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Ellagic acid dose and time-dependently abrogates D-galactose-induced animal model of aging: Investigating the role of PPAR-γ

Vafa Baradaran Rahimi[a,b], Vahid Reza Askari[c,a,b], Seyed Hadi Mousavi[d,*]

[a] Pharmacological Research Center of Medicinal Plants, Mashhad University of Medical Sciences, Mashhad, Iran
[b] Student Research Committee, Department of Pharmacology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[c] Neurogenic Inflammation Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
[d] Medical Toxicology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

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ABSTRACT

Aims: The world's population is becoming aged and the proportion of older persons is growing in almost every country in the world. Ellagic acid (EA) shows abundant pharmacological properties. Therefore, we aimed to determine the mechanism of anti-aging effects of low and high doses of EA.

Main methods: Aging model was induced by D-galactose (DG), and the anti-aging effect of EA alone or in the presence of PPAR-γ antagonist GW9662, and in combination with metformin were evaluated. The activities of ALT, AST, and AChE, the levels of FBS, HbA1c, testosterone and DHEA-SO_4, MDA, GSH, TNF-α, IL-6, advanced glycation end products (AGEs), and BDNF were measured in serum, liver or brain.

Key findings: DG led to increasing in the levels of IL-6, TNF-α, MDA, AChE, AGEs, ALT, AST, FBS, and HbA1c, in which decrease in the levels of body weight, GSH, BDNF, DHEA-SO4 and testosterone. Metformin (300 mg/kg) abrogated the effects of DG-induced aging model. We also found that the low dose of EA (30 mg/kg) decreases the deteriorative effects of DG-induced aging at 10 weeks of treatment only, however, high dose of EA (100 mg/kg) was effective at both 6 and 10 weeks of treatment. The addition of GW9662 completely reversed the effects of the low dose of EA, but not for the high dose, on DG-induced aging model.

Significance: We revealed that daily and oral administration of EA provides anti-aging effects at low dose in a PPAR-γ receptor-dependent fashion, but not at the high dose.

1. Introduction

Increasingly, the world's population is becoming aged and the proportion of older persons is growing in almost every country in the world. According to the United Nations World Population Prospects; the 2017 revision, the number of population aged ≥60 is rising from 962 million globally in 2017 to 2.1 billion in 2050 and 3.1 billion in 2100. It has been reported that Europe and Northern America possess the greatest percentage of people aged 60 or over (25% and 22%, respectively). Globally, people aged 60 or over is growing faster than all younger age groups [1]. Nowadays, due to the increased rate of old people and their age-related disorders and complications, the anti-aging studies have come to the forefront of attention [2].

Aging can be defined as progressive, accumulative, time-related and natural phenomenon that leads to irreversible deterioration of the physiological functions of an organism. Several lines of evidence mentioned that aging is associated to the development and pathogenesis of several age-related disorders including osteoporosis, cardiovascular diseases, liver and kidney failure, immune system dysfunction and neuro-degenerative disorders especially Alzheimer's and Parkinson's

Abbreviation list: AChE, acetyl cholinesterase; AGEs, advanced glycation end products; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; AST, aspartate aminotransferase; BDNF, brain-derived neurotrophic factor; CAT, catalase; DHEA-SO_4, dehydroepiandrosterone sulfate; DG, D-galactose; DTNB, 2,2′-dinitro-5,5′-dithiodibenzoic acid; EA, ellagic acid; EDTA, ethylene diamine tetra acetic acid; Erk1/2, disodium salt, extracellular signal-regulated kinase1/2; FBS, fasting blood sugar; FRAP, ferric reducing antioxidant potential; GSH, glutathione; GSH-Px, glutathione peroxidase; HO-1, heme oxygenase; HbA1c, hemoglobin A1C; IL-6, interleukin 6; MDA, malondialdehyde; NGF, nerve growth factor; NO, nitric oxide; NF-κB, nuclear factor-kappa B; PPAR-γ, peroxisome proliferator-activated receptor-gamma; PMSF, phenylmethanesulfonyl fluoride; KPE, potassium phosphate buffer; ROS, reactive oxygen species; RAGE, receptor of advanced glycation end products; SASP, senescence-associated secretory phenotype; Sirt1, sirtuin 1; SOD, superoxide dismutase; TBA, thiobarbituric acid; T-AOC, total antioxidant capacity; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha

* Corresponding author at: Department of Pharmacology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.
E-mail address: mousavih@mums.ac.ir (S.H. Mousavi).

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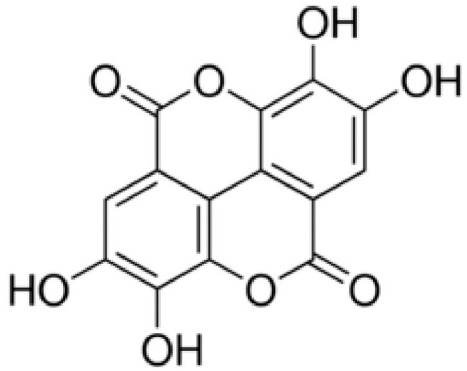

Fig. 1. The chemical structure of EA [11].

diseases [3,4].

Previous studies demonstrated that chronic exposure to the D-galactose (DG) mimics human natural aging and is commonly used the anti-aging studies [5]. DG, a reducing sugar, is present in various foods such as cheese, butter, honey, milk, cherries, and plums. Normal levels of DG can be completely metabolized to glucose, although its overload speeds up the generation of reactive oxygen species (ROS), oxidative stress, apoptosis and inflammation [6]. Inflammation and oxidative stress play a crucial role in aging development. Senescence-associated secretory phenotype (SASP) is the final feature of senescent cells which consist of chemokines, growth factors and cytokines such as Interleukin 6 (IL-6), IL-8 and tumor necrosis factor alpha (TNF-α). It has been emphasized that chronic inflammation caused by these cytokines is responsible for almost every age-related disorders [7,8].

Ellagic acid (EA, 2,3,7,8-tetrahydroxy-benzopyranol (5,4,3-cde) benzopyran-5,10-dione, Fig. 1) is a natural polyphenolic compound, which is available in several nuts and fruits including walnuts, almonds, pecans, cranberries, raspberries, strawberries, grapes, green tea and pomegranate. It has been supported that EA shows abundant pharmacological properties such as anti-inflammatory, anti-apoptotic, anti-carcinogenic, neuroprotective and antioxidant activities [9]. Recently, it was reported that EA (100 and 150 mg/kg) exerts anti-oxidant, anti-inflammatory and anti-apoptotic effects on liver and brain of DG induced-aging in rats [10], but not reporting the specific aging-related markers. On the other hand, in our previous study, we demonstrated that EA (0.01–10 μM) propagates cell proliferation and glutathione (GSH) level, while diminishes ROS, malondialdehyde (MDA), TNF-α, β-galactosidase and advanced glycation end products (AGEs) levels following DG-induced aging. Intriguingly, the effect of low concentration of EA (1 μM) was stronger than its high concentration (10 μM) and metformin (2.5 mM). We also revealed that the anti-aging effect of EA at low concentration is through peroxisome proliferator-activated receptor gamma (PPAR-γ) signaling pathway due to the addition of a PPAR-γ antagonist (GW9662) blocked its effects [11].

Therefore, in this study, we aimed to determine the anti-aging effect of EA alone or in combination with metformin following DG-induced aging in mice. Furthermore, the possible involvement of PPAR-γ signaling pathway in the anti-aging effects of EA was evaluated using a pharmacological PPAR-γ antagonist GW9662.

2. Material and methods

2.1. Drugs and chemicals

Metformin, ellagic acid (EA), D-galactose (DG) and GW9662 were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). DTNB (2,2′-dinitro-5,5′-dithiodibenzoic acid), potassium phosphate buffer, ethylene diamine tetraacetic acid (EDTA) disodium salt, KCl, thiobarbituric acid (TBA), phenylmethanesulfonyl fluoride (PMSF) and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals or reagents were also provided at analytical grades from Santa Cruz Biotechnology (Santa Cruz, USA).

2.2. Animals

Male albino mice weighing 25–30 g with age of 3 months were obtained from the animal care center, faculty of medicine, Mashhad University of Medical Sciences, Mashhad, Iran. The mice were housed in separated standard cages and a silent and ventilated room with controlled temperature (21 ± 2 °C) and humidity (60 ± 3%). They had free access to standard animal chew and tap water with a 12-h light/dark schedule. The study was executed in conformity with ethical guidelines approved by the *Animal Care Use Committee of Mashhad University of Medical Sciences* (No. IR.MUMS.fm. REC.1396.462).

2.3. Study design and monitoring

2.3.1. Protocol 1

This experiment was included control, model (DG-induced aging), metformin (300 mg/kg, oral gavage [12]) and EA (30 and 100 mg/kg, oral gavage) groups. All groups received DG subcutaneously (s.c.) at the dose of 500 mg/kg [13,14] once daily for 10 weeks except the control group (Table 1). Control group mice were injected with saline (s.c.) with the same volume.

2.3.2. Protocol 2

To have a better understanding about the EA mechanism of action, a PPAR-γ antagonist GW9662 (1 mg/kg; i.p. [15,16]) was injected 1 h

Table 1
Study design for evaluating the anti-aging effects of EA.

Protocol	Group	Treatment	Duration of treatment	
			6 weeks	10 weeks
1	1	Control group: Saline (s.c.)	N = 7–8	N = 7–8
	2	Model group: DG (500 mg/kg s.c.)	N = 7–8	N = 7–8
	3	Metformin (300 mg/kg oral gavage) + DG (500 mg/kg s.c.)	N = 7–8	N = 7–8
	4	EA 30 mg/kg (oral gavage) + DG (500 mg/kg s.c.)	N = 7–8	N = 7–8
	5	EA 100 mg/kg (oral gavage) + DG (500 mg/kg s.c.)	N = 7–8	N = 7–8
2	1	Control group: Saline (s.c.) + GW9662 (1 mg/kg, i.p.)	N = 7–8	N = 7–8
	2	Model group: DG (500 mg/kg; s.c.) + GW9662 (1 mg/kg, i.p.)	N = 7–8	N = 7–8
	3	EA 30 mg/kg (oral gavage) + DG (500 mg/kg s.c.) + GW9662 (1 mg/kg, i.p.)	N = 7–8	N = 7–8
	4	EA 100 mg/kg (oral gavage) + DG (500 mg/kg s.c.) + GW9662 (1 mg/kg, i.p.)	N = 7–8	N = 7–8
3	1	Control group: Saline (s.c.)	N = 7–8	N = 7–8
	2	Model group: DG (500 mg/kg s.c.)	N = 7–8	N = 7–8
	3	EA 30 mg/kg (oral gavage) + DG (500 mg/kg s.c.) + metformin (300 mg/kg, oral gavage)	N = 7–8	N = 7–8

before EA (30 and 100 mg/kg; oral gavage), control and model (DG-induced aging) groups (Table 1). There were no statistical differences between control and model groups of protocol one and two.

2.3.3. Protocol 3

According to the result of protocol two, in this set of experiment, we assessed the combination of EA (30 mg/kg) with metformin (300 mg/kg) as a PPAR-γ and AMP-activated protein kinase (AMPK) activator, respectively (Table 1). There were no statistical differences between control and model groups of protocol one and three.

The male mice were randomly divided into eight groups (n = 15–16 per group). The body weight was measured at weeks 0, 6 and 10. The half number of each group (n = 7–8) were euthanized at the end of 6 and 10 weeks of treatment. Mice were deeply anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) [17,18], and then the blood sample, whole brain, and liver were isolated for further investigations.

2.4. Sample preparation

Blood samples (1.5 ml per mice) were collected by cardiac puncture, and then immediately centrifuged at 3000 rpm for 10 min at 4 °C and the supernatant was separated for further assays. Brain and liver homogenate (10% w/v) were prepared in 5% potassium chloride, 0.5 mM PMSF along with the protease inhibitor cocktail, then centrifuged at 3000 rpm for 10 min at 4 °C. The supernatants were separated and stored at −80 °C for further measurements. The total protein concentrations were measured according to Bradford's method [19].

2.5. Assessment of alanine aminotransferase (ALT), aspartate aminotransferase (AST), fasting blood sugar (FBS) and hemoglobin A1C (HbA1c) in serum

The activity of ALT and AST, and the levels FBS and HbA1c were measured using the commercially available kits from Pars Azmoon® Co., Iran, according to the manufacturer's manual.

2.6. Assessment of dehydroepiandrosterone sulfate (DHEA-SO_4) and testosterone in serum

The levels of testosterone (CSB-E05101m) and DHEA-SO_4 (CSB-E08228m) were measured by the sandwich enzyme-linked immunosorbent assay (ELISA) method according to the manufacturer's instruction. The kits were from CUSABIO Co., China.

2.7. Measurement of oxidative and anti-oxidative factors in liver and brain: MDA and GSH

As an index of the antioxidant system, the total level of sulfhydryl (–SH) groups was measured using DTNB which reacts with the –SH moieties and produces a yellow complex dye with a peak absorbance at 412 nm. Twenty μl of each sample was used for the assessment. Sample absorbance was read out at 412 nm against KPE buffer (0.05 M potassium phosphate buffer pH = 7.2 containing. 1 mM EDTA) alone (A1). Then, 120 μl DTNB reagent (2 mg/3 ml KPE) was added to the mixture, and absorbance was read again after 2 min away from the light at room temperature (22–25 °C) (A2). The absorbance of the DTNB reagent alone was also read as a blank (B). Total thiol concentration (nM/mg protein) was calculated using the following equation [20,21]; Total thiol concentration (nM/mg protein) = $(A_2 - A_1 - B) \times \frac{1.07}{0.05} \times 13.6$.

To determine the level of malondialdehyde (MDA) as an oxidative marker and lipid peroxidation, the sample was combined with a solution containing HCl and TBA (Thiobarbituric acid). Briefly, one ml of samples were added to two ml of HCl and TBA mixture and heated in a water bath at 50 °C for 40 min. After cooling and reaching the room temperature (22–25 °C), it was centrifuged at 1000 rpm for 10 min. The absorbance was read out at 535 nm and then the MDA concentration was achieved by the following equation; C (M) = (A/1.65) × 10^5, where C and A represented the concentration and absorbance, respectively [22,23].

2.8. Detection of inflammatory cytokines in serum, liver, and brain: IL-6 and TNF-α

As indexes of inflammation, the concentration of TNF-α and IL-6 were measured in the serum, liver, and brain using the commercially available ELISA kits according to the manufacturer's instruction [24,25]. The kits were obtained from Abcam ®, Cambridge, MA, USA.

2.9. Assessment of brain-derived neurotrophic factor (BDNF), acetylcholinesterase (AChE), and advanced glycation end products (AGEs) levels in the brain

In the brain context, the levels of BDNF (CYT306, Millipore ®, Germany), AChE (ab138871, Abcam ®, Cambridge, MA, USA), and AGEs (E0589Mo, BT-Laboratory, Shanghai Korain Biotech Co., China) were measured using the sandwich ELISA method according to the manufacturer's protocol.

2.10. Statistical analysis

The results were presented as means ± SEM. Firstly, the normality of data distribution was done using Kolmogorov-Simonov test. After that, the comparison between the results was performed using two-way ANOVA with Tukey-Kramer's *post hoc* test. The probability (p) value < 0.05, 0.01, and 0.001 were found statistically significant.

3. Results

3.1. The effects of EA and metformin on the body weight

3.1.1. At the end of 6 weeks

DG treatment caused a significant reduction in body weight in comparison to the control group ($p < 0.001$, Fig. 2). In contrast, the body weight was significantly increased in EA (100 mg/kg) treated group compared to the DG group ($p < 0.001$ for both cases, Fig. 2). Furthermore, the combination of EA (30 mg/kg) and metformin (300 mg/kg) notably improved the body weight in comparison with EA (30 mg/kg) alone and DG groups ($p < 0.001$ for both cases, Fig. 2).

3.1.2. At the end of 10 weeks

DG treatment significantly decreased body weight in comparison to the control group ($p < 0.001$, Fig. 2). However, the body weight was enhanced in metformin and EA (30 and 100 mg/kg) treated groups in comparison with the DG group ($p < 0.001$–0.01, for all cases, Fig. 2). The addition of GW9662 markedly reversed the increasing effects of EA (30 mg/kg) on body weight compared to EA (30 mg/kg) alone ($p < 0.001$, Fig. 2). Moreover, the combination of metformin (300 mg/kg) with EA (30 mg/kg) meaningfully elevated the body weight in comparison with metformin, EA (30 mg/kg) alone and DG groups ($p < 0.001$, $p < 0.01$, and $p < 0.001$, respectively, Fig. 2).

3.2. The effects of EA and metformin on biochemical factors in serum

3.2.1. At the end of 6 weeks

We found that the serum levels of ALT, AST, FBS, and HbA1c are notably elevated, while the levels of DHEA-SO_4 and testosterone are reduced by DG treatment compared to the control group ($p < 0.001$ for all cases, Fig. 3A–F). Metformin and EA (100 mg/kg) treatments remarkably decreased the serum ALT, AST, FBS, and HbA1c levels, while enhanced DHEA-SO_4, testosterone levels compared to the DG group ($p < 0.001$ and $p < 0.01$, Fig. 3A–F). The combination of GW9662 with EA (30 mg/kg) considerably regurgitated the protective

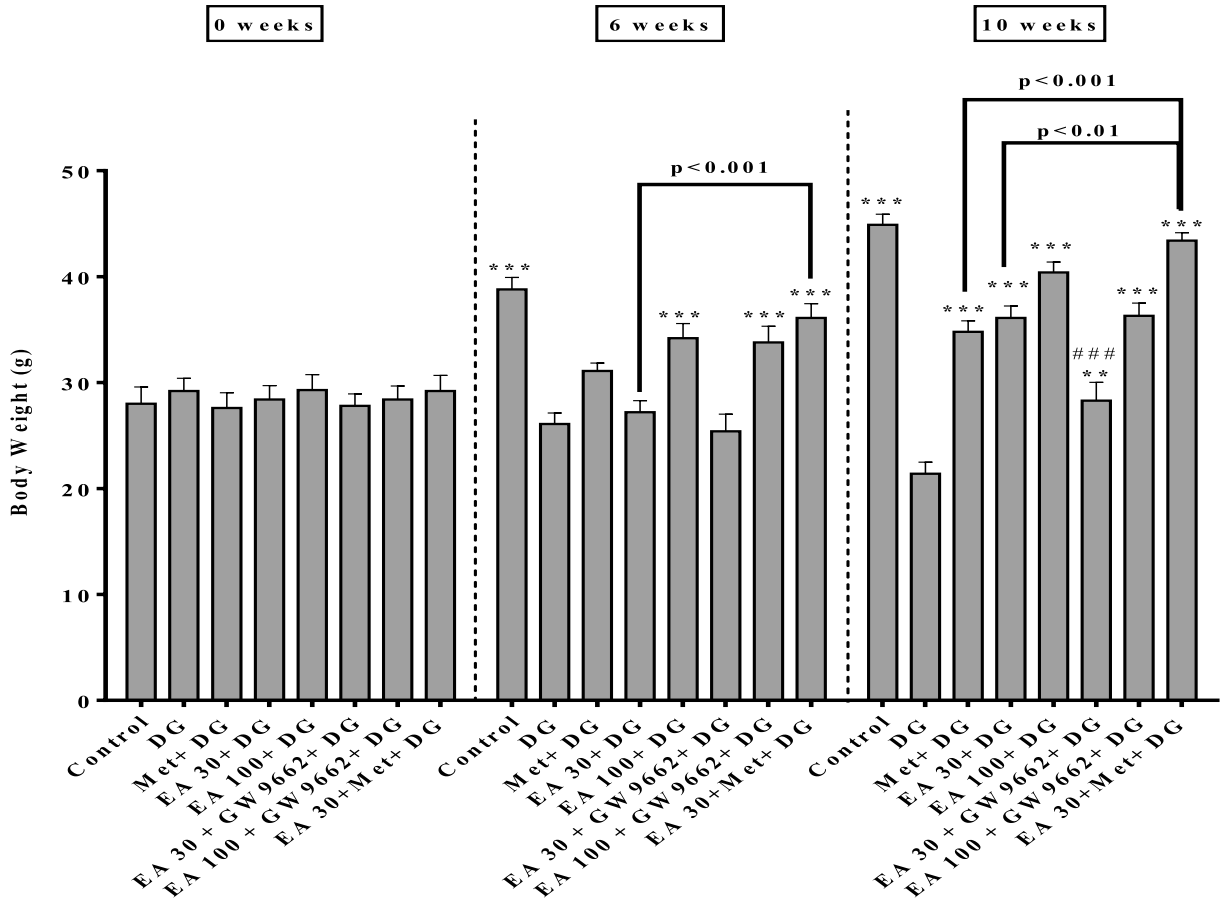

Fig. 2. The effects of EA (30 and 100 mg/kg) and metformin (300 mg/kg) on body weight following DG (500 mg/kg) induced aging in the presence and absence of GW9662 (1 mg/kg). Data were presented as mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to DG group, $\#\#\#p < 0.001$ compared to EA (30 mg/kg) in the absence of GW9662. Data were analyzed using Two-way ANOVA with Tukey-Kramer's *post hoc* test.

effects of EA (30 mg/kg) on serum ALT level in comparison with EA (30 mg/kg) alone ($p < 0.05$, Fig. 3A). Furthermore, the combination of metformin (300 mg/kg) with EA (30 mg/kg) significantly reduced the levels of ALT, AST, FBS, and HbA1c, while increased the levels of DHEA-SO_4 and testosterone in serum compared to EA (30 mg/kg) treated alone and DG groups ($p < 0.001$ for all cases, Fig. 3A-F).

3.2.2. At the end of 10 weeks

DG treatment meaningfully stimulated the levels of ALT, AST, FBS, and HbA1c, while prevented the levels of DHEA-SO_4 and testosterone in serum in comparison with the control group ($p < 0.001$ for all cases, Fig. 3A–F). In contrast, ALT, AST, FBS and HbA1c levels in serum were notably diminished by either metformin or EA (30 and 100 mg/kg) treatments in comparison with the DG group ($p < 0.001$ for all cases, Fig. 3A–F). Furthermore, the levels of DHEA-SO_4 and testosterone in serum were markedly improved in metformin and EA (30 and 100 mg/kg) groups compared to the DG group ($p < 0.001$ for all cases, Fig. 3A–F). In the presence of GW9662, the reducing effects of EA (30 mg/kg) on serum ALT, AST, testosterone, FBS and HbA1c levels, were meaningfully reversed in comparison with EA (30 mg/kg) treatment alone ($p < 0.001$ for all cases, Fig. 3A–F). Moreover, GW9662 remarkably decreased the protective effects of EA (100 mg/kg) on serum ALT, testosterone and HbA1c levels compared to EA (100 mg/kg) treatment alone ($p < 0.01$, $p < 0.001$ and $p < 0.05$, respectively, Fig. 3A, D and F). The combination of metformin (300 mg/kg) with EA (30 mg/kg) considerably mitigated the serum ALT, AST, FBS, and HbA1c levels, while significantly propagated the levels of DHEA-SO_4 and testosterone in serum in comparison with EA (30 mg/kg) treated alone and DG groups ($p < 0.05$ and $p < 0.001$for all cases, Fig. 3A–F).

3.3. The effects of EA and metformin on oxidative stress factors in liver and brain

3.3.1. At the end of 6 weeks

The exposure with DG led to an increase in the level of MDA and a decrease in GSH content compared to the control group ($p < 0.001$ for both cases, Fig. 4A–D).

Treatment with metformin and EA (100 mg/kg) significantly attenuated MDA level in brain and liver homogenates ($p < 0.001$ for all cases, Fig. 4A and B), however, low dose of EA (30 mg/kg) significantly reduced the level of MDA in brain only ($p < 0.001$ for all cases, Fig. 4B), compared to the DG group. Furthermore, the combination of metformin with EA (30 mg/kg) made a significant decrement in MDA level in both liver and brain homogenates, in comparison with EA (30 mg/kg) treatment alone and DG groups ($p < 0.001$ for both, Fig. 4A and B). Accompanying metformin (300 mg/kg) and EA (30 mg/kg) meaningfully stimulated the GSH level in liver and brain compared to EA (30 mg/kg) treated alone and DG groups ($p < 0.05$ to 0.001, Fig. 4C and D).

3.3.2. At the end of 10 weeks

In the presence of DG administration, the levels of MDA and GSH content were significantly increased and reduced, respectively, compared to the control group ($p < 0.001$ for both cases, Fig. 4A–D).

The levels of MDA and GSH were significantly alleviated and elevated, respectively, in metformin and EA (30 and 100 mg/kg) groups in both liver and brain homogenates compared to the DG group ($p < 0.001$ to 0.01, Fig. 4A–D). The addition of GW9662 to EA (30 mg/kg) remarkably regurgitated its protective effects on MDA and GSH

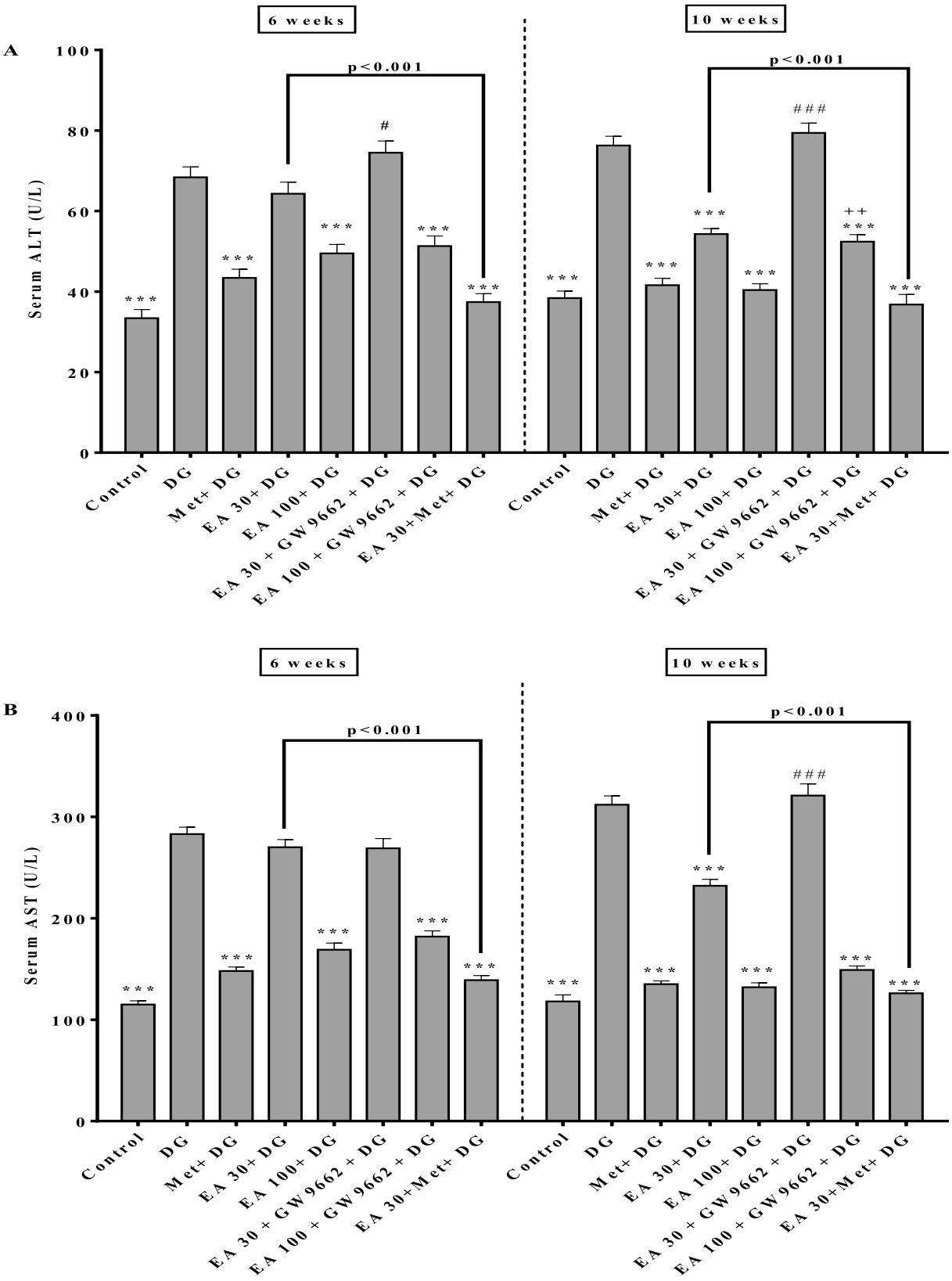

Fig. 3. The effects of EA (30 and 100 mg/kg) and metformin (300 mg/kg) on biochemical factors following DG (500 mg/kg) induced aging in the presence and absence of GW9662 (1 mg/kg); A) ALT, B) AST, C) DHEA-SO_4, D) Testostoron, E) FBS, and F) HbA1c. Data were presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to DG group, $^{###}p < 0.001$ compared to EA (30 mg/kg) in the absence of GW9662, $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ compared to EA (100 mg/kg) in the absence of GW9662. Data were analyzed using Two-way ANOVA with Tukey-Kramer's *post hoc* test.

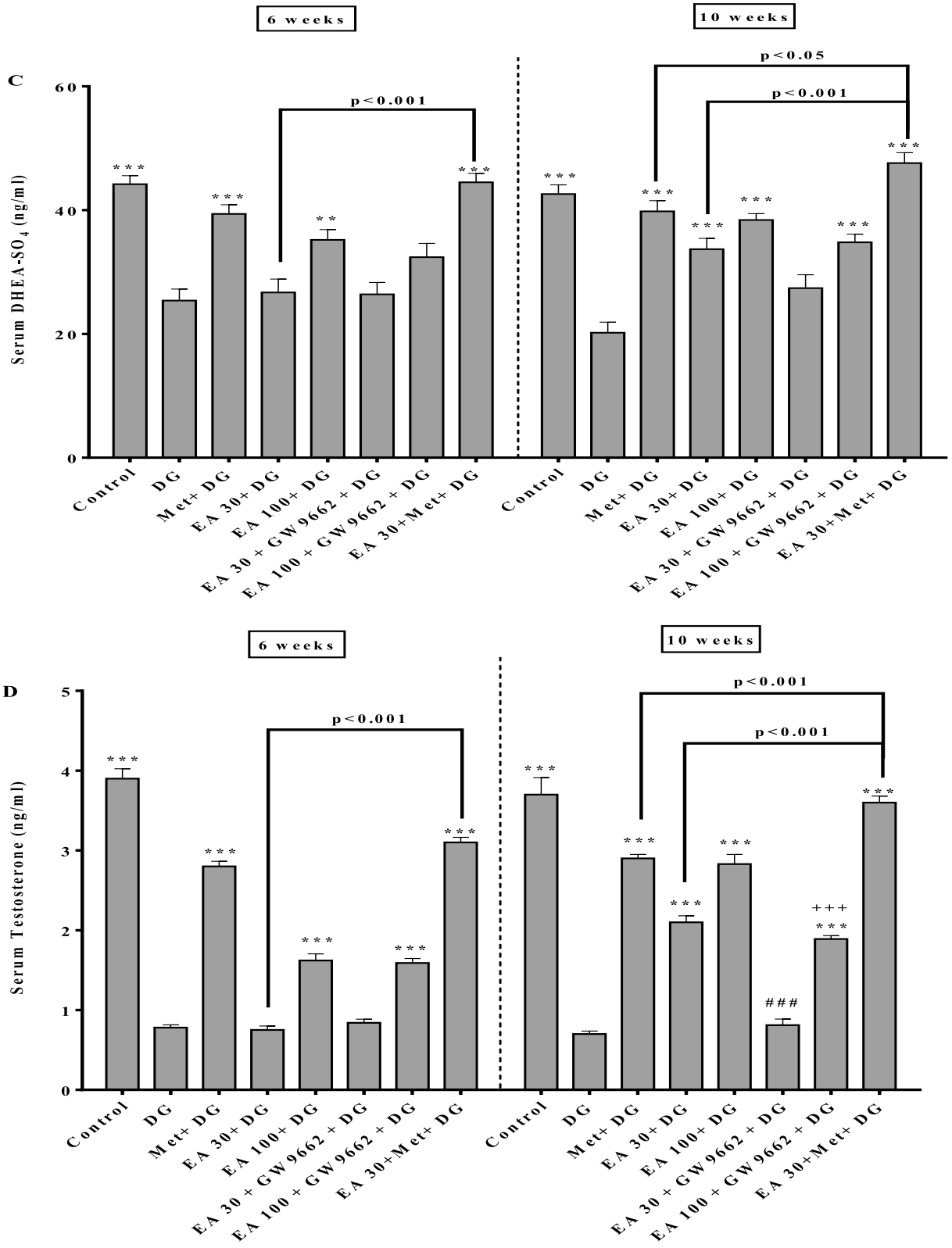

Fig. 3. (*continued*)

levels in liver and brain, in comparison with EA (30 mg/kg) treated alone ($p < 0.05$ and $p < 0.001$, Fig. 4A–D). The combination of metformin with EA (30 mg/kg) considerably dwindled MDA level and appended GSH level in liver and brain homogenates, compared to both EA (30 mg/kg) and metformin-treated alone as well as DG groups ($p < 0.001$ to 0.05, Fig. 4A–D).

3.4. EA and metformin significantly mitigated IL-6 level in serum, and homogenates of liver and brain

3.4.1. At the end of 6 weeks

DG dramatically increased the level of IL-6 in serum, and homogenates of liver and brain, compared to the control group ($p < 0.001$

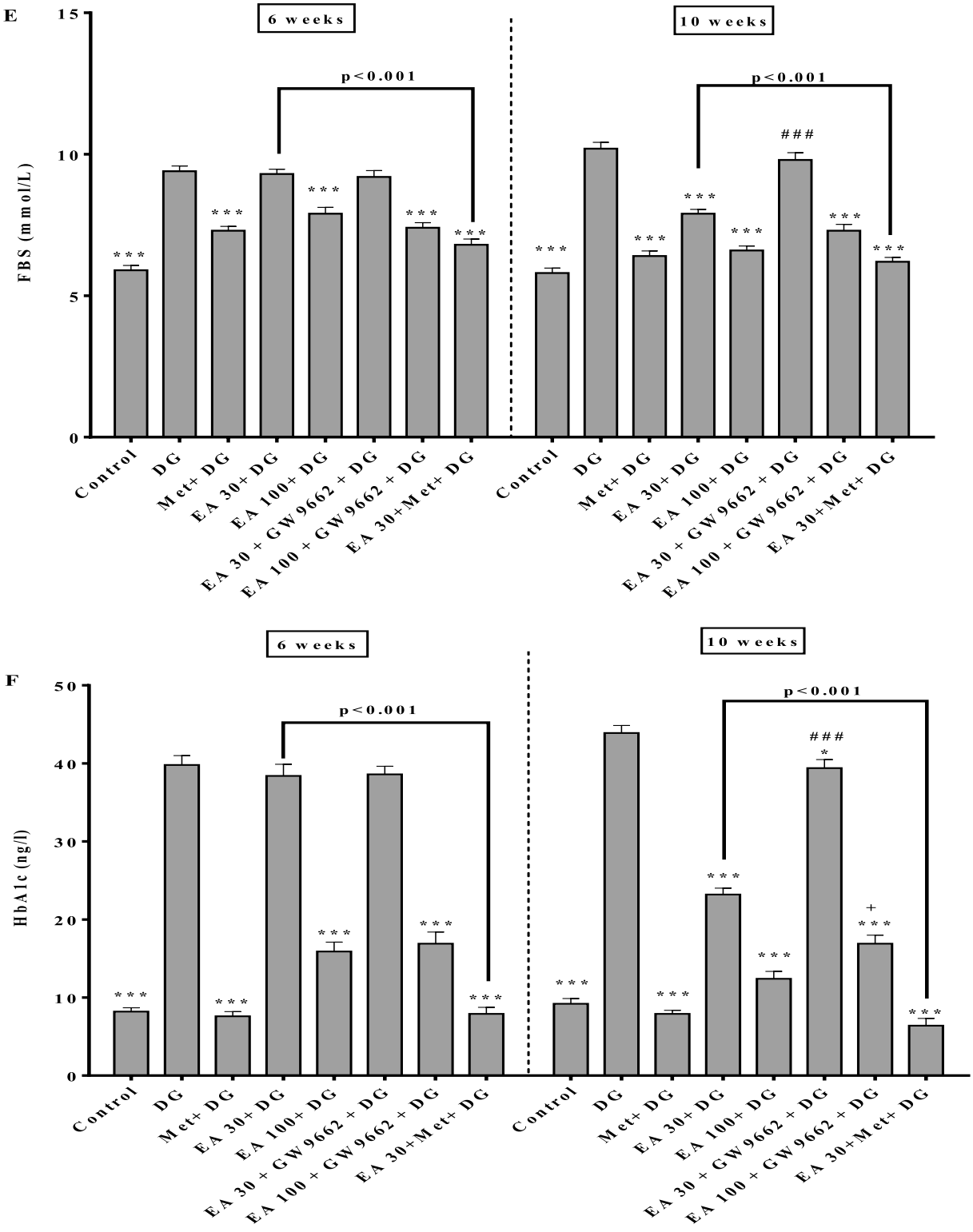

Fig. 3. (*continued*)

for all cases, Fig. 5A–C). Metformin markedly reduced the levels of IL-6 in serum, liver, and brain compared to DG group following DG-induced aging ($p < 0.001$ to 0.05, for all cases, Fig. 5A–C). EA (100 mg/kg) notably attenuated the level of IL-6 in serum and brain, compared to the DG group ($p < 0.01$ and $p < 0.05$, respectively, Fig. 5A and C). The addition of metformin (300 mg/kg) to EA (30 mg/kg) notably diminished the levels of IL-6 in serum, liver, and brain, in comparison with EA (30 mg/kg) alone and DG groups ($p < 0.001$ for all cases, Fig. 5A–C).

3.4.2. At the end of 10 weeks

DG significantly increased the level of IL-6 in serum, and homogenates of liver and brain, compared to the control group ($p < 0.001$ for all cases, Fig. 5A–C). The levels of IL-6 were meaningfully attenuated in metformin and EA (30 and 100 mg/kg) groups in serum, liver, and brain, compared to the DG group ($p < 0.001$ for all cases,

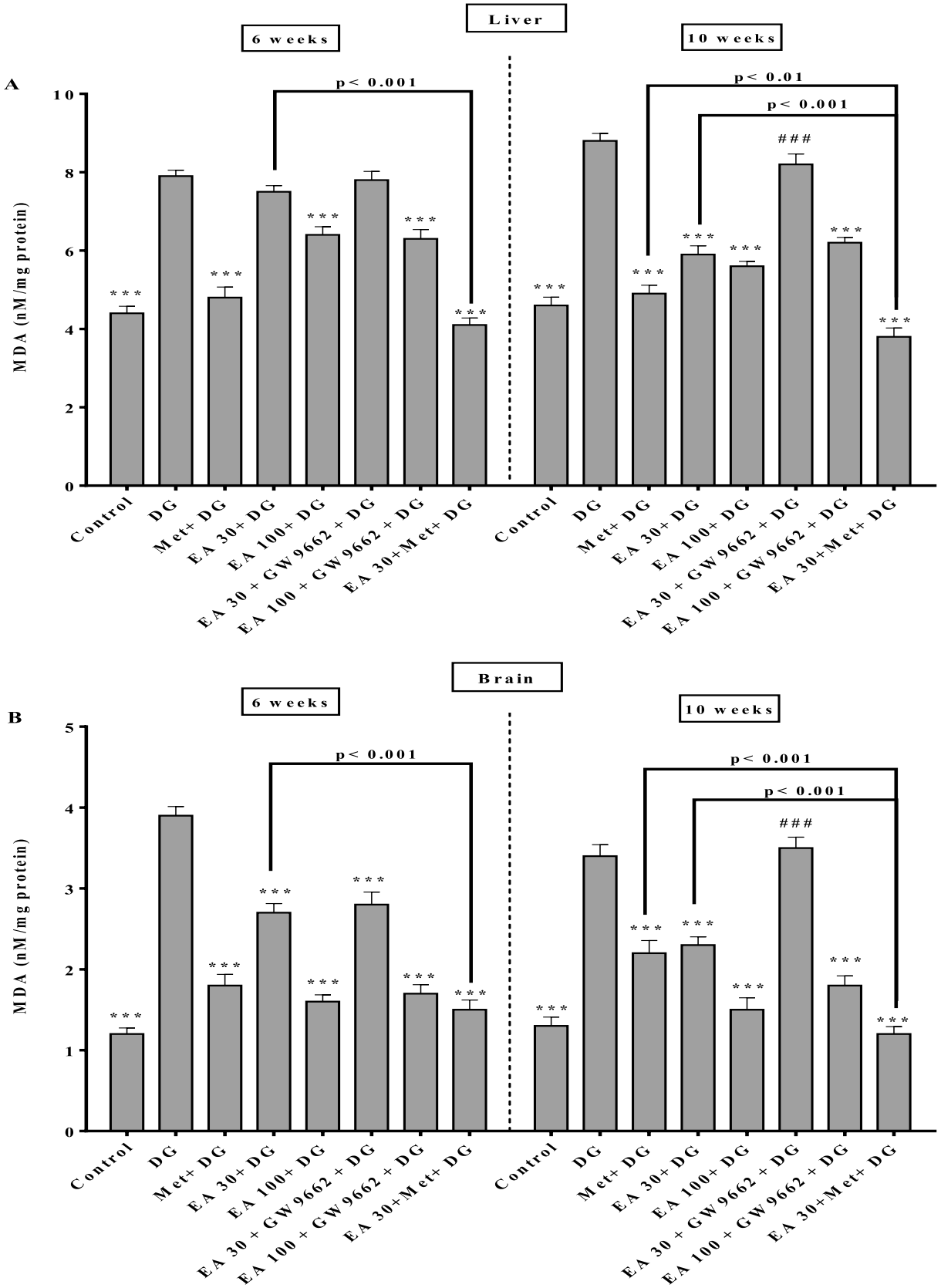

Fig. 4. The effects of EA (30 and 100 mg/kg) and metformin (300 mg/kg) on oxidative stress factors in liver and brain following DG (500 mg/kg) induced aging in the presence and absence of GW9662 (1 mg/kg); A) liver MDA, B) brain MDA, C) liver GSH, and D) brain GSH. Data were presented as mean ± SEM. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared to DG group, $\#p < 0.05$ and $\#\#\#p < 0.001$ compared to EA (30 mg/kg) in the absence of GW9662. Data were analyzed using Two-way ANOVA with Tukey-Kramer's *post hoc* test.

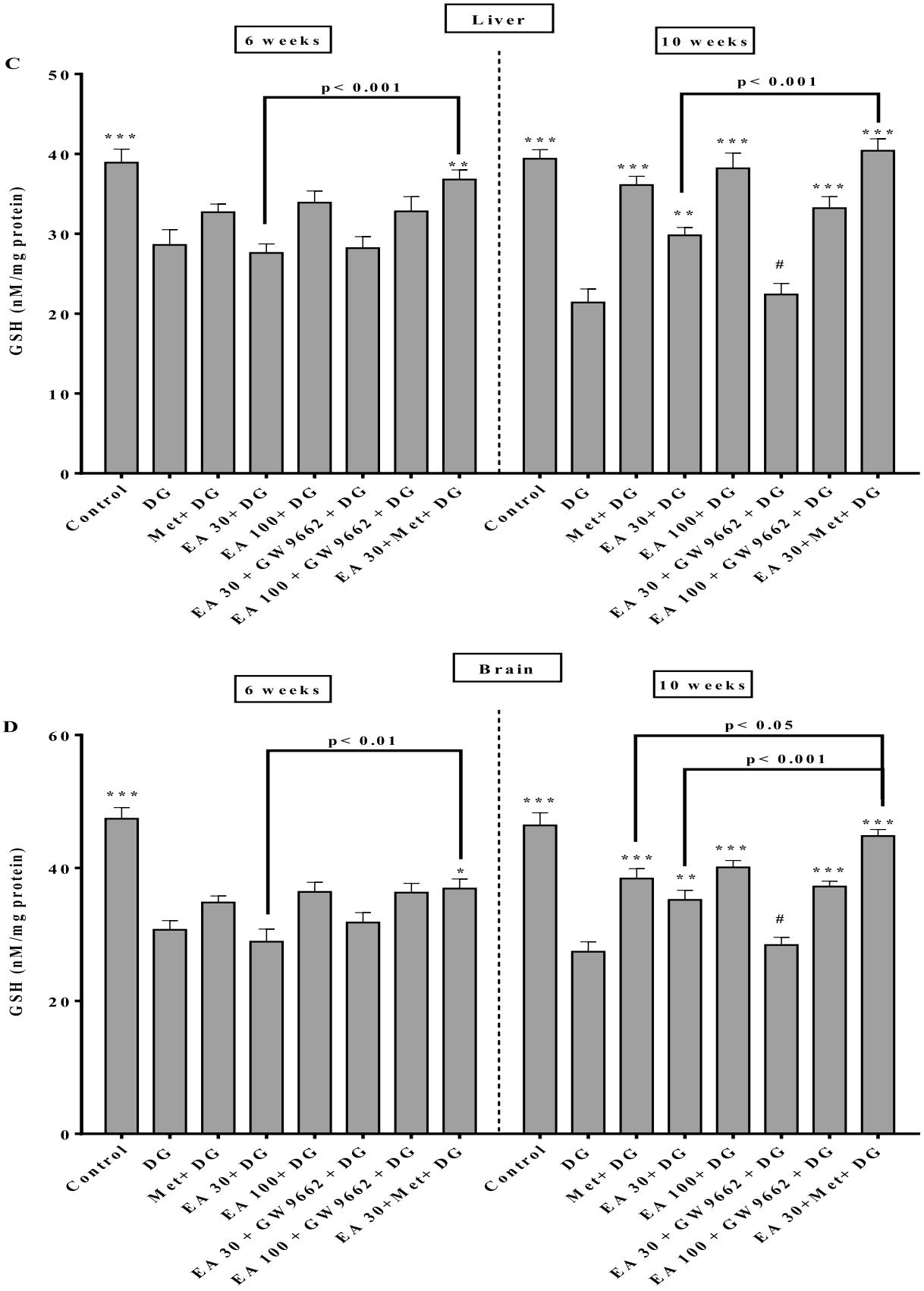

Fig. 4. (*continued*)

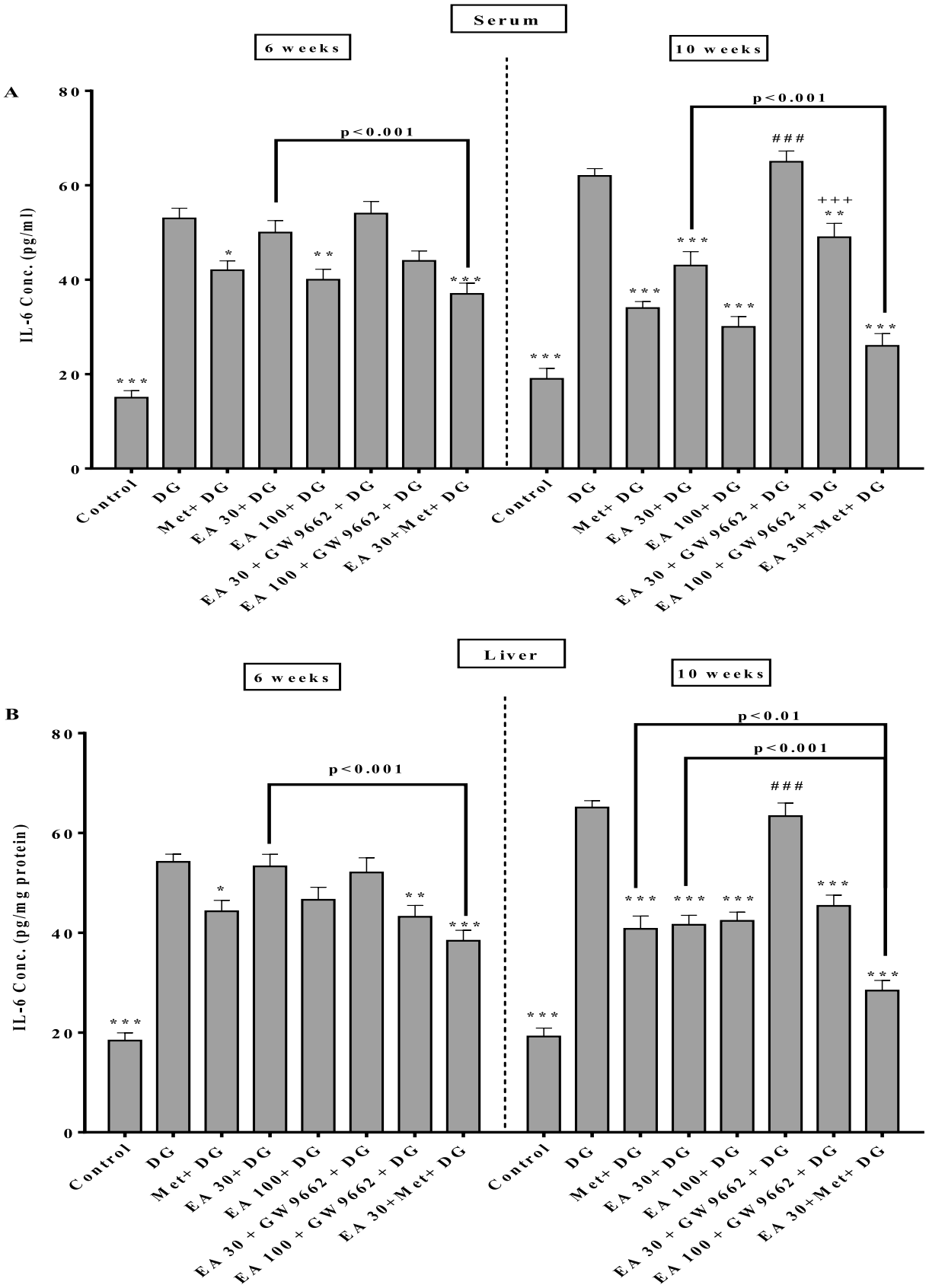

Fig. 5. The effects of EA (30 and 100 mg/kg) and metformin (300 mg/kg) on IL-6 in A) serum, B) liver and C) brain following DG (500 mg/kg) induced aging in the presence and absence of GW9662 (1 mg/kg). Data were presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to DG group, ##$p < 0.01$ and ###$p < 0.001$ compared to EA (30 mg/kg) in the absence of GW9662, + +$p < 0.01$ and + + +$p < 0.001$ compared to EA (100 mg/kg) in the absence of GW9662. Data were analyzed using Two-way ANOVA with Tukey-Kramer's *post hoc* test.

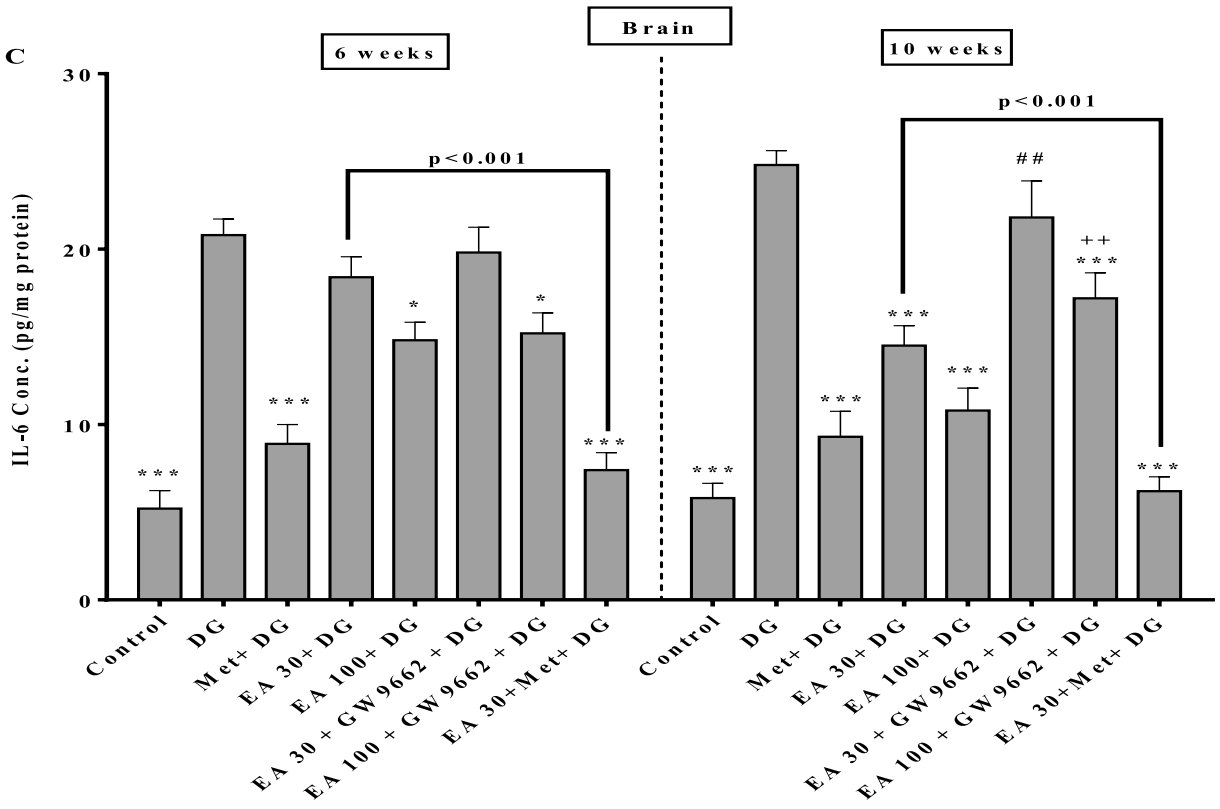

Fig. 5. (*continued*)

Fig. 5A–C). The combination of EA (30 mg/kg) with GW9662 completely reversed the level of IL-6 in serum, liver, and brain, in comparison with EA (30 mg/kg) alone ($p < 0.001$ to 0.01, Fig. 5A–C). however, the addition of GW9662 to EA (100 mg/kg) considerably reduced the protective effects of EA (100 mg/kg) on IL-6 levels in serum and brain, compared to EA (100 mg/kg) treated alone ($p < 0.001$ and $p < 0.01$, respectively, Fig. 5A and C). The combination of metformin with EA (30 mg/kg) significantly alleviated the level of IL-6 in serum, liver, and brain compared to EA (30 mg/kg) treated alone and DG groups ($p < 0.001$ for all cases, Fig. 5A–C). Furthermore, the decreasing effect of the combination of metformin with EA (30 mg/kg) on IL-6 level was significantly greater than the metformin-treated alone group in the liver ($p < 0.01$, Fig. 5B).

3.5. EA and metformin notably attenuated TNF-α level in serum, liver, and brain

3.5.1. At the end of 6 weeks

The level of the inflammatory cytokine TNF-α was significantly stimulated by DG treatment, in comparison to the control group ($p < 0.001$, Fig. 6A–C). Treatment with each of metformin and EA (100 mg/kg) group considerably abolished TNF-α level in serum, liver and brain tissue, compared to the DG group ($p < 0.001$ to 0.05, for all cases, Fig. 6A–C). Moreover, the combination of EA (30 mg/kg) with metformin markedly decreased TNF-α level in serum, liver and brain tissue, in comparison with EA (30 mg/kg) alone and DG groups ($p < 0.001$ for all cases, Fig. 6A–C).

3.5.2. At the end of 10 weeks

The administration of DG led to a significant increase in the level of TNF-a in serum, liver and brain tissue, compared to the control group ($p < 0.001$ for all cases, Fig. 6A–C). The level of TNF-α was meaningfully reduced by treating either metformin or EA (30 and 100 mg/kg) treatments in serum, liver and brain tissue, in comparison with the DG ($p < 0.001$ for all cases, Fig. 6A–C). The combination of GW9662 with EA (30 mg/kg) remarkably enhanced TNF-α level in serum, liver, and brain, compared to EA (30 mg/kg) treatment alone ($p < 0.001$ for all cases, Fig. 6A–C). The addition of GW9662 to EA (100 mg/kg) considerably regurgitated the decreasing effects of EA on serum TNF-α level, in comparison with EA (100 mg/kg) treatment alone ($p < 0.01$, Fig. 6A). Furthermore, the combination of metformin with EA (30 mg/kg) significantly diminished TNF-α level in serum, liver and brain tissue, compared to EA (30 mg/kg) treated alone and DG groups ($p < 0.001$ for all cases, Fig. 6A–C), however, it was further found a significant difference comparing to metformin-treated alone group in brain only ($p < 0.05$, Fig. 6C).

3.6. The effects of EA and metformin on AChE, BDNF and AGEs levels in the brain

3.6.1. At the end of 6 weeks

Following the DG administration, the levels of AChE and AGEs were meaningfully elevated, while the level of BDNF was firmly reduced, compared to the control group ($p < 0.001$ for all cases, Fig. 7A–C). In contrast, metformin and EA (100 mg/kg) significantly diminished AChE level in the brain, in comparison with the DG group ($p < 0.001$ for both cases, Fig. 7A). Additionally, accompanying GW9662 to EA (30 mg/kg) notably stimulated AChE level in the brain, compared to EA (30 mg/kg) alone treatment ($p < 0.05$, Fig. 7A). The combination of metformin with EA (30 mg/kg) notably showed stronger effects on the brain AChE, AGEs, and BDNF levels, compared to EA (30 mg/kg) treated alone and DG groups ($p < 0.001$ to 0.05, for all cases, Fig. 7A–C). Moreover, this combination also provided a significant reducing effect on the level of AChE in the brain, compared to metformin-treated alone ($p < 0.001$, Fig. 7A).

3.6.2. At the end of 10 weeks

In the presence of DG treatment, the levels of AChE and AGEs were

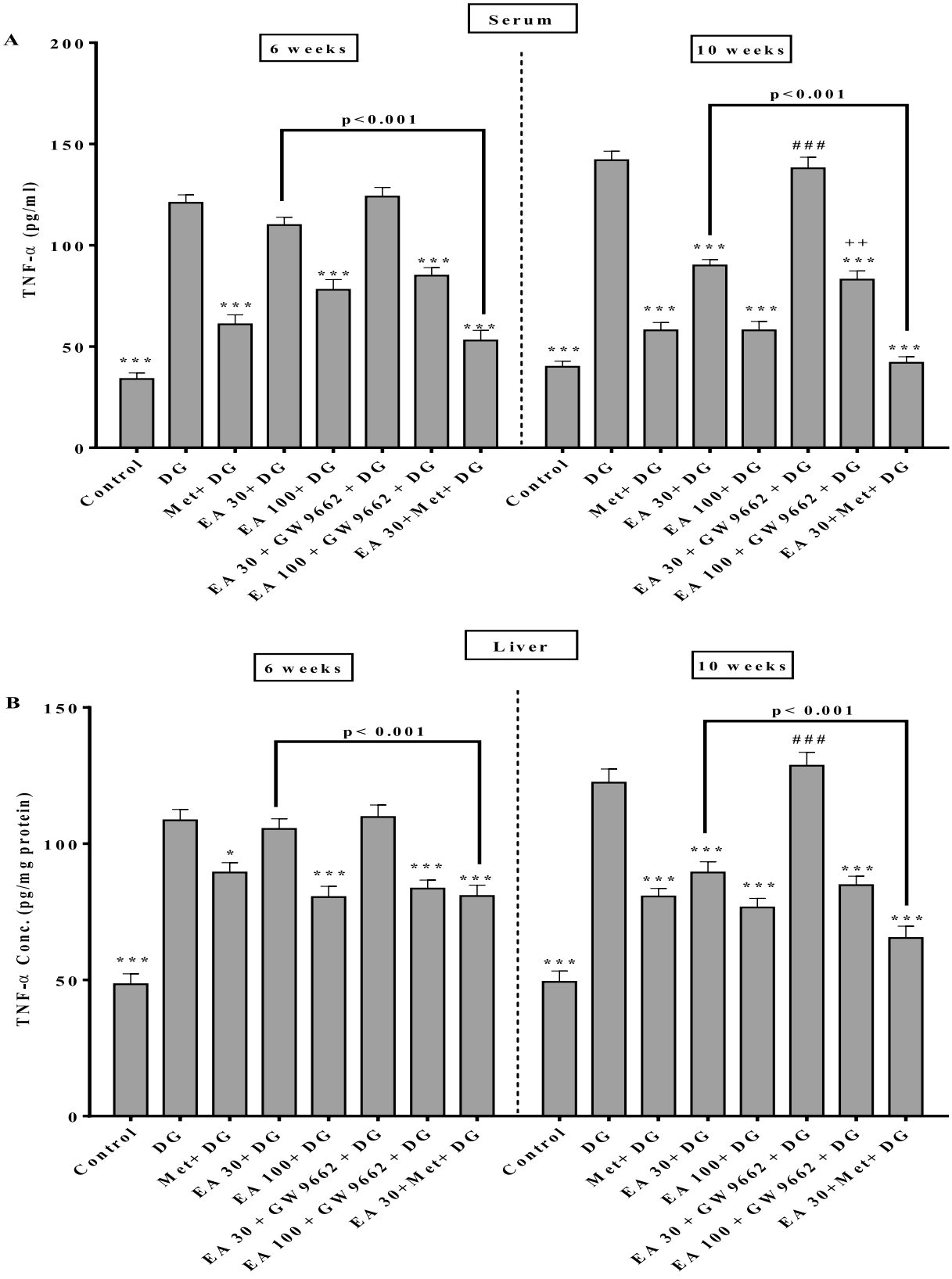

Fig. 6. The effects of EA (30 and 100 mg/kg) and metformin (300 mg/kg) on TNF-α level in A) serum, B) liver and C) brain following DG (500 mg/kg) induced aging in the presence and absence of GW9662 (1 mg/kg). Data were presented as mean ± SEM. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to DG group, $\#\#\#p < 0.001$ compared to EA (30 mg/kg) in the absence of GW9662, $++p < 0.01$ compared to EA (100 mg/kg) in the absence of GW9662. Data were analyzed using Two-way ANOVA with Tukey-Kramer's *post hoc* test.

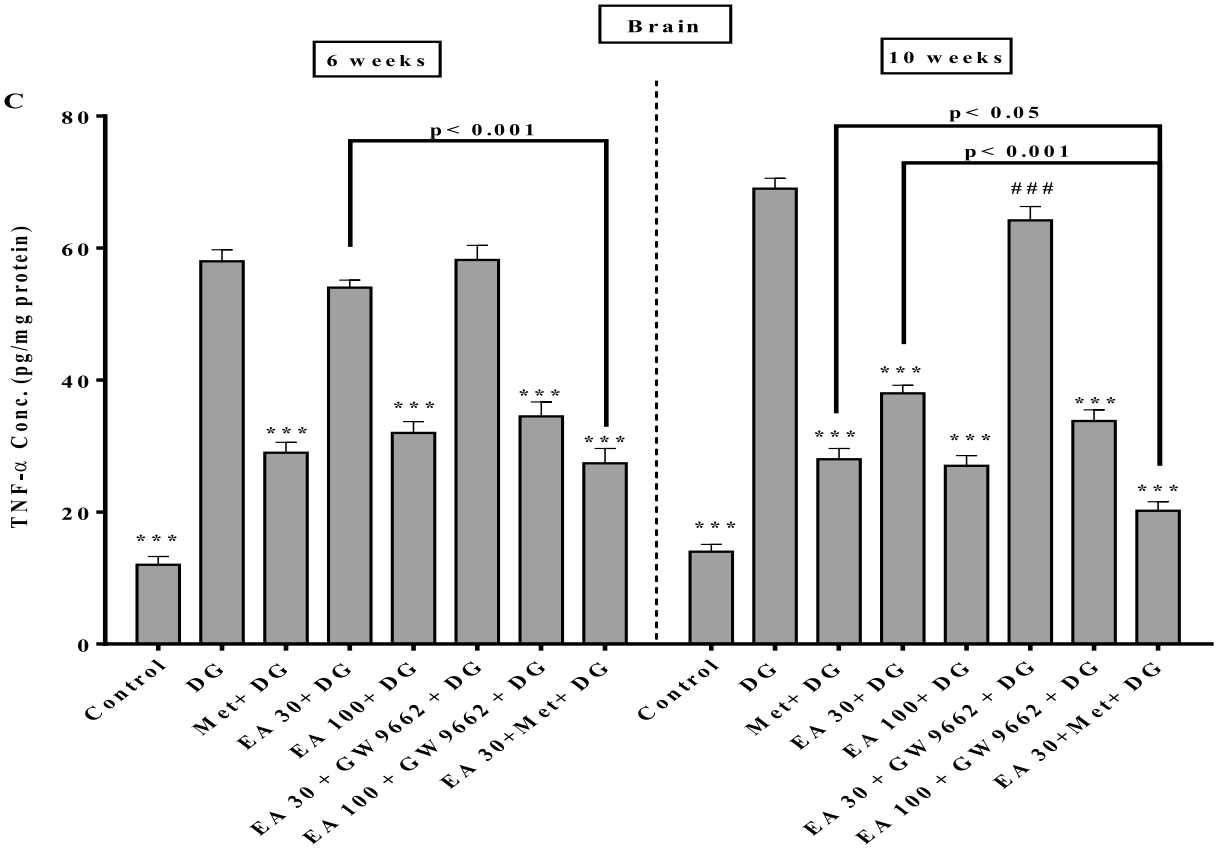

Fig. 6. (*continued*)

strikingly increased, while the level of BDNF was significantly decreased, compared to the control group ($p < 0.001$ for all cases, Fig. 7A–C). Metformin and EA (30 and 100 mg/kg) treatments remarkably attenuated AChE and AGEs level, which in turn propagated BDNF level in the brain, compared to the DG group ($p < 0.001$ to 0.01, for all cases, Fig. 7A–C). The addition of GW9662 to EA (30 mg/kg) completely blocked the effects of EA on the brain AChE, BDNF, and AGEs levels, compared to EA (30 mg/kg) treated alone ($p < 0.001$ to 0.05 for all cases, Fig. 7A–C). Nevertheless, the combination of GW9662 with EA (100 mg/kg) significantly elevated AChE levels in the brain, in comparison with EA (100 mg/kg) treated alone ($p < 0.01$, Fig. 7A). Accompanying metformin and EA (30 mg/kg) markedly decreased and increased the levels of AChE and BDNF, respectively, in the brain compared to EA (30 mg/kg) treated alone and DG groups ($p < 0.001$ and $p < 0.01$, respectively, Fig. 7A and B). Furthermore, this combination also provided a significant reducing effect on the brain level of AGEs in comparison to the DG group ($p < 0.001$, Fig. 7C).

4. Discussion

To the best of our knowledge, this is the first mechanistic study on the protective effects of EA on DG-induced model of aging. As results, daily administration of DG (s.c.) to animals for both 6 and 10 weeks led to increase in the levels of IL-6, TNF-α, MDA, AChE, AGEs, ALT, AST, FBS, and HbA1c, in which decrease in the levels of body weight, GSH and BDNF as well as serum DHEA-SO_4 and testosterone levels. In contrast, metformin (300 mg/kg) as the positive control abrogated the effects of DG-induced aging model. Furthermore, we found that low dose of EA (30 mg/kg) decreases the deteriorative effects of DG-induced aging at 10 weeks of treatment only, however, high dose of EA (100 mg/kg) was effective at both 6 and 10 weeks of treatment. Interestingly, we observed that the addition of GW9662, a PPAR-γ antagonist, completely reversed the protective effects of the low dose of EA, but not for the high dose, on DG-induced aging model. As another finding of the present study, the combination of metformin (300 mg/kg) and a low dose of EA promoted their protective effects in comparison to each one alone.

DG is a reducing sugar which exists in plenty of foods and is metabolized into glucose by galactokinase and uridyl-transferase in normal concentrations [2]. However, the oversupply of DG accumulates in the body and forms advanced glycation end products (AGEs) through the reaction with amines of peptides and proteins. It has been emphasized that AGEs bind to the receptor of advanced glycation end products (RAGE) and cause oxidative stress and inflammatory responses which is responsible for aging and age-related disorders [26]. There are several experiments notion that the chronic administration of DG mimics the human natural aging and is commonly used for developing the accelerated animal model of aging [5,11,27,28]. Accordingly, our results revealed that both 6 and 10 weeks administration of DG (500 mg/kg/day; s.c.) increased IL-6, TNF-α, MDA, AChE, AGEs, ALT, AST, FBS, and HbA1c levels, and attenuated body weight, GSH, BDNF, DHEA-SO_4 and testosterone levels. In line with our results, Majdi et al reported that DG (500 mg/kg/day; s.c. for 6 weeks) impairs spatial and episodic memory, and enhances ROS production, Bax/Bcl2 ratio and caspase-3 levels as well as diminishes BDNF and nerve growth factor (NGF) levels, in the brain tissue of mice [29]. In another study, 8 weeks administration of DG (500 mg/kg/day; s.c.) increased AGEs, and nitric oxide (NO) levels as well as nitric oxide synthase activity in the brain tissue [30]. Moreover, DG (1000 mg/kg/day; s.c. for 8 weeks) alleviates the total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px) and SOD levels as well as elevates MDA levels in serum, liver, and brain of C57BL/6 mice. DG also reduces heme Oxygenase (HO-1), and Klotho as well as enhances iNOS expression in liver and brain of C57BL/6 mice [31]. These studies could support our results regarding the effects of chronic administration of DG as an appropriate model for accelerated aging.

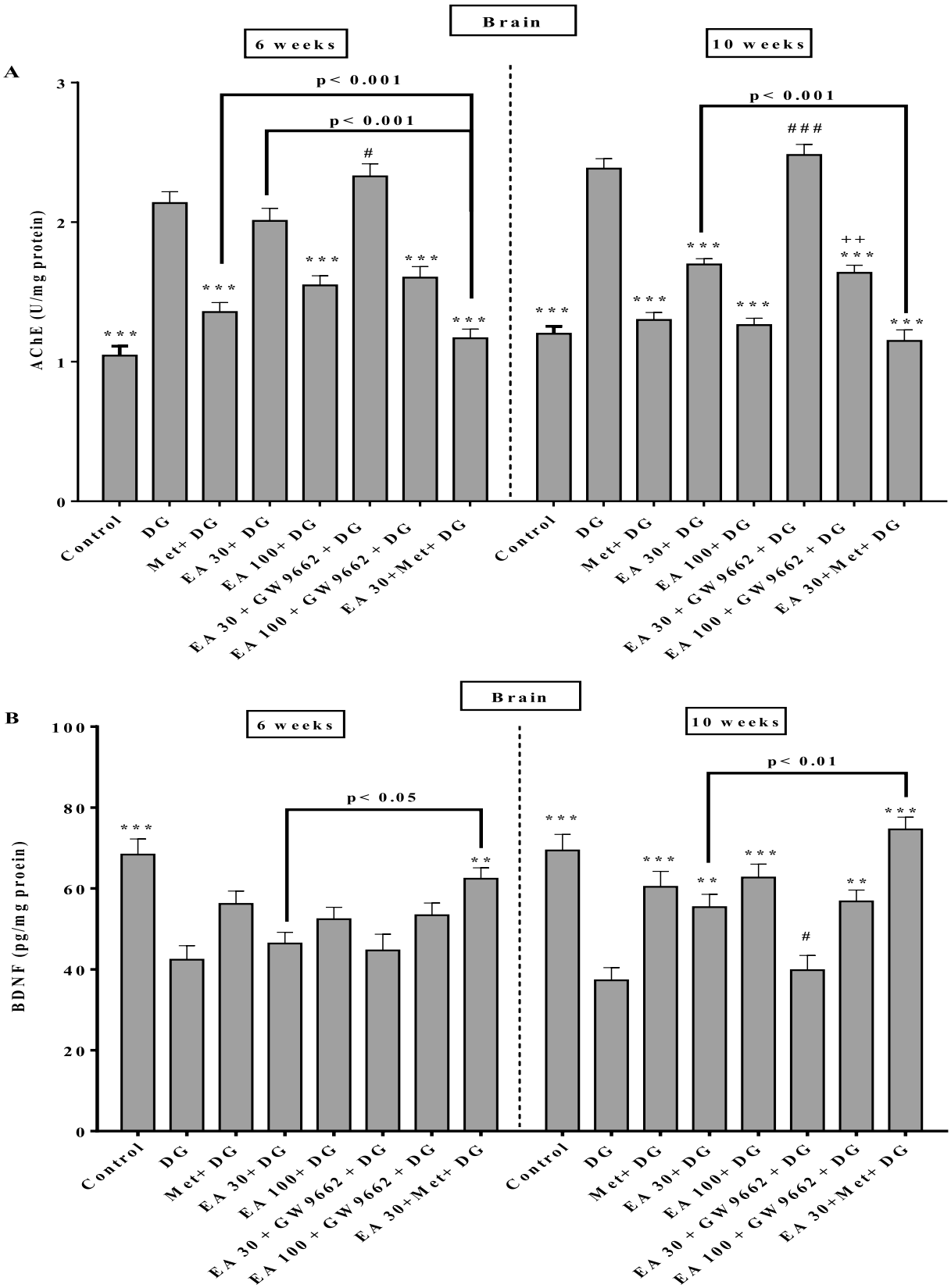

Fig. 7. The effects of EA (30 and 100 mg/kg) and metformin (300 mg/kg) on A) AChE, B) BDNF and C) AGEs levels in brain following DG (500 mg/kg) induced aging in the presence and absence of GW9662 (1 mg/kg). Data were presented as mean ± SEM. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared to DG group, $\#p < 0.05$ and $\#\#\#p < 0.001$ compared to EA (30 mg/kg) in the absence of GW9662, $++p < 0.01$ compared to EA (100 mg/kg) in the absence of GW9662. Data were analyzed using Two-way ANOVA with Tukey-Kramer's *post hoc* test.

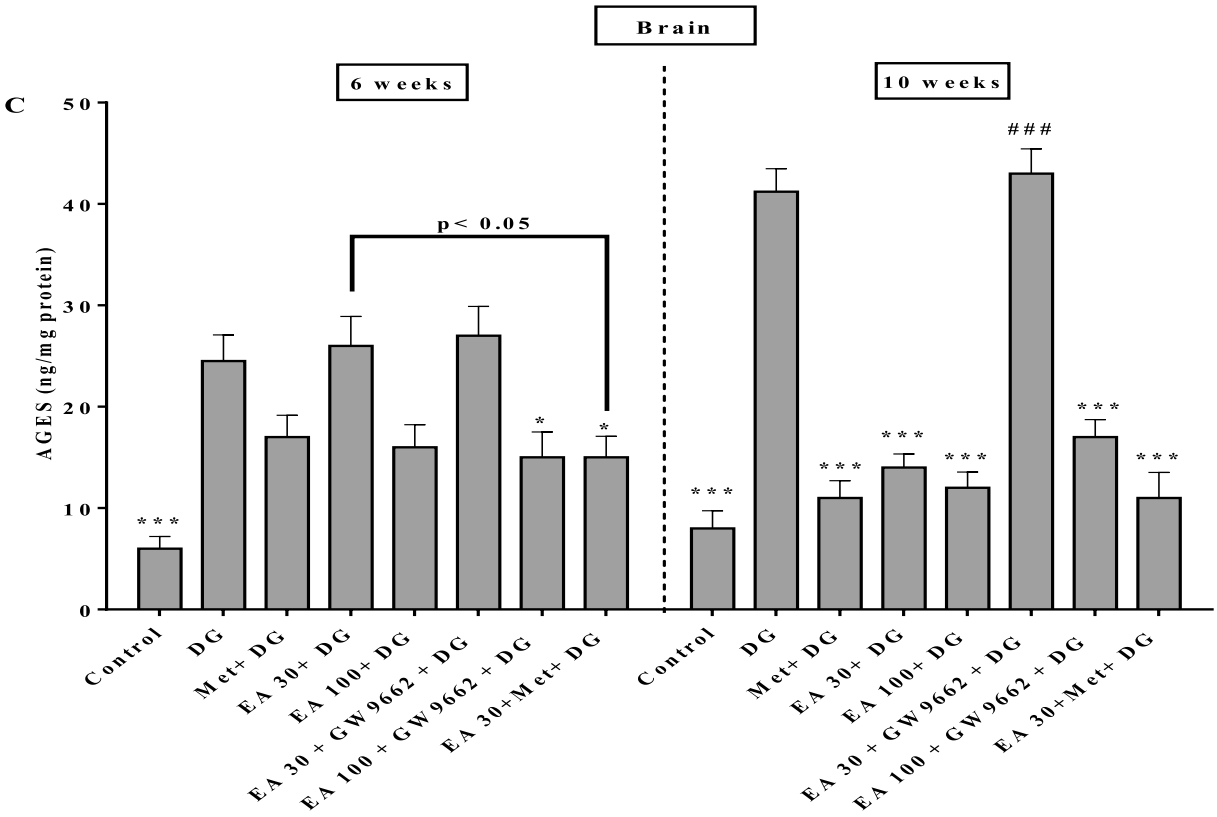

Fig. 7. (*continued*)

Metformin is a widely-used drug which is commonly prescribed for the treatment of hyperglycemia in type II diabetes and metabolic syndrome. It propagates insulin sensitivity and glycolysis and inhibits gluconeogenesis in the liver [32]. The mechanism of action responsible for the therapeutic effects of metformin involves increasing the AMPK activity. However, other beneficial properties of metformin have been proved including anti-oxidant, anti-inflammation and neuroprotection [33]. Recently, anti-aging and longevity effects of metformin have been emphasized on different animal experiments consisting of worms, flies, mice, and rats [34]. Moreover, MILES clinical trial [35] supported the anti-aging effects of metformin. In our previous study, we emphasized the anti-aging effects of metformin on DG-induced aging in SH-SY5Y human neuroblastoma cells through increasing cell viability and GSH level, while decreasing ROS, MDA and TNF-α levels [11]. Given the anti-aging effects of metformin and due to the inexistence of standard positive control for aging studies, we used metformin as positive anti-aging control in our experiment. As results, we showed that both 6 and 10 weeks administration of metformin (300 mg/kg/day, orally) alleviates IL-6, TNF-α, MDA, AChE, AGEs, ALT, AST, FBS, and HbA1c levels as well as propagates body weight, GSH, BDNF, DHEA-SO_4 and testosterone levels following the DG-induced aging in mice. In acknowledging our results, Garg et al. also demonstrated the effects of metformin (300 mg/kg, orally for 6 weeks) on naturally aged and DG-induced accelerated aged rats. They showed that metformin augments ferric reducing antioxidant potential (FRAP), GSH and AChE levels as well as diminishes MDA, protein carbonyl, ROS and NO levels in both naturally aged and DG-induced aging groups, compared to the age groups. Furthermore, metformin down-regulated the expression levels of IL-6, TNF-α and sirtuin-2 as well as up-regulated Beclin-1 expression level, in both mentioned aging models [12]. In another study, Kenawy et al. evaluated and compared the effects of metformin (500 mg/kg/day; orally for 90 days, about 13 weeks) and saxagliptin (1 mg/kg/day; orally) in DG-induced aging in rats. They reported that metformin and saxagliptin, with a preference to metformin, improve memory and learning function and body weight as well as reduce HbA1c level in serum following DG-induced aging. Furthermore, metformin reduced MDA, NO, TNF-α, insulin, RAGE and phosphorylated tau protein levels as well as enhanced GSH, phosphorylated insulin receptors, acetylcholine and glutamate levels in the brain tissue of rats [36]. In fact, the results of these studies are in line with our results regarding the anti-aging effects of metformin and may support our procedure of the present study.

EA is a natural polyphenolic compound with various pharmacological properties including antioxidant, anti-inflammatory, and neuroprotective effects as well as hepato-protection [37,38]. In our previous study, we showed that EA (0.1–10 μM) possesses anti-aging properties through increasing cell viability and GSH levels, while decreases the levels of ROS, MDA, TNF-α, β-GAL, and AGEs following the DG-induced aging in SH-SY5Y human neuroblastoma cells model. Furthermore, we found that the lower concentration of EA (1 μM) exerts more potent anti-aging effects compared with the higher concentration (10 μM) on SH-SY5Y cells [11]. Therefore in this study, we evaluated the anti-aging effects of low and high doses of EA (30 and 100 mg/kg, respectively) in mice. Our results revealed that low dose of EA (30 mg/kg) were also able to mitigate IL-6, TNF-a, MDA, AChE, AGEs, ALT, AST, FBS, and HbA1c levels as well as propagates body weight, GSH, BDNF, DHEA-SO_4 and testosterone levels following DG-induced aging in mice after 10 weeks of treatment. However, a high dose of EA (100 mg/kg) showed its anti-aging effects at both 6 and 10 weeks of treatment. Indeed, we realized that the protective effects of EA at a high dose (100 mg/kg) are initiated earlier than its low dose (30 mg/kg). Recently, it has been published a similar experiment about anti-inflammatory and anti-oxidant effects of EA on DG-induced aging model in rat. Chen et al. showed that EA (50–150 mg/kg/day, for 8 weeks) improves the levels of body weight, catalase (CAT), GSH, MDA, SOD, T-AOC, and Bcl-2 as well as reduces caspase-3 and Bax levels and Bax/Bcl-2 ratio in liver and brain following the DG-induced aging in rats. Furthermore, EA diminished the levels of TNF-α, IL-6, IL-1β, ALT, and AST

in serum [10]. The results of this study are in line with our results. However, we studied not only the anti-aging effects of a low dose of EA (30 mg/kg) but also a longer period of treatment (10 weeks) was evaluated. In fact, Chen et al. measured only the anti-oxidative, inflammatory and apoptotic markers, while we evaluated more specific markers of aging (AGEs), brain damages (AChE and BDNF) and male sex hormones (DHEA-SO_4 and testosterone), which they are strikingly impressed in aging.

In agreement with our findings, there are several lines of evidence that show the effectiveness of EA at low doses and concentrations. Contextually, Goyal et al. reported the hepatoprotective effects of EA (10 mg/kg/day; orally for 6 weeks) in cisplatin-induced hepatotoxicity in mice [39]. In another study, EA (20 and 40 mg/kg; orally for 11 days) also reduced ROS generation, apoptosis and inflammatory markers (IL-1β, TNF-α, INF-γ) in arsenic-induced neuro-inflammation in rats [40]. Moreover, EA (17.5 and 35 mg/kg; orally for 28 days) improved cognitive deficit and GSH levels, while diminished TBARS and TNF-α levels in streptozotocin-induced memory deficit in rats [41]. EA (10 and 30 mg/kg; orally for 5 days) also ameliorated kidney function and decreased serum and kidney inflammatory and apoptotic markers in cisplatin-induced nephrotoxicity in rats [42]. Furthermore, EA (25 and 50 mg/kg/day, orally for 13 days) attenuated AChE and MDA levels and enhanced GSH level, SOD and CAT activity in scopolamine-induced memory dysfunction in rats [43]. In fact, these studies support our finding regarding the beneficial effects of oral administration of EA at a low dose, in inflammatory situations and related diseases. Alternatively, we recently demonstrated that EA at low concentrations protects the neuronal cells (SH-SY5Y) against DG-induced cellular aging model. Indeed, findings of the present study are in line with our previous report on EA, and mutually support them.

By founding the controversial results regarding the effectiveness of both low and high doses of EA, we performed a new experiment for a better insight into details of the involved mechanism(s). In our previous experiment, we observed that the use of GW9662, a PPAR-γ antagonist, completely abolishes the anti-aging effects of low concentrations of EA (0.1 and 1 μM) following DG-induced aging in SH-SY5Y cells. However, it could not affect the anti-aging effects of a high concentration of EA (10 μM) [11]. Based on this evidence, in the present study, we applied GW9662 before both low (30 mg/kg) and high (100 mg/kg) doses of EA in DG-induced aging model in mice. Interestingly, we found that the protective effects of the low dose of EA are PPAR-γ receptor-mediated only, because the addition of a PPAR-γ antagonist GW9662 completely blocked the effects of the low dose of EA, but not at the high dose. This finding is also in concordance with our previous report. In line with our results, Nankar et al. reported that EA (0.25–5 μM) propagates the expression of PPAR-γ in L6 myoblast cells treated with insulin [44]. In another study, the effects of EA (30 mg/kg; orally), pioglitazone (10 mg/kg; orally) and their combination (10 mg/kg for both cases) were evaluated on nicotinamide and streptozotocin-induced diabetes in rats. They suggested that EA (2.22 ± 0.24 fold changes) and pioglitazone (1.38 ± 0.29 fold changes), with a preference to EA, enhance the level of PPAR-γ gene expression compared to diabetic control (0.30 ± 0.01) in skeletal muscle tissue. Moreover, the combination of EA and pioglitazone showed an additive effect on the level of PPAR-γ gene expression (3.37 ± 0.40 fold changes) in diabetic rats [45]. These studies reported that EA increases the expression of PPAR-γ and may support our results. In fact, it may be concluded that PPAR-γ receptor and its expression level play a crucial role during the protective effects of EA at a low dose. Therefore, the promising protection of EA is likely mitigated by deletion or in the presence of pharmacological antagonist, which we showed in the current experiment. On the other hand, several studies indicated that the PPAR-γ receptor activation provides anti-aging effects by different mechanisms. In one study, Shen et al. showed that the use of pioglitazone, a known PPAR-γ agonist, attenuates aging-related disorders in aged apolipoprotein E deficient mice. They described that pioglitazone leads to up-regulation of PPAR-γ receptor, mRNA levels of anti-aging genes Sirtuin1 and Klotho, and decreased the p53 protein level and inflammatory markers [46]. Furthermore, it has been reported that the use of pioglitazone PPAR-γ receptor dependently led to the reduction in the mitochondrial apoptotic pathway and mito-oxidative damage in the DG-induced mouse model, since the addition of BADGE (PPAR-γ antagonist) with pioglitazone abolished the protective effect of pioglitazone [47]. Taken together, PPAR-γ receptor activation *via* EA or pioglitazone provides anti-aging effects.

Given the protective effects of AMPK in the regulation of energy metabolism, the control of many cellular functions and prevention of aging [48], and the crucial role of PPAR-γ activators in the prevention of aging and its related disorders, we postulated a new experiment by metformin and low dose of EA as an AMPK and PPAR-γ activator, respectively. Indeed, in this step, we evaluated the pharmacological interaction of EA and metformin. Collectively, we observed that the combination of EA with metformin slightly and mutually potentiates their anti-aging effects during six and ten weeks of treatment, especially by reducing the inflammatory and oxidative stress marker and increasing the testosterone and DHEA-SO_4. It has been demonstrated that EA is able to inhibit the activation of extracellular signal regulated kinase1/2 (Erk1/2) and exerts its anti-oxidant effect in high glucose-induced endothelial cells toxicity [49]. In fact, Erk1/2 axis is known as an inhibitory signaling pathway for AMPK activation, which its inhibition could potentiate the protective effects of AMPK activators such as metformin [48]. Additionally, there are several studies indicating that EA reduced the level of NF-κB, which is a downstream target of the AMPK signaling cascade. In one study, Ellagic acid prevents carrageenan-induced acute local inflammation in rats through inhibition of NF-κB, cyclooxygenase-2 and pro-inflammatory cytokines TNF-α and IL-β production and enhancement of IL-10 *via* an antioxidant mechanism [50]. These studies may support the results of EA and metformin combination in the present study. We reported the protective effects of the combination of a low dose of EA and metformin for the first time. However, it seems that further investigations are required regarding this combination even with higher doses of EA, which was not performed in our study, to calculate its combination index and report the suitable pharmacologic terms antagonism, addition, or synergism. Additionally, we suggest that protein or mRNA levels of PPAR-γ and AMPK/p-AMPK are measured in future studies, although we did not perform this assay as another limitation of the study.

5. Conclusion

In conclusion, for the first time, we reported the anti-aging effects of EA on DG-induced model of aging and the possible involvement of the PPAR-γ receptor. We found that the low dose of EA (30 mg/kg) decreases the deteriorative effects of DG-induced aging at 10 weeks of treatment only, however, high dose of EA (100 mg/kg) was effective at both 6 and 10 weeks of treatment. We revealed that daily and oral administration of EA provides anti-aging effects at low dose in a PPAR-γ receptor-dependent fashion, but not at the high dose. We also showed that the combination of EA with metformin reciprocally promotes their anti-aging effects by six and ten weeks of treatment.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Acknowledgments

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