall, our findings suggest that D3G is capable of extending the lifespan and improving health through its antioxidant property.

INTRODUCTION

Numerous studies have investigated the antioxidant, antimicrobial, anti-inflammatory, and anticancer properties of crude anthocyanin extracts from various plant sources [1–3]. Some of these studies have highlighted the potential of anthocyanin to attenuate aging and reduce aging-associated diseases [4,5]. Delphinidin is one of the most common classes of anthocyanin, but only a few studies have explored its potential as a pure compound, such as delphinidin-3-glucoside (D3G).

Previous research has identified D3G in various berries, grapes, eggplants, and beans. Studies have demonstrated that this compound can inhibit platelet activation and thrombosis [6], suppress breast cancer via the Akt pathway [7], and attenuate lipid accumulation in HepG2 cells [8]. Despite these findings, more research is necessary to understand the effects of D3G on

the aging process of multicellular organisms. To the best of our knowledge, there is no study that has investigated the impact of D3G on the lifespan, healthspan, and stress tolerance of a multicellular organism.

Therefore, our study aimed to investigate the effects of D3G on lifespan extension and health indicators such as egg-laying ability and pharyngeal pumping rate. First, we observed that heat, ultraviolet (UVA), and oxidative stress could impact the lifespan of *Caenorhabditis elegans* (*C. elegans*). We then assessed the effects of D3G on the lifespan and health indicators of *C. elegans* under heat, UVA, and oxidative stress.

MATERIALS AND METHODS

Procurement and storage of the compounds

We purchased D3G (>97%) and coenzyme Q10 (coQ10) (>98%) from AS polyphenols (Sandnes, Norway) and ApexBio (TX, USA), respectively. We used freshly prepared D3G and CoQ10 (positive control) solutions by reconstituting these compounds to the desired concentration with distilled water. We stored the freshly prepared solutions at 4°C until used.

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Preparation of nematode growth medium and maintenance of the nematode

We followed the protocol described in Nas *et al.* [9] to prepare the nematode growth medium (NGM) and maintain the *C. elegans*. To prepare the NGM, we dissolved 750 mg NaCl, 4.5 g bacteriological agar, and 625 mg peptone in 250 ml distilled water, and autoclaved the mixture at 121°C for 15 minutes. After the mixture cooled, we added 125 µl each of 1 M CaCl₂, 1 M MgSO₄, and 5 mg/ml cholesterol in absolute ethanol, and then added 3.125 ml of 1 M KPO₄ before pouring the mixture into small Petri plates. We seeded each plate with 100 µl of *Escherichia coli* (*E. coli*) strain OP50 to serve as a food source for the *C. elegans* N2 strain obtained from the Caenorhabditis Genetic Center (MN, USA).

To age-synchronize the *C. elegans*, we collected the eggs laid by an adult worm after 1 hour of egg-laying on the NGM plate. We assumed that the age difference of each nematode was ± 1 hour. We maintained the nematodes at a constant temperature of 20°C and replenished their food source with freshly prepared *E. coli* OP50 and treatment solutions on the NGM plates.

Lifespan and healthspan assay without stress

We followed the protocol of Nas *et al.* [10] and Park *et al.* [11] with slight modifications for our assay. Briefly, we placed 30 L4-stage *C. elegans* on freshly prepared NGM plates containing *E. coli* OP50 coated with either coQ10 (175 µM) or D3G (0, 25, 50, and 100 µM). We monitored the number of live, dead, and missing worms daily using a bright-field stereomicroscope. We classified the nematodes as alive if they responded to a light poke with a nichrome wire. We also counted the number of eggs laid on the plates daily and divided this by the total number of alive individuals to determine egg-laying capacity [12].

To measure the pharyngeal pumping rate, we counted the number of pumps per minute and recorded it daily using an Amscope MD500 camera (7.5 fps, 35 mm, 1080p HD) (Amscope, CA, USA) and stereomicroscope. We transferred each nematode daily to new NGM plates to avoid bacterial depletion. This assay was performed thrice, using a different set of worms in each trial.

Lifespan and healthspan assay with stressors

We conducted an experiment to test the effects of coQ10 (175 µM) or D3G (0, 25, 50, or 100 µM) on 30 L4 nematodes exposed to daily stressors such as heat, UVA, or oxidative stress, following the published protocol of Nas *et al.* [13]. Each stressor was applied to a different set of nematodes throughout the assay.

To induce heat stress, we placed the live worms in an incubator at a constant temperature of 30°C for 30 minutes daily. For UV stress, we exposed the nematodes to ultraviolet (UVA) light (1,300 µW/cm² intensity) at 365 nm for 2 minutes every day using a UV-GL-58 handheld lamp (Analytik Jena, CA, USA), which was positioned 3 inches above the base of the plates. To induce oxidative stress, we followed a modified protocol [14] and administered 100 µM of freshly prepared hydrogen peroxide (H₂O₂) solution (PHILUSA Corp.,

Philippines) to the live worms by dispensing about 0.2 µl of the solution to their head. We then placed the worms on NGM plates filled with water to remove the H₂O₂ solution from their body before transferring them to a newly prepared NGM plate containing the different treatments. We repeated all assays three times, using different sets of worms in each trial.

Statistical analysis

We conducted all experiments using two independent trials and presented the data as mean \pm SEM. To analyze the lifespan data, we used OASIS 2 (<https://sbi.postech.ac.kr/oasis2>) and performed the log-rank test. For the analysis of free radical scavenging activity, EC50, the mean number of eggs laid, and average pharyngeal pumping rate of *C. elegans*, we used GraphPad Prism version 7 (GraphPad Software, CA, USA) and performed one-way analysis of variance with *post-hoc* Tukey's multiple comparisons tests. We considered results statistically significant at $\alpha \leq 0.05$.

RESULTS

D3G enhances the lifespan and pharyngeal pumping of *C. elegans*

We found that supplementation with 100 µM of D3G led to a significant 23.4% increase ($p \leq 0.05$) in the mean lifespan of *C. elegans*, as illustrated in Figure 1A and B. This increase is comparable to the effect observed with coQ10 supplementation. However, D3G and coQ10 intake did not affect the average number of eggs laid by the nematode, as shown in Figure 1C and D. Moreover, *C. elegans* supplemented with 100 µM of D3G exhibited an average pharyngeal pumping rate approximately 8.5% higher ($p \leq 0.05$) than the control group receiving only distilled water, but this effect was similar to that observed with coQ10 supplementation, as shown in Figure 1E and F.

D3G does not improve the tolerance of *C. elegans* against heat stress

We disrupted the normal physiology of *C. elegans* by subjecting them to elevated temperatures for a short period every day. While we observed significant increases in the mean lifespan and pharyngeal pumping rate of *C. elegans* supplemented with D3G under normal conditions, we did not observe similar effects under heat stress conditions. Specifically, we did not find significant changes ($p > 0.05$) in the lifespan, number of eggs laid, or pharyngeal pumping rate of *C. elegans* exposed to heat stress, as shown in Figure 2A–F.

D3G does not enhance the tolerance of *C. elegans* against UVA-induced stress

We examined the effects of D3G supplementation in *C. elegans* exposed to UVA light for a short period every day. Our results, as depicted in Figure 3A and B, show that the mean lifespan of *C. elegans* fed with varying concentrations of D3G was not significantly different ($p > 0.05$) from that of untreated worms. Moreover, we did not observe any significant changes ($p > 0.05$) in the number of eggs laid or the pharyngeal pumping rate of *C. elegans* supplemented with D3G, as shown in Figure 3C and F.

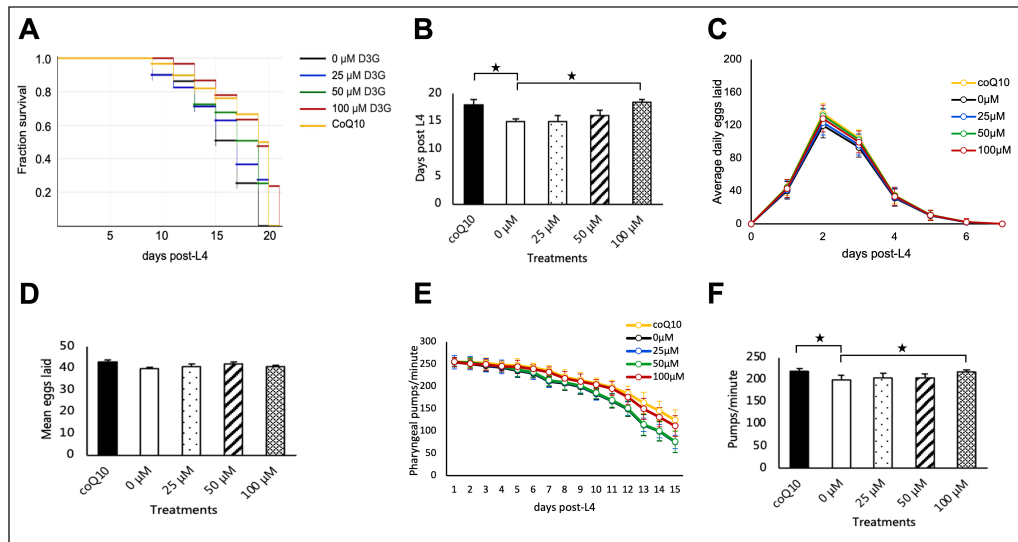


Figure 1. D3G prolongs the lifespan and enhances the pharyngeal pumping in *C. elegans* without stress. We nourished the nematodes with varying concentrations of D3G (0, 25, 50, and 100 μM) and we used coQ10 (175 μM) as the positive control. We measured the **A** – Daily survival rate, **B** – Mean lifespan, **C** – Daily eggs laid, **D** – Mean number of eggs laid, **E** – Daily pharyngeal pumping rate, and **F** – Average pharyngeal pumping rate of the nematodes. Each column represents mean \pm SEM from three independent trials. Significance was set at $p \leq 0.05$ represented by *.

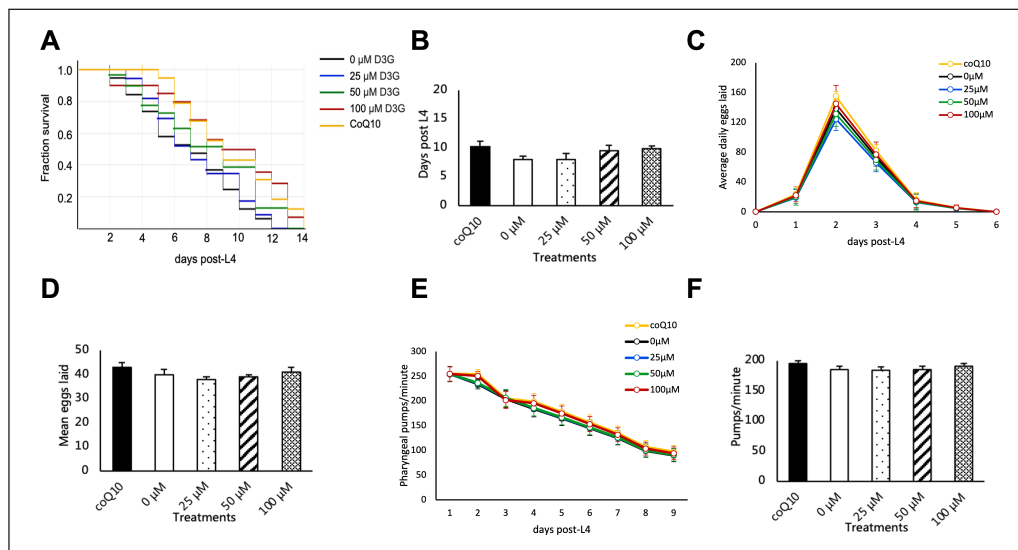


Figure 2. D3G did not affect the lifespan and health indicators of *C. elegans* under heat stress. We supplemented the nematodes with varying concentrations of D3G (0, 25, 50, and 100 μM) and we used coQ10 (175 μM) as the positive control. We incubated the *C. elegans* at 30°C for 30 minutes every day. We measured the **A** – Daily survival rate, **B** – Mean lifespan, **C** – Daily eggs laid, **D** – Mean number of eggs laid, **E** – Daily pharyngeal pumping rate, and **F** – Average pharyngeal pumping rate of the nematodes. Each column represents mean \pm SEM from three independent trials. Significance was set at $p \leq 0.05$ represented by *.

D3G enhances lifespan and pharyngeal pumping of *C. elegans* under H_2O_2 -induced stress

We determined that H_2O_2 at concentrations greater than 100 μM is toxic to *C. elegans*. To evaluate the protective effects of D3G, we supplemented the nematodes with D3G and exposed them to 100 μM of H_2O_2 daily. Our findings shown in Figure 4A and B demonstrate that 100 μM D3G extended the lifespan and

increased the mean lifespan of *C. elegans* by 32.5% ($p \leq 0.05$). Similarly, the pharyngeal pumping rate of *C. elegans* supplemented with 100 μM D3G under oxidative stress was also enhanced by 8.13% ($p \leq 0.05$), as shown in Figure 4E–F. However, the number of eggs laid by the nematodes was still unaffected, as shown in Figure 4C and D. The observed increase in the mean lifespan and average pharyngeal pumping rate were all comparable to coQ10.

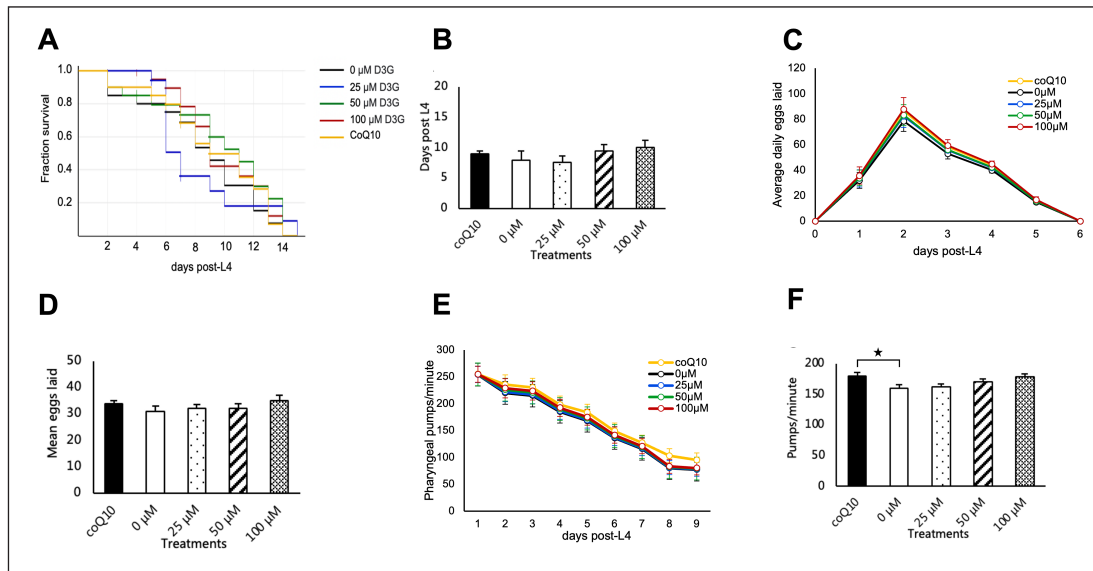


Figure 3. D3G did not affect the lifespan and health indicators of *C. elegans* treated with UVA. We fed the nematodes with varying concentrations of D3G (0, 25, 50, and 100 μ M) and we used coQ10 (175 μ M) as the positive control. We measured the **A** – Daily survival rate, **B** – Mean lifespan, **C** – Daily eggs laid, **D** – Mean number of eggs laid, **E** – Daily pharyngeal pumping rate, and **F** – Average pharyngeal pumping rate of the nematodes. Each column represents mean \pm SEM from three independent trials. Significance was set at $p \leq 0.05$ represented by *.

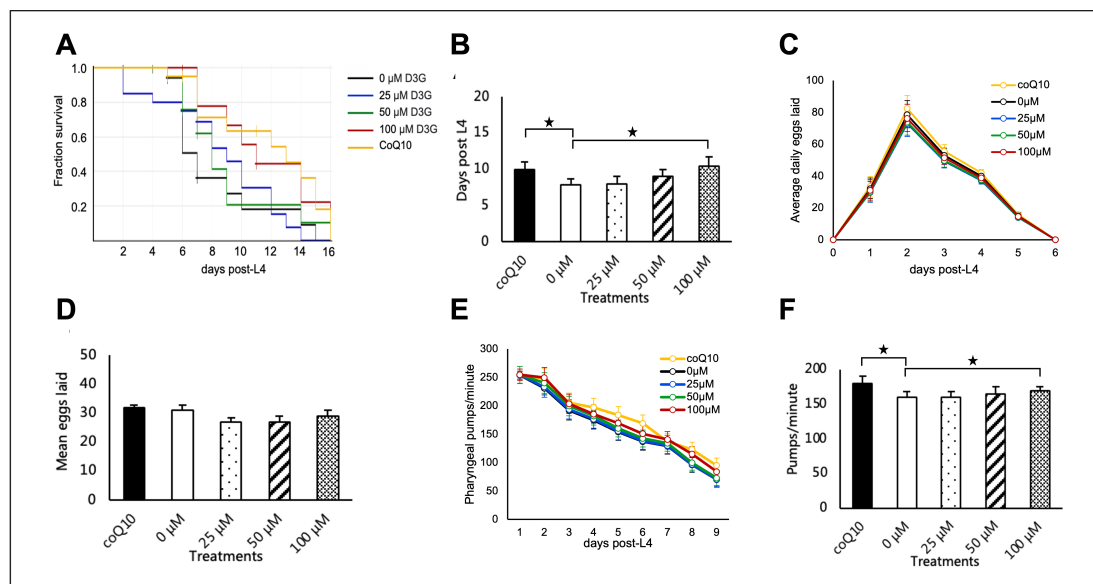


Figure 4. D3G increased the mean lifespan and pharyngeal pumping rate of *C. elegans* fed with H_2O_2 . We nourished the nematodes with varying concentrations of D3G (0, 25, 50, and 100 μ M) and we used coQ10 (175 μ M) as the positive control. We measured the **A** – Daily survival rate, **B** – Mean lifespan, **C** – Daily eggs laid, **D** – Mean number of eggs laid, **E** – Daily pharyngeal pumping rate, and **F** – Average pharyngeal pumping rate of the nematodes. Each column represents mean \pm SEM from three independent trials. Significance was set at $p < 0.05$ represented by *.

DISCUSSION

The findings of our study suggest that D3G supplementation could promote healthy aging and improve physiological function in *C. elegans*. Specifically, our results showed that D3G was able to increase the mean lifespan of the nematodes by a significant amount, which is a promising result

for the potential use of D3G as an anti-aging supplement. The observed increase in lifespan was comparable to that seen with coQ10, a well-known antioxidant and anti-aging supplement. Our findings align with previous research demonstrating the lifespan-extending effects of anthocyanin extracts from various sources such as purple wheat, blueberry, and acai berry [15–17].

Interestingly, D3G supplementation did not affect the average number of eggs laid by the nematodes. This finding suggests that D3G may not directly impact the deterioration of the muscles involved in egg laying in *C. elegans*. Further studies are needed to investigate the underlying mechanisms behind this observation.

Our study also demonstrated that D3G supplementation could increase the average pharyngeal pumping rate of *C. elegans*, an indicator of physiological function. In a study on blueberry polyphenols, Wilson *et al.* [17] reported that the extract enhanced pharyngeal pumping in *C. elegans*. This result is significant, as it suggests that D3G supplementation could improve the overall healthspan of the nematodes, possibly by enhancing their ability to maintain proper nutrient uptake and digestion.

However, our results also indicate that the protective effects of D3G were not observed in *C. elegans* exposed to heat stress or UVA radiation. These findings suggest that the effects of D3G may be context-dependent, and its potential benefits may vary in different physiological contexts. Further research is needed to fully understand the mechanisms underlying the effects of D3G and its potential applications.

The results of our study suggest that D3G has a protective effect on *C. elegans* under oxidative stress induced by H_2O_2 . This is evidenced by the significant increase in mean lifespan and average pharyngeal pumping rate of the nematodes supplemented with 100 μM of D3G, compared to the control group exposed to H_2O_2 only. Interestingly, the protective effect of D3G on mean lifespan and pharyngeal pumping rate was similar to that observed with coQ10 supplementation, a well-known antioxidant with reported anti-aging properties. However, it is important to note that while the supplementation of D3G showed positive effects under oxidative stress, it was not sufficient to fully reverse the detrimental impact of H_2O_2 . Despite the administration of D3G supplementation, the mean lifespan and average pharyngeal pumping of the nematodes did not exhibit a comparable outcome to those worms that did not receive H_2O_2 treatment.

It is worth noting that the average number of eggs laid by *C. elegans* was not affected by D3G supplementation under oxidative stress, indicating that the protective effects of D3G may be specific to certain physiological functions. Further studies are needed to explore the underlying mechanisms of D3G's protective effects and to determine its potential as an anti-aging supplement.

Overall, our study provides evidence for the potential use of D3G as a protective agent against oxidative stress and highlights its comparable effectiveness to coQ10 in improving lifespan and pharyngeal pumping rate in *C. elegans*. These findings may have implications for developing new therapeutic strategies for age-related diseases and improving human healthspan.

It is worth noting that previous studies on the effects of crude anthocyanins on the survival of *C. elegans* have presented conflicting claims. For example, the study on blueberry polyphenols suggested that this extract could not defend *C. elegans* from paraquat and H_2O_2 [17]. On the other hand, other studies demonstrated that anthocyanins from acai,

bilberry, and mulberry attenuated reactive oxygen species (ROS) and protected *C. elegans* from oxidative damage [16,18–20]. These inconsistencies may be due to differences in the types and concentrations of anthocyanins used and the specific stress conditions applied in each study.

Oxidative damage is a well-established contributor to the aging process and age-related diseases. Our findings revealed that D3G exhibited the ability to slow down aging and mitigate the functional decline observed in the nematodes exposed to oxidative stress. This suggests that D3G may hold potential benefits for promoting healthy aging by reducing oxidative damage. Although the exact mechanism by which D3G confers these benefits is yet to be fully understood, we have put forth several hypotheses. It is possible that D3G acts by neutralizing ROS, which are known to cause oxidative damage. By scavenging ROS, D3G may prevent their harmful effects on cellular components. Furthermore, D3G might activate inherent antioxidant defense pathways, enhancing the cellular antioxidant capacity. This can help counteract the detrimental effects of oxidative stress and contribute to maintaining cellular health and function. Additionally, D3G may stimulate genes involved in cellular repair and maintenance, potentially aiding in the restoration of damaged cellular components and promoting longevity. By reducing oxidative damage, D3G has the potential to confer benefits to cellular health and function, which are critical factors for healthy aging. Further research is warranted to elucidate the specific mechanisms through which D3G exerts its effects and to explore its potential applications for promoting healthy aging in diverse model systems and human studies.

CONCLUSION

In conclusion, our study investigated the effects of D3G supplementation on the lifespan and healthspan of *C. elegans* under various conditions. We found that D3G supplementation significantly increased the mean lifespan and average pharyngeal pumping rate of *C. elegans* under oxidative stress induced by H_2O_2 . However, supplementation did not significantly affect the nematodes exposed to elevated temperatures or UVA light. Additionally, D3G supplementation did not affect the average number of eggs laid by the nematodes. Our results suggest that D3G has the potential as a protective agent against oxidative stress-induced damage in *C. elegans*. Further studies are needed to determine the underlying mechanisms of D3G's protective effects and its potential application as a human dietary supplement.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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