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Research Article

Evaluation of empagliflozin efficacy as a promising anti-aging treatment in mice: In-vivo study

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Abstract

Aim: Evaluation of the anti-aging properties of Empagliflozin (EMP) associated with the aging process in mice.

Methods: The mice were allocated into four groups: negative control received normal saline without receiving D-galactose (DGA); all the three other groups received DGA (200 mg/kg/day orally) for eight weeks; the second group received normal saline; the third group received vitamin C, the final group received EMP and continued for another eight weeks.

Results: Treatment with EMP reduced the levels of TNF-α, IL-1β, and MDA levels significantly compared to induction group (91.7±9.6 ng/ml, 30.6±5.5 pg/ml, and 66.7±8.3 ng/ml vs. 304.0±102.9 ng/ml, 70.2±6.8 pg/ml, and 204.7±56.9 ng/ml; respectively), while levels of GSH-Px were significantly increased (3.3±0.6 ng/ml vs. 0.3±0.2 ng/ml). In addition, EMP increases the level of both COL-1 and COL-3 compared to the induction group (1,783.6±186.9, and 1,583.6±186.9, vs. 885.7±242.5, and 685.7±242.5 pg/ml; respectively).

Conclusion: EMP positively affects several aging parameters in mice.

Keywords

Aging, antioxidant, empagliflozin, heart, inflammation

Introduction

Aging is a biological process that is inherited and manifests as changes in the makeup and function of cell and extracellular constituents. These changes are further influenced by numerous injuries an individual may suffer throughout their lifetime; their cumulative effects ultimately lead to the gradual disruption of the organism's regulatory mechanisms responsible for maintaining homeostasis (Xing et al. 2023). It is imperative to comprehend that the aging process should not be classified as an illness, as the progression of aging is comprehensive and intricate. At the same time, diseases typically exhibit more confined manifestations (Bulterijs et al. 2015). Several common characteristics of aging in mammals have been observed. These factors encompass heightened death rates upon reaching adulthood and modifications in the biochemical makeup of tissues, such as significant reductions in lean body mass and overall bone mass in humans and elevations in lipofuscin (often referred to as age pigment).

Furthermore, it is seen that there is a gradual decrease in physiological functioning as individuals grow older. As individuals age, there are notable declines in glomerular filtration rate, maximal heart rate, and vital capacity, along with a diminished capability to respond effectively to environmental stimuli; an increased inclination and

vulnerability to sickness (St-Onge and Gallagher 2010; Guo et al. 2022; Hernández-Álvarez et al. 2023).

The aging process is influenced by various factors, with the primary factor being the progressive accumulation of random molecular damage that remains unrepaired throughout time; this ultimately results in cellular abnormalities, leading to impaired tissue function and the aging process (Maynard et al. 2015; Gladyshev et al. 2021). These mechanisms include Genomic Instability (Tiwari and Wilson 2019), Telomere Attrition (Shay 2016), Epigenetic alterations (Kane and Sinclair 2019), and Loss of proteostasis (Koga et al. 2011). These mechanisms operate collaboratively within a multi-layered framework, ultimately leading to the progression of the aging process (Mc Auley et al. 2017).

The existing hypotheses regarding the aging process in humans span a range of biological and molecular views. Within this context, two notable hypotheses, namely the Inflammatory-Aging theory and the Oxidative Stress or Free Radical theory, have garnered significant attention. The Inflammatory-Aging hypothesis suggests a direct association between age and the activation of macrophages, sometimes referred to as MACROPH-AGING. On the other hand, the Oxidative Stress or Free Radical theory was offered by Harman. The latter hypothesis posits that reactive oxygen species (ROS) are produced as an inevitable result of metabolic processes (Jin 2010; Zuo et al. 2019).

The physiological aging process significantly impacts the many biological organ systems inside the human body (Khan et al. 2017). The impact of aging on the skin has been a prominent topic of discussion across various disciplines for an extended period (Bonté et al. 2019). The dermal collagen content experiences an annual reduction of 1% during adulthood. Moreover, collagen transforms as skin ages, transitioning from structured and thin reticulated fibers in youthful skin to fragmented and disorganized fibers in older skin. An elevation in the levels of metalloproteinases and collagen-degrading enzymes accompanies this process. Additionally, the interconnections between collagen and elastin fibers, which facilitate the skin's ability to regain its shape after deformation, diminish over time. Consequently, these alterations ultimately form wrinkles in adult skin (Quan et al. 2010; Pittayapruek et al. 2016).

Empagliflozin is a novel anti-hyperglycemic drug that acts as a competitive, reversible, and highly specific inhibitor of the Sodium-glucose cotransporter-2 (SGLT2). It belongs to a relatively recent class of medications used for managing type 2 diabetes; the inhibition of SGLT2 by empagliflozin reduces the reabsorption of glucose into the bloodstream; this subsequently enhances glucose filtration via the kidneys, leading to its excretion in the urine and ultimately reducing glucose levels. Importantly, this effect is independent of insulin action (Grempler et al. 2012; Neumiller 2014).

Empagliflozin (EMP) has been acknowledged as a powerful antioxidant drug that protects tissues by acting as free radical scavengers, thereby mitigating oxidative damage (Tsai et al. 2021). EMP has an anti-inflammatory effect due to its anti-inflammatory properties, which decrease the synthesis of PGE2 and proinflammatory cytokines. This effect is achieved by inhibiting COX-2, iNOS, cytokine, and chemokine mRNA expression in RAW 264.7 macrophages (Lee et al. 2021). The objective of this study is to evaluate the impact of EMP in mitigating the effects of aging on multiple variables in mice.

Method

Study design

A sample of Swiss albino male mice, with an average weight range of 25–35 g and an age range of 4–8 months, was randomly allocated into four groups. Each group consisted of six animals, resulting in 24 mice. The mice used in this study were sourced from the National Drug Control Laboratory in Baghdad, Iraq. They were kept in a polypropylene cage in a controlled setting with an ambient temperature of 21±4 °C. The lighting conditions were set to a regular 12 h light/12 h dark cycle. Before the commencement of the study, the mice had been habituated for two weeks at the Animal Facility of the Al-Mustafa University College in Baghdad, Iraq. The animals were provided with a regular pellet meal and unrestricted access to water, which was given by the Animal Facility at Al-Mustafa University College. The study was prepared following the ARRIVE guidelines 2.0.

The mice were allocated into four groups: negative control received normal saline without receiving D-galactose (DGA) (G1), all the three other groups received DGA (200 mg/kg/day orally) for eight weeks (this is the induction phase) (Chogtu et al. 2018; Martinovic et al. 2023); the second group after the induction phase; received orally normal saline for eight weeks; named induction group (G2), the third group after the induction phase; starting next day received 100 mg/kg/day vitamin C and continued for another eight weeks (G3) (Li et al. 2019), the final group after the induction phase; starting next day received 1 mg/kg/day EMP and continued for another eight weeks (G4) (Han et al. 2017), all oral drugs administer utilizing gastric gavage, as illustrated in Table 1 and Fig. 1.

A successful induction is characterized by ragged fur and a more overall plump physical look. Additionally, older mice may exhibit reduced alertness, decreased activity levels, wrinkly skin, and diminished responsiveness or increased hesitancy in their movements compared to their younger counterparts (Toth 2018).

Table 1. Animal allocation for each group.

	D-galactose[a]	Drug received (eight weeks)	Duration
G1	Did not receive	normal saline by gastric gavage	16 weeks
G2 (Chogtu et al. 2018; Martinovic et al. 2023)	Received	normal saline by gastric gavage	16 weeks
G3 (Li et al. 2019)	Received	Vitamin C (100 mg/kg/day) by gastric gavage	16 weeks
G4 (Han et al. 2017)	Received	EMP (1 mg/kg/day) by gastric gavage	16 weeks

[a] dosage of 200 mg/kg/day of DGA orally by gastric gavage for eight weeks.

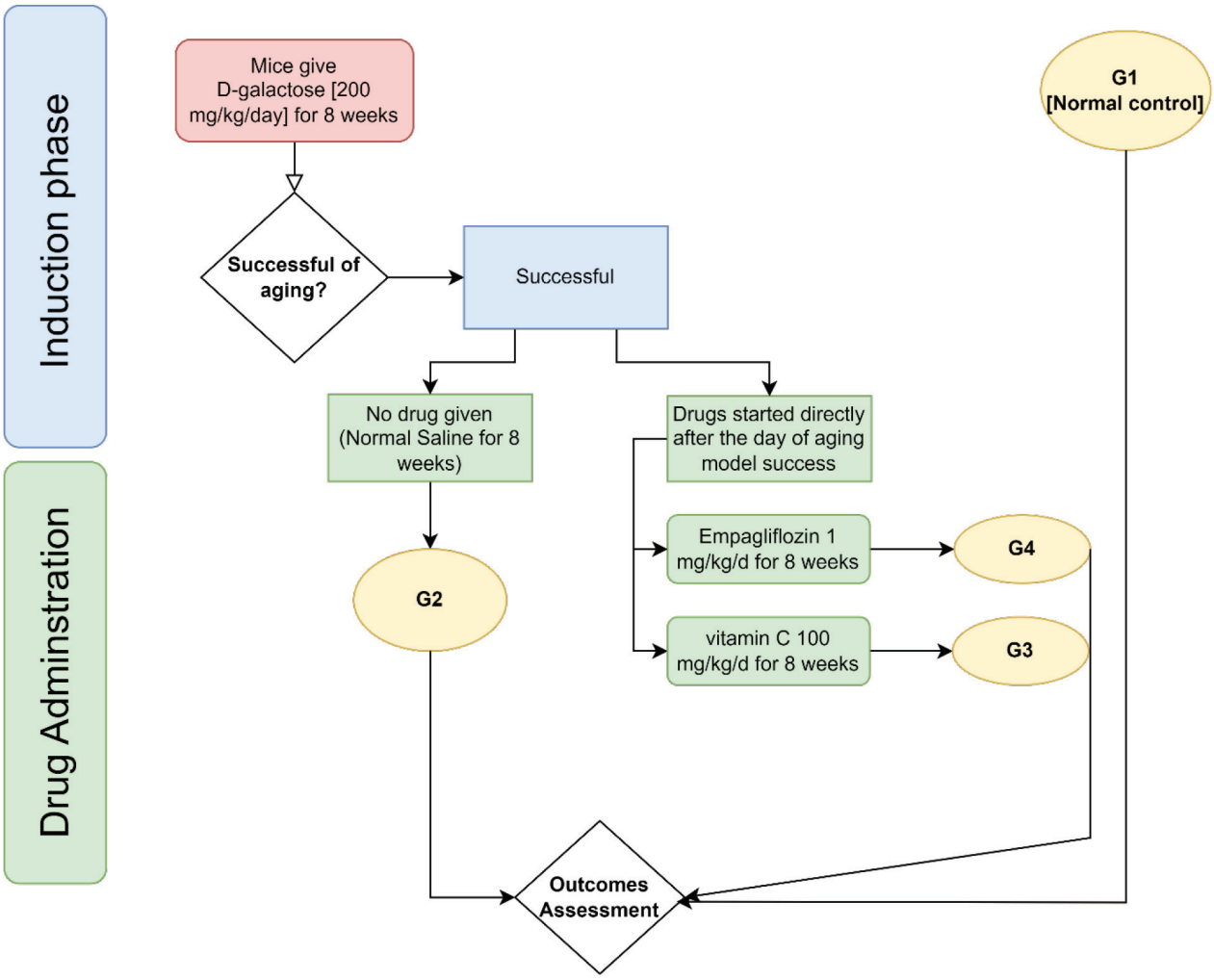

Figure 1. Flow chart of the study.

Materials

DGAL was purchased from Sigma Aldrich, USA (CAS no. 59-23-4). EMP was purchased from Shanghai Biolang Biotechnology Co., Ltd., China (CAS no. 864070-44-0). Vitamin C was purchased from Hangzhou Hyper Chemicals Limited, China (CAS no.86404-04-8).

Animal allocations

For sample size computation, program G Power was utilized (Faul et al. 2007) based on Cohen's principles (Charan and Kantharia 2013). The groupings were constructed randomly using a table of random integers. The mice were systematically allocated into properly marked boxes and individually identified with tail tags to mitigate the occurrence of misinterpretation (Festing 2006).

The study modules employed a randomized block design. The mice were categorized into four distinct blocks. In block one, G1 received their treatment plan. Block two (G2) commenced treatment in the subsequent week. Block three (G3) commenced treatment in the subsequent week. Finally, block four (G4) commenced treatment in the subsequent week.

Outcome measures

Weight measurements were conducted for all mice at the beginning of the study and before their euthanasia. After the medication delivery period, all mice were subjected to euthanasia, a process that occurred after 16 weeks. After the conclusion of the therapeutic intervention, all animals had a period of fasting lasting 10 hours. Subsequently, they were subjected to intraperitoneal (IP) anesthesia with a dosage of 80 mg/kg of ketamine and 10 mg/kg of xylazine. After undergoing complete anesthesia, the mice were euthanized using carbon dioxide (Underwood and Anthony 2020; Yaribeygi et al. 2023).

After the conclusion of every treatment period for every group, a dissection procedure was conducted on deceased animals. The objective of this dissection was to extract the heart, which was subsequently weighed to calculate the organ index (Chen et al. 2021).

$$Organ\ index\ (\%) = \frac{\text{organ weight (g)}}{\text{body weight (g)}}\ x\ 100\%$$

The heart tissue was subjected to histological investigation after being rinsed with phosphate-buffered sa-

line (PBS) at a pH of 7.4. Subsequently, the conventional processing protocol utilizes the paraffin-embedded technique (Sadeghipour and Babaheidarian 2019). An additional cardiac and cutaneous tissue sample was collected and subjected to a cold PBS (pH 7.4) rinse. Subsequently, the material was dried using filter paper for ELISA evaluation using an ELISA reader from Diagnostic Automation / Cortez Diagnostics, California, USA. Additionally, the tissue was measured using a sensitive balance. In the ELISA procedure, 50 mg of tissue was placed in an Eppendorf tube (Eppendorf, Hamburg, Germany) with 0.45 ml of cooled PBS. The tissue was then finely chopped into small fragments. Subsequently, the tissue sample tube was placed in a beaker filled with ice to maintain a low temperature. The homogenization was done using an electrical tissue homogenizer machine (Staruar, England). The resulting homogenate was then centrifuged at a temperature of four degrees Celsius and a speed of 2000 revolutions per minute for 20 minutes. This centrifugation was performed using a cold centrifuge manufactured by Thermos Scientific in the United States. The supernatant was obtained using a micropipette (Bioevopeak, China) and stored at -20 °C until the analysis day (Saja Majeed and Sarmed Hashim 2022).

Assessment of heart tissue histopathology

A photograph was produced for every mouse using a digital camera and a mounted light microscope. The histopathologist assessed hypertrophic cells in heart tissue using the H&E stain. The morphology of the cardiac myocytes was visualized using a light microscope (Olympus BX51 Microscope, Olympus Corporation, Japan). Five areas of a slide corner and the central region were randomly observed at a magnification level of X40.

Biochemical analysis

The supernatant obtained from the homogenized heart and skin tissues of the tested animals was warmed up and subjected to biochemical testing using the double-sandwich ELISA method. The levels of Tumor necrosis factor-alpha (TNF-α), Interleukin-1Beta (IL-1β), Glutathione peroxidase (GSH-px), Malondialdehyde (MDA), Collagen I (Col-I), and Collagen III (Col-III) were determined using specific ELISA kits (Mouse Tumor Necrosis Factor A, TNF-A ELISA KIT, product ID SL0547Mo; Mouse Interleukin one beta, IL-1beta ELISA Kit, product ID SL0316Mo; Mouse Glutathione Peroxidase, GSH-Px ELISA Kit, product ID SL0241Mo; Mouse Malondialdehyde (MDA) ELISA Kit, product ID SL0370Mo; Mouse Collagen Type I,(Col-I) ELISA Kit, product ID SL0141Mo; Mouse Collagen Type III (COL-III) ELISA Kit, product ID: SL0942Mo; Sunlong biotech, China).

Statistical analysis

Statistical analysis was performed utilizing GraphPad Prism version 10.0.1, and one-way ANOVA was used to assess the difference in groups with the Tukey test as a post hoc for pair-wise comparison. The P-value is considered to be significant if ≤0.05.

Results

TNF-α, IL-1β, and MDA levels were significantly higher in G2 compared to the other groups; there was no significant difference in levels between G1, G3, and G4. Meanwhile, levels of GSH-Px were significantly lower in G2 compared to the other groups; no significant difference in levels between G1, G3, and G4, as illustrated by Table 2 and Fig. 2.

COL-1 and COL-3 levels were significantly lower in G2 compared to other groups; levels in G4 were significantly lower than in G3 and G1. Regarding heart index, G2 showed significantly higher levels compared to other groups; levels in G4 were significantly higher than G1, as demonstrated in Table 3 and Fig. 3.

Normal cardiac cells are illustrated in Fig. 4A, which shows a syncytium composed of cardiac fibers exhibiting central nuclei. Certain fibers have intercalated discs that are faintly pink in color. Red blood cells are observed to be arranged linearly within capillaries amidst the fibers. Fig. 4B for animals that received DGAL shows bizarre, irregular, and hyperchromatic nuclei. In contrast, Fig. 3C for animals received 1 mg/kg EMPA showing some irregular and hyperchromic nuclei with the start of healing to normal heart tissue.

Table 2. Evaluation of study agents on inflammatory mediators and oxidative stress markers.

Groups	TNF-α (ng/ml)	IL-1β (pg/ml)	GSH-Px (ng/ml)	MDA (ng/ml)
G1	28.0±1.1^{b}	16.0±2.5^{a}	5.1±0.6^{c}	23.5±8.4^{b}
G2	304.0±102.9^{a}	70.2±6.8^{a}	0.3±0.2^{a}	204.7±56.9^{a}
G3	87.0±10.1^{b}	29.5±5.5^{b}	3.7±0.6^{b}	58.6±9.1^{b}
G4	91.7±9.6^{b}	30.6±5.5^{b}	3.3±0.6^{b}	66.7±8.3^{b}
p-value	<0.001	<0.001	<0.001	<0.001

Columns with different litters indicate a significant difference (p-value≤0.05).
Parameters presented as mean ± standard deviation.
G1: Normal control, G2: Induction with DGAL, G3: vitamin C 100 mg/kg, G4: EMP 1 mg/kg.

Table 3. Evaluation of heart index and skin collagen.

Groups	COL-1 (pg/ml)	COL-III (pg/ml)	Heart index (%)
G1	3,062.2±343.3^{a}	2,862.2±343.3^{a}	0.37±0.10^{c}
G2	885.7±242.5^{d}	685.7±242.5^{d}	0.85±0.05^{a}
G3	2,604.1±310.6^{b}	2,204.1±310.6^{b}	0.55±0.07^{b}
G4	1,783.6±186.9^{c}	1,583.6±186.9^{c}	0.55±0.09^{b}
p-value	<0.001	<0.001	<0.001

Columns with different litters indicate a significant difference (p-value≤0.05).
Parameters presented as mean ± standard deviation.
G1: Normal control, G2: Induction with DGAL, G3: vitamin C 100 mg/kg, G4: EMP 1 mg/kg.

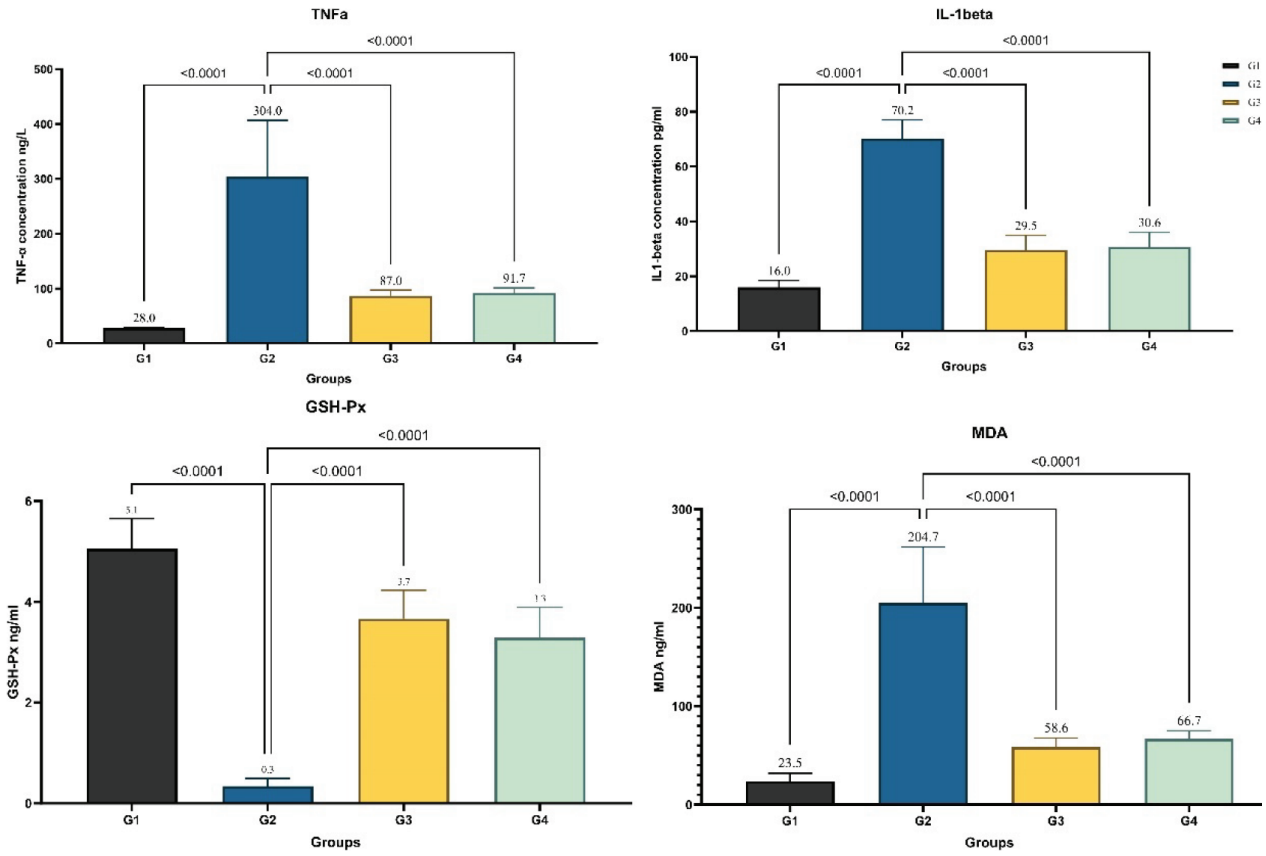

Figure 2. Assessment of inflammatory and oxidative stress markers.

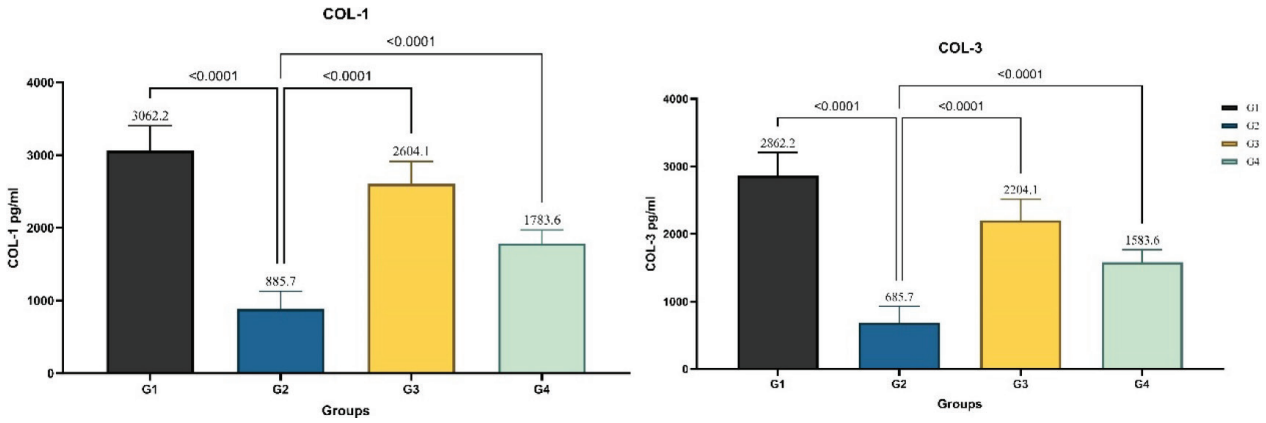

Figure 3. Assessment of COL-1 and -3 levels.

Discussion

The current investigation observed that the administration of DGAL led to the development of cardiac hypertrophy. However, when the mice were treated with 1 mg/kg EMP, this impact was mitigated, resulting in a lower heart index in G4 than in G2. According to a study conducted by Refaie et al., it was observed that there was a considerable rise in heart weights in the Cadmium cardiotoxic group as compared to the control group. In contrast, the administration of Cd in combination with dapagliflozin resulted in a notable reduction in heart weight compared to the group that received Cd alone (Refaie et al. 2022); these findings agree with the results of the current study.

Multiple studies have demonstrated the potential of this class of drugs to improve heart morphological alterations, such as cardiac hypertrophy and fibrosis. Sodium-glucose co-transporter two inhibitors (SGLT2i) have been observed to reduce both cardiac preload and afterload by reducing intracellular sodium (Na^+) and calcium (Ca^{2+}) loading. These findings suggest that EMP, a specific SGLT2i, may have a preventive effect on cardiac hypertrophy (Lahnwong et al. 2018).

One potential mechanism is the beneficial impact of EMP on cardiomyocytes, which may be achieved by the

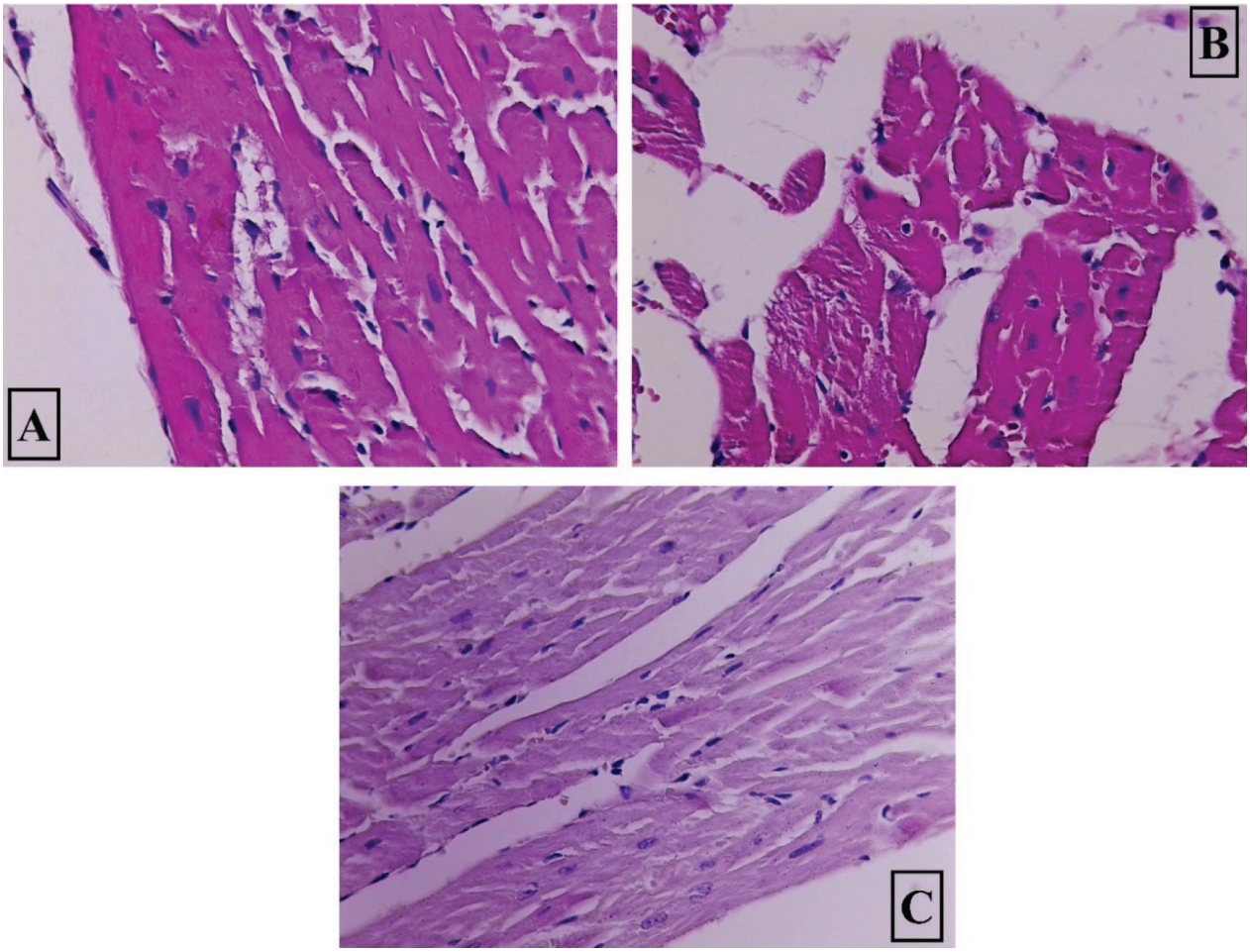

Figure 4. Cross-sections of heart tissue of mice. **A:** In mice received normal saline only (G1); **B:** In mice received DGAL only (G2); **C:** In mice received DGAL and EMP 1 mg/kg after the end of induction (G4) (Olympus BX51microscpose and a software DP controller X40).

upregulation of SIRT-1 expression (Yang et al. 2023). In the context of cardiomyocytes, the nuclear isoform of SIRT-1 serves as a protective mechanism against myocyte damage caused by oxidative stress. This protective mechanism is achieved by upregulating MnSOD production and promoting increased antioxidants, including catalase. The overexpression of SIRT-1 in cardiac tissue resulted in the mitigation of age-related elevations in ventricular hypertrophy (Alcendor et al. 2007; Chong et al. 2012).

One of the most notable changes with advancing age is the immune response disruption, leading to a persistent systemic inflammatory condition (Chung et al. 2019). The current study assessed many inflammatory markers derived from the cardiac homogenate content in distinct groups under investigation. The findings revealed a considerable enhancement of TNF-α and IL-1β in mice treated with EMP. In a study conducted by El-Mahdy et al., rats were subjected to a high carbohydrate, high-fat diet (HFHC). The results indicated a significant increase in levels of TNF-α and IL-1β in rats that were exclusively fed the HFHC diet compared to the control group. In rats administered oral dapagliflozin throughout the trial, a notable decrease in levels of TNF-α and IL-1β was observed when compared to rats on an HFHC diet, which aligns with the present study's findings (El-Mahdy et al. 2020).

EMP can potentially mitigate calcium excess, reducing inflammation and modulating various proinflammatory cytokines, including the IL-1β pathway (Shibusawa et al. 2019). SGL2i was found to induce a phenotypic shift of M1 macrophages, which are known to mediate inflammatory responses, towards an M2 macrophage phenotype. The results of this study suggest that EMP has direct anti-inflammatory effects that are not dependent on glucose concentrations. These effects are achieved by suppressing TLR-4 expression and NF-kB activation and inhibiting proinflammatory mediator production (Abdollahi et al. 2022). Furthermore, the administration of SGL2i treatment resulted in elevated levels of the anti-inflammatory (IL-10), which controls both acute and chronic inflammation by inhibiting the production of proinflammatory cytokines from immune cells such as tumor necrosis factor-alpha (TNF-α) (García-Ropero et al. 2019).

The current study assessed two oxidative stress markers in heart homogenate content across different groups under investigation. The findings revealed that the amount of GSH-Px was increased, while the level of MDA was lowered in mice treated with EMP compared to the group subject-

ed to induction. The present study's findings are consistent with prior research conducted on mice, which demonstrates that SGL2i effectively decreases the levels of MDA in the cardiac tissue homogenates of diabetic animals compared to untreated diabetic animals. In contrast to the group of individuals with untreated diabetes mellitus, the groups who received treatment exhibited a significant augmentation in the levels of the antioxidant GPx (El-Shafey et al. 2022). According to a study conducted by Kıngır et al., the administration of dapagliflozin led to a decrease in MDA levels and an increase in GSH levels. These findings align with the results of the present investigation (Kingir et al. 2019).

SGLT2 inhibitors safeguard mitochondrial activity by preserving a balanced redox state. Moreover, the induction of normoglycemia by SGLT2 inhibitors has been found to decrease the levels of advanced glycation end products (AGEs), significantly contributing to the creation of free radicals; this is because hyperglycemia serves as a strong stimulus for the generation of AGEs and intensifies the interaction between AGEs and the receptor for AGEs (RAGE), known as the AGEs-RAGE axis (Habibi et al. 2017). Moreover, it has been observed that SGLT2 inhibitors could mitigate insulin resistance, a condition intricately associated with oxidative stress, in individuals with diabetes; this implies that these inhibitors may indirectly influence the reduction of oxidative stress (Rosenstock and Ferrannini 2015; Shin et al. 2016).

The current investigation showed that EMP exhibited notably elevated levels of COL-I and COL-III in the skin compared to the induction group. The study conducted by Horikawa et al. demonstrated that diabetic mice treated with dapagliflozin had elevated levels of total skin collagen in comparison to diabetic mice that were not treated. This finding aligns with the findings of the present investigation (Horikawa et al. 2022). Activated mast cells have been observed to enhance the production of MMP-1, an enzyme known for its collagenolytic properties. Consequently, this process results in a decrease in collagen levels. Conversely, the administration of DAPA has been found to effectively decrease the levels of both MMP-1 and mast cells (Horikawa et al. 2022).

The accumulation of reactive oxygen species (ROS) from free radicals is widely acknowledged as a prominent factor contributing to skin aging (Piotrowska and Bartnik 2014). The elevation of reactive oxygen species (ROS) production commonly leads to mitogen-activated protein kinase (MAPK) activation. The loss in collagen formation with aging can be attributed to the activation of MAPK, which subsequently stimulates AP-1 (activated protein 1), increasing MMP expression (Son et al. 2011). The long-term oxidative damage in cells and tissues influences the aging process. As a result, intervention strategies can be employed to specifically address this damage and potentially mitigate the detrimental effects associated with aging (Reilly and Lozano 2021). The current investigation showed that the administration of EMP in mice significantly reduced levels of GSH-Px. Additionally, there was a large increase in levels of MDA in mice treated with EMP, consistent with earlier findings reported in this study. These results provide partial insight into the mechanism underlying the anti-aging effect exhibited by EMP.

Study limitations

The current study focuses on assessing the anti-aging effect in mice models based on inflammatory and oxidative stress theory; however, aging involves more than these pathways, like genetic and molecular pathways, which we could not assess in the current work to analyze the effects of EMP comprehensively. Additionally, our findings focus on two organs, namely, the heart and the skin, and we could not examine the other organs, like kidneys, which could benefit from the effect of EMP.

Conclusion

EMP positively affects several aging parameters in mice, as shown by this study. It decreased myocytic weight, restoring them to normal size, improved skin vitality by improving the level of collagen, decreased the burden of inflammatory mediators, and improved antioxidants' impact.

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