Avicenna Journal of Phytomedicine (AJP)

Original Research Article

Protective effects auraptene alone and in combination with estradiol on intestine injury induced by traumatic brain injury in male rats: The role of oxidative stress

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Article history:
Received: Aug 09, 2024
Received in revised form: Mar 31, 2025
Accepted: Apr 01, 2025
AJP, Vol. 16, No. 3, May-June 2026, 577-589.
https://dx.doi.org/10.22038/ajp.2025.26871

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Keywords:
TBI
Auraptene
Estrogen
Oxidative Stress
Neuroprotection

Abstract

Objective: This investigation aimed to evaluate how varying doses of auraptene (AUR) alone and in combination with estradiol (E2) on various parameters after traumatic brain injury (TBI) in rats.

Materials and Methods: The rats were divided into twelve groups, including a sham group and eleven TBI groups. The TBI groups consisted of three vehicle groups (DMSO, Oil, and DMSO+Oil), an E2 group, three AUR groups with varying doses (4, 8, and 25 mg/kg), and three combination groups of AUR and E2 (AUR 4+E2, AUR 8+E2, and AUR 25+E2).AUR was administered for five consecutive days) ip). TBI was induced 30 minutes after the last injection on the fifth day.E2 and Oil were injected 30 minutes post-TBI.

Results: AUR at 25 mg/kg and in combination with E2 significantly reduced brain water content. Nitric Oxide (NO) and Malondialdehyde (MDA) levels were lower in AUR 8 and 25, and all AUR+E2 groups. Glutathione peroxidase (GPX) and Catalase (CAT) activity increased in all AUR+E2 groups .TBI increased interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) levels in the intestine, which were reduced by AUR alone and AUR+E2

Conclusion: These findings support AUR, particularly with E2, as a promising therapeutic strategy for managing oxidative stress, inflammation, and tissue damage in TBI cases.

Please cite this paper as:
Amiresmaili S, Seyedjoodaki A, Vosughi Motlagh A, Afshari A.R, Maghool F, Keshavarzi Z. Protective effects auraptene alone and in combination with estradiol on intestine injury induced by traumatic brain injury in male rats: The role of oxidative stress. Avicenna J Phytomed, 2026; 16(3): 577-589.

Introduction

Traumatic brain injury (TBI) often leads to severe disabilities and, in extreme cases, life-threatening consequences. While clinical care focuses on managing brain symptoms, TBI also affects other organs, particularly the intestine (Keshavarzi et al. 2017; Hanscom et al. 2021). TBI pathology has two phases: the initial injury response and the secondary injury response, involving inflammation, apoptosis, and oxidative stress (Khaksari et al. 2013a; Cheng et al. 2018). Research suggests that TBI triggers notable alterations in the gastrointestinal system, creating a feedback loop that worsens brain damage via the brain-gut axis (El Baassiri et al. 2024, Hanscom et al. 2021;DeSana et al. 2024) .Key antioxidants are glutathione peroxidase (GPx) and superoxide dismutase (SOD)(Shandilya et al. 2022). Severe trauma can cause excessive cytokine production, intestinal dysfunction, and neuroinflammation, with increased Tumor necrosis factor alpha (TNF-α), Interleukin-6 (IL-6), and Interleukin-1 beta (IL-1β) levels post-TBI (Grotz et al. 1999; Cannon et al. 2023).

Medicinal plants, especially those with antioxidant properties, are increasingly used for health benefits (Frozandeh et al. 2021; Keshavarzi et al. 2019). Auraptene (AUR), found in citrus fruits, has anti-inflammatory and anti-carcinogenic properties, suppresses the activation of microglial cells and reduces the expression of Cyclooxygenase-2 (COX-2) in astrocytes (Bibak et al. 2019; Okuyama et al. 2015).

E2, also known as 17-β estradiol, is widely acknowledged for its antioxidant properties which are facilitated through both classical estrogen receptors (ERα and ERβ) and non-classical pathways such as G protein-coupled estrogen receptor (GPER). Studies have highlighted these mechanisms (Naderi et al. 2015) (Kövesdi et al. 2020). The preventive impact of E2 on liver activity is linked to the selective expression of estrogen receptor (ER) subtypes in different tissues (Amiresmaili et al. 2021). Specifically, estrogen treatment has been found to increase the expression of ER mRNA and protein in the brain, indicating the neuroprotective role of estrogen (Rafie et al. 2022). Research by de Medina et al. (2010) demonstrated that AUR binds to ERs (ER-α and ER-β) and influences gene expression by employing an estrogen receptor (ER -sensitive reporter and the cell's genetic elements involved in regulating inflammatory responses (de Medina et al. 2010).

Researches have also indicate that both E2 and AUR exhibit neuroprotective properties, positively impacting brain and intestinal functions in rat models (Keshavarzi et al. 2023;Khaksari et al. 2013). This research investigates the preventive roles of E2 and AUR on intestinal oxidative stress and cytokine levels post-TBI.

Materials and Methods

Animals

Adult male Wistar rats, weighing 210–250 g, were procured from the animal care unit of Kerman University of Medical Sciences, Iran, for use in this study. Throughout the research, they were able to obtain food and water while being preserved under thermoneutral conditions (21-25°C) and a 12-hr light/dark cycle. The North Khorasan University of Medical Sciences' Research and Ethics Committee in Bojnurd, Iran, signed the lab protocol (Ethics code: IR.NKUMS.REC.1400.108).

Drugs

AUR (LKT Laboratories, USA) was dissolved in 10% dimethylsulfoxide (DMSO) and administered to rats at 4, 8, and 25 mg/kg. E2 purchased from Aburaihan Pharmaceutical in Tehran, Iran.

Both drugs were injected intraperitoneally (IP).

Methodology for experiment

The rats were divided into 12 groups, with a total of 7 rats in each group. (1) Sham group: Skin incision only; (2) TBI group: Brain trauma, no medication; (3) TBI + DMSO group: DMSO injection (AUR solvent)(Okuyama et al. 2015); (4) TBI + E2 group: E2 injection (33.3 µg/kg) 30 min post-TBI (Wang et al. 2012), (5) TBI + Oil group: Sesame oil injection (E2 solvent) 30 min post-TBI (Naderi et al. 2015), (6) TBI + Low dose AUR group: AUR injection (4 mg/kg); (7) TBI + Medium dose AUR group: AUR injection (8 mg/kg); (8) TBI + High dose AUR group: Administration of AUR (25 mg/kg) via injection , (9) TBI + AUR 4+ E2 group: AUR (4 mg/kg) + E2 (33.3 µg/kg); (10) TBI + AUR 8+ E2 group: AUR (8 mg/kg) combined with E2 (33.3 µg/kg); (11) TBI + AUR 25+ E2 group: AUR (25 mg/kg) + E2 (33.3 µg/kg); and (12) TBI + DMSO + Oil group: DMSO + sesame oil.

AUR was administered for five consecutive days (4, 8, and 25 mg/kg, IP ~~ip~~). TBI was induced 30 min after the last injection on the fifth day (Okuyama et al. 2015).

Diffuse TBI model

TBI was induced in anesthetized rats using the Marmarou et al method. A 3 mm-thick, 10 mm-diameter steel plate was attached to the skull, and a 300-g mass was released from a height of 2 m. Post-injury, the rats were placed on a respiratory pump until regular respiration resumed, then placed in individual cages for recovery(Marmarou et al. 1994).

Measurement of cerebral edema

Cerebral edema was assessed by determining brain water content (BWC) 24 hr post diffuse TBI induction. After confirming under full anesthesia (ketamine/xylazine (80/10 mg/kg, IP), the animal's brain was extracted and initially weighed to record its wet weight. The samples were then subjected to a 100°C drying process for 24 hr before being reweighed to obtain the dry weight. BWC was determined using the formula:

BWC (%) = [(wet weight - dry weight) / wet weight] × 100 (Amirkhosravi et al. 2021).

Assessment of MDA level

To measure MDA, 0.5 ml of thiobarbituric acid was added to a mixture of 125 µl serum and 1.5 ml trichloroacetic acid (Sigma Chemical Co.). The test tubes were boiled for 45 min and cooled, and 1 ml of n-butanol was added. After centrifuging at 750 g for 10 min, the pink phase was separated, and absorbance was measured at 534 nm. MDA content was determined using a standard curve of tetra ethoxy propane (Rao et al. 1989).

Assessment of NO levels

Nitric oxide (NO) levels were assessed by mixing deprotonated plasma with Griess Reagent, consisting of 2% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-Naphthyl) ethylenediamine hydrochloride dissolved in deionized water. Plasma proteins were precipitated using $ZnSO_4$ and 0.3 M NaOH. The optical density (OD) was then measured at 540 nm(Yucel et al. 2012)~~.~~

Evaluation of CAT and SOD activities

The activities of catalase (CAT) and superoxide dismutase (SOD) in intestinal tissue were analyzed using a Randox assay kit (London, England), following the guidelines provided by the manufacturer.

Evaluation of the intestine cytokines

Cytokines (TNF-α and IL-6) oncentrations in intestinal tissue were measured using widely accessible ELISA kits from Hangzhou Eastbiopharm, China (Talmac et al. 2019)

Data analysis evaluation

Data were analyzed with GraphPad Prism 6.00. Descriptive statistics, including mean and standard error, were applied after confirming data normality using the

Shapiro-Wilk test. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test. The results are presented as mean ± SEM, with a significance level set at $p<0.05$.

Results

The effects of AUR and E2 on brain edema

Figure 1 illustrates variations in BWC 24 hr post TBI. (A) Compared to the sham group (70.18±0.38 %), BWC was significantly elevated in the TBI, Oil, and DMSO groups ($p<0.001$). However, the E2 group (70.83±0.38 %) exhibited a reduction in BWC relative to the Oil group ($p<0.001$). (B) In the AUR 25 group (79.61 ± 0.69%), BWC was lower than in the DMSO group ($p<0.05$). Additionally, the AUR 4+E2 ($p<0.05$), AUR 8+E2, and 25+E2 ($p<0.001$) groups showed a notable decline in BWC compared to the DMSO + Oil group. Moreover, a significant reduction in BWC was observed in the AUR 8+E2 group (72.86 ± 0.45 %) compared to the 8 group ($p<0.01$), and in the 25+E2 group (71.71 ± 0.45 %) compared to the AUR 25 group ($p<0.05$) (Figure 1).

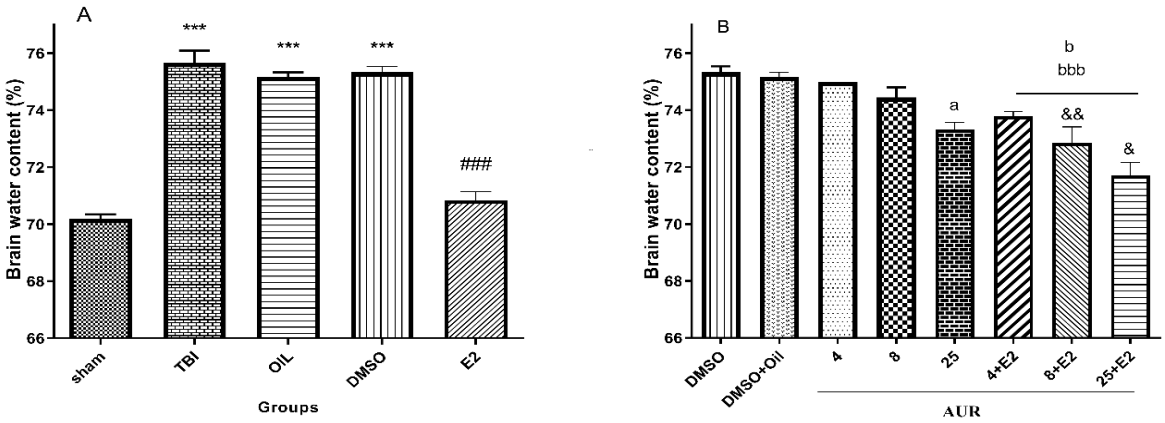

Figure 1. Comparison of BWC (%) after TBI among the different groups**.** (n =7 in each group). The data are presented as mean ± SEM. (A), $^{***}p<0.001$, TBI, Oil and DMSO vs. Sham. $^{###}p<0.001$, E2 vs. Oil. (B), $^{a}p<0.05$, 25 vs. DMSO. $^{b}p<0.05$, AUR 4 + E2 vs. DMSO + Oil. $^{bbb}p<0.001$, AUR 8 + E2 and AUR 25 + E2 vs. DMSO + Oil. $^{\&\&}p<0.01$, 8 + E2 vs.8, $^{\&}p<0.05$, 25 + E2 vs. 25.

The effects of AUR and E2 on levels of intestine MDA and NO

Figure 2 displays the comparison of malondialdehyde (MDA) and nitric oxide (NO) levels in the intestine among the experimental groups. Twenty-four hours after TBI, both MDA and NO concentrations were significantly higher in the TBI, Oil, and DMSO groups compared to the sham group ($p<0.001$). As depicted in Figure 2A, the E2 group (1.25±0.09~~0.27 ± 0.01~~) showed a significant reduction in ~~MDA~~ NO levels compared to the Oil group ($P<0.001$). Figure 2B demonstrates that NO levels in the AUR 8 (1.41 ± 0.05 µ/mg) and AUR 25 (1.26 ± 0.05 µ/mg) groups were lower than those in the DMSO group. The 4+E2, 8+E2, and 25+E2 groups also displayed a substantial decrease in NO levels compared to the DMSO + Oil group ($p<0.001$). Furthermore, NO levels in the AUR 4+E2 group (1.20 ± 0.05 µ/mg) were reduced compared to the 4 group ($p<0.001$), in the AUR 8+E2 group (1.08 ± 0.05 µ/mg) compared to the 8 group ($p<0.001$), and in the AUR 25+E2 group (0.97 ± 0.05 µ/mg) compared to the AUR 25 group ($P<0.001$). Additionally, NO levels in the AUR 25+E group were significantly lower than in the AUR 4+E2 group ($p<0.01$).

Figure 2C emphasizes a notable decrease in MDA activity in the E2 group (0.27 ± 0.01 nanomol/mg) relative to the Oil group ($p<0.001$). Similarly, Figure 2D shows that MDA concentrations in the AUR 25 group (0.30±0.01 nanomol/mg) were significantly

reduced compared to the DMSO group ($p<0.001$). The AUR 4+E2, AUR 8+E2, and AUR 25+E2 groups exhibited a significant reduction in MDA levels compared to the DMSO + Oil group ($p<0.001$). Furthermore, MDA concentrations were notably lower in the AUR 4+E2 group (0.24± 0.01 nanomol/mg) relative to the 4 group ($p<0.001$), in the AUR 8+E2 group (0.23± 0.01 nanomol/mg) compared to the 8 group ($p<0.001$), and in the nanomol/mg 25+E2 group (0.19 ± 0.01) compared to the 25 group ($p<0.001$). Additionally, the 25+E group demonstrated a significant decrease in MDA levels compared to the AUR 4+E2 group ($p<0.01$). Figure 2E depicts the percentage changes in oxidative stress markers relative to the vehicle group for both the E2 and AUR 25+E groups. The changes in MDA and NO levels in the E2-treated rats (-0.23± 0.04 and -0.24 ± 0.03 nanomol/mga, respectively) were lower than those in the AUR 25+E2-treated rats ($p<0.001$) (Figure 2).

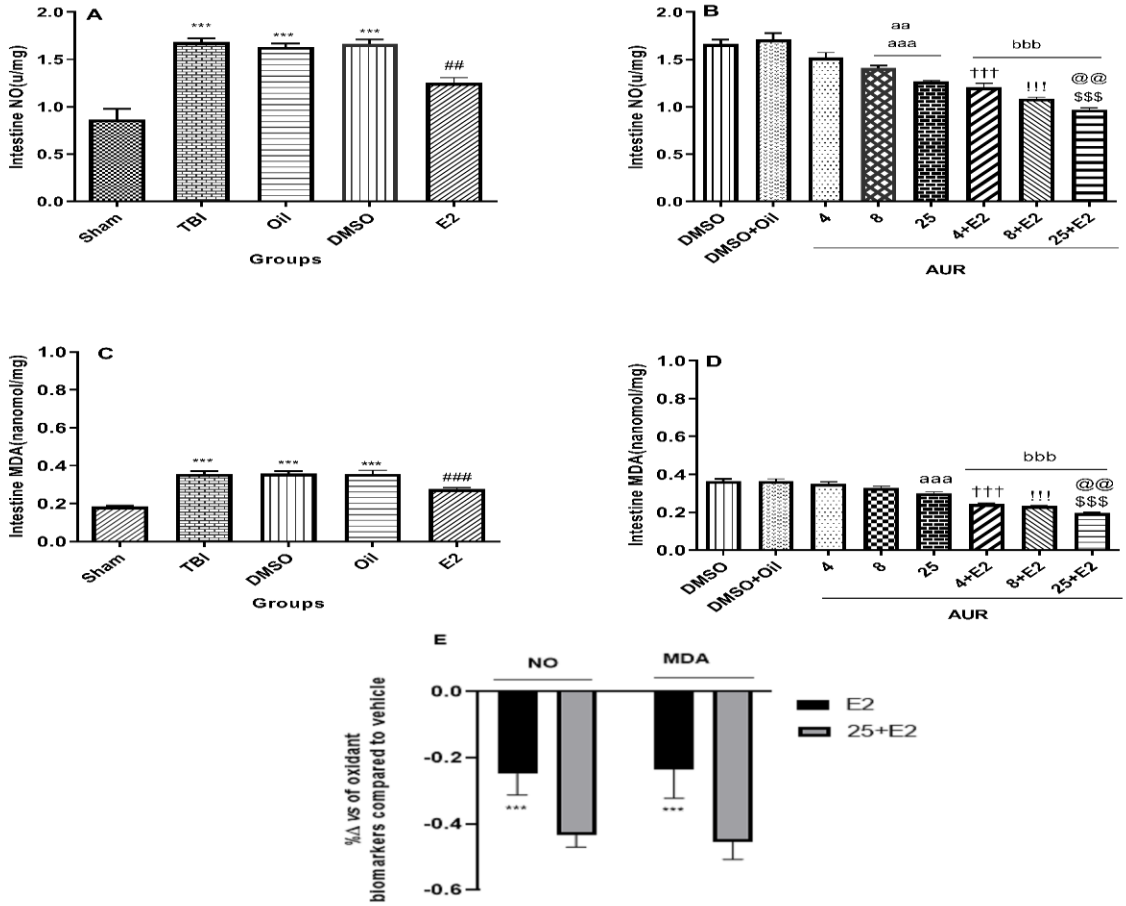

Figure 2. (A-B) Comparison of intestine NO (μ/mg) after TBI in the different groups. (n =7 in each group). The data are presented as mean ± SEM. (A), $^{***}p<0.001$, TBI, Oil and DMSO vs. Sham. $^{\#\#}p<0.01$, E2 vs. Oil. (B), $^{aaa}p<0.001$, AUR 25 vs. DMSO, $^{aa}p<0.01$, AUR 8 vs. DMSO, $^{bbb}p<0.001$, AUR 4 + E2, AUR 8 + E2 and AUR 25 + E2 vs. DMSO + Oil. $^{\dagger\dagger\dagger}p<0.001$, AUR 4 + E2 vs.4,$^{!!!}p<0.001$, AUR 8 + E2 vs.8, $^{\$\$\$}p<0.001$, AUR 25 + E2 vs.25. $^{@@}p<0.01$, AUR 25 + E vs. AUR 4 + E2. (C-D) Comparison of intestine MDA (nanomol/mg) after TBI in the different groups. (n =7 in each group). (C), $^{***}p<0.001$, TBI, Oil and DMSO vs. Sham. $^{\#\#\#}p<0.001$, E2 vs. Oil. (D), $^{aaa}p<0.001$, 25 vs. DMSO, $^{bbb}p<0.001$, AUR 4 + E2, AUR 8 + E2 and AUR 25 + E2 vs. DMSO + Oil. $^{\dagger\dagger\dagger}p<0.001$, AUR 4 + E2 vs. AUR 4,$^{!!!}p<0.001$, AUR 8 + E2 vs.8, $^{\$\$\$}p<0.001$, AUR 25 + E2 vs. AUR 25. $^{@@}p<0.01$, 25 + E vs. 4 + E2. (E) Comparison of the effect of E2 and 25 + E2 on the changes of MDA and NO (%) after TBI. $^{***}p<0.001$, E2 vs. AUR 25 + E2.

The effects of AUR and E2 on the levels of intestine GPX and CAT

Figure 3 compares the GPX and CAT levels in the intestines among different study groups. "Levels of intestinal GPX and CAT were significantly reduced in the TBI, Oil, and DMSO groups 24 hr post-TBI compared to the sham group ($p<0.001$). As shown in Figure 3A, GPX activity was markedly higher in the E2 group (9.70 ± 0.49 U/mg) than the Oil group ($p<0.001$). Figure 3B indicates that GPX levels in the AUR 25 group (9.49 ± 0.28 U/mg) were elevated compared to the DMSO group. The AUR 4+E2, AUR 8+E2 ($p<0.01$), and AUR 25+E2 ($p<0.001$) groups also showed a significant increase in GPX levels relative to the DMSO + Oil group. Additionally, GPX levels were higher in the AUR 4+E2 group (8.80 ± 0.28 U/mg) compared to the 4 group ($p<0.01$), in the AUR 8+E2 group (8.79 ± 0.28 U/mg) compared to the AUR 8 group ($p<0.01$), and in the AUR 25+E2 group (10.27 ± 0.28 U/mg) compared to the AUR 25 group ($p<0.001$). The GPX level in the AUR 25+E group was also higher than the 4+E2 group ($p<0.001$). Figure 3C reveals that CAT activity in the E2 group (9.88 ± 0.29) was significantly increased compared to the Oil group ($p<0.001$). Similarly, CAT levels in the AUR 25 group (8.99 ± 0.29) were higher than the DMSO group ($p<0.001$). The AUR 4+E2, AUR 8+E2, and 25+E2 groups also exhibited a significant rise in CAT levels compared to the DMSO + Oil group ($p<0.001$). Furthermore, CAT levels were notably higher in the AUR 4+E2 group (9.30 ± 0.26 U/mg) compared to the AUR 4 group ($p<0.001$), in the AUR 8+E2 group (10.17 ± 0.26 U/mg) compared to the AUR 8 group ($p<0.001$), and in the AUR 25+E2 group (10.82 ± 0.26 U/mg) compared to the AUR 25 group ($p<0.001$). The CAT level in the AUR 25+E group was also higher than the AUR 4+E2 group ($p<0.01$). Figure 3E illustrates the percentage changes in antioxidant levels relative to the vehicle group for the E2 and AUR 25+E groups. No significant differences were observed between rats treated with E2 and those treated with AUR 25+E2 (Figure 3).

The effects of AUR and E2 on the levels of intestinal TNF-α and IL-1β

Figure 4 presents a comparison of TNF-α and IL-1β levels in the intestines following TBI across different experimental groups. Both TNF-α and IL-1β concentrations were significantly elevated in the TBI, Oil, and DMSO groups compared to the sham group ($p<0.001$).

As shown in Figure 4A, the E2 group (181.6±14.94 pg/ml) exhibited significantly lower TNF-α levels compared to the Oil group ($p<0.001$). However, TNF-α levels were notably higher in the AUR 8+E2 and AUR 25+E2 groups relative to the DMSO+Oil group ($p<0.001$). Additionally, the AUR 8+E2 group (160.4 ± 22.39 pg/ml) showed higher TNF-α levels compared to the AUR 8 group ($p<0.001$), and the AUR 25+E2 group (140.8±22.39 pg/ml) had higher levels compared to the AUR 25 group ($p<0.001$). The AUR 25+E2 group also displayed higher TNF-α levels than the AUR AUR 4+E2 group ($p<0.05$). Figure 4B indicates that IL-1β levels in the E2 group (42.51±5.60 pg/ml) were significantly higher than the Oil group ($p<0.001$). In contrast, the AUR 25 group (57.51 ± 4.94 pg/ml) showed lower IL-1β levels compared to the DMSO group ($p<0.01$). IL-1β concentrations were significantly reduced in the AUR 4+E2, AUR 8+E2 ($p<0.001$), and AUR 25+E2 ($p<0.01$) groups compared to the DMSO+Oil group. Furthermore, IL-1β levels were lower in the AUR 4+E2 group (55.85 ± 4.94 pg/ml) compared to the AUR 4 group ($p<0.05$), in the AUR 8+E2 group (54.23 ± 4.94 pg/ml) compared to the 8 group ($p<0.05$), and in the AUR 25+E2 group (34.23 ± 4.94 pg/ml) compared to the AUR 25 group ($p<0.001$). The AUR 25+E2 group also exhibited lower IL-1β levels than the AUR 4+E2 group ($p<0.01$). Figure 4C illustrates the percentage changes in TNF-α and IL-1β levels in the E2 and AUR 25+E2 groups relative to the vehicle group. Rats treated with E2 showed a smaller change in TNF-α levels compared to those treated with AUR 25+E2 ($p<0.05$).

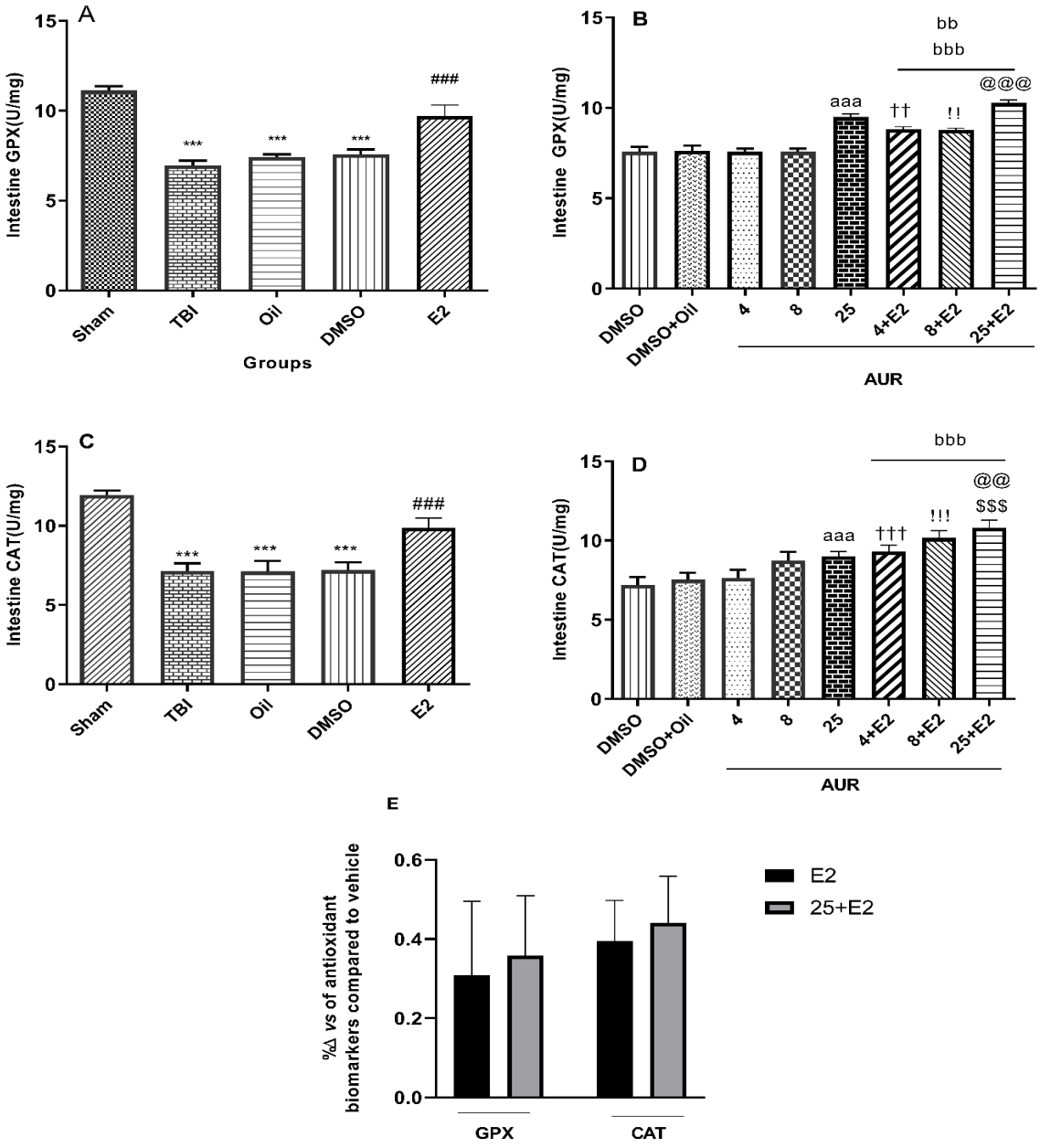

Figure 3. (A-B) Comparison of GPX (U/mg~~μ/mg~~) after TBI among the different groups. (n =7 in each group). The data are presented as mean ± SEM. (A), ***p<0.001, TBI, Oil and DMSO vs. Sham. $^{###}$p<0.001, E2 vs. Oil. (B) ^{aaa}p< 0.001, AUR 25 vs. DMSO, ^{bbb}P< 0.001, AUR 25 + E2 vs. DMSO + Oil. ^{bb}p< 0.01, AUR 4 + E2, and AUR 8 + E2 vs. DMSO + Oil. ††p< 0.001, AUR 4 + E2 vs.4, $^{!!}$p< 0.001, AUR 8 + E2 vs. AUR 8, $^{@@@}$p< 0.01, AUR 25 + E vs. AUR 4 + E2. (C-D) Comparison of intestine CAT (U/mg) after TBI among the different groups. (n =7 in each group). The data are presented as mean ± SEM. (C), ***p<0.001, TBI, Oil and DMSO vs. Sham. $^{###}$p<0.001, E2 vs. Oil. (D), ^{aaa}p< 0.001, AUR 25 vs. DMSO, ^{bbb}p< 0.001, AUR 4 + E2, AUR 8 + E2 and AUR 25 + E2 vs. DMSO + Oil. †††p< 0.001, AUR 4 + E2 vs. AUR 4, $^{!!!}$p< 0.001, 8 + E2 vs.8, $^{\$\$\$}$p< 0.001, AUR 25 + E2 vs. AUR 25. $^{@@}$p< 0.01, AUR 25 + E vs. AUR 4 + E2. (E) Comparison effect of E2 and AUR 25 + E2 on GPX and CAT (%) after TBI.

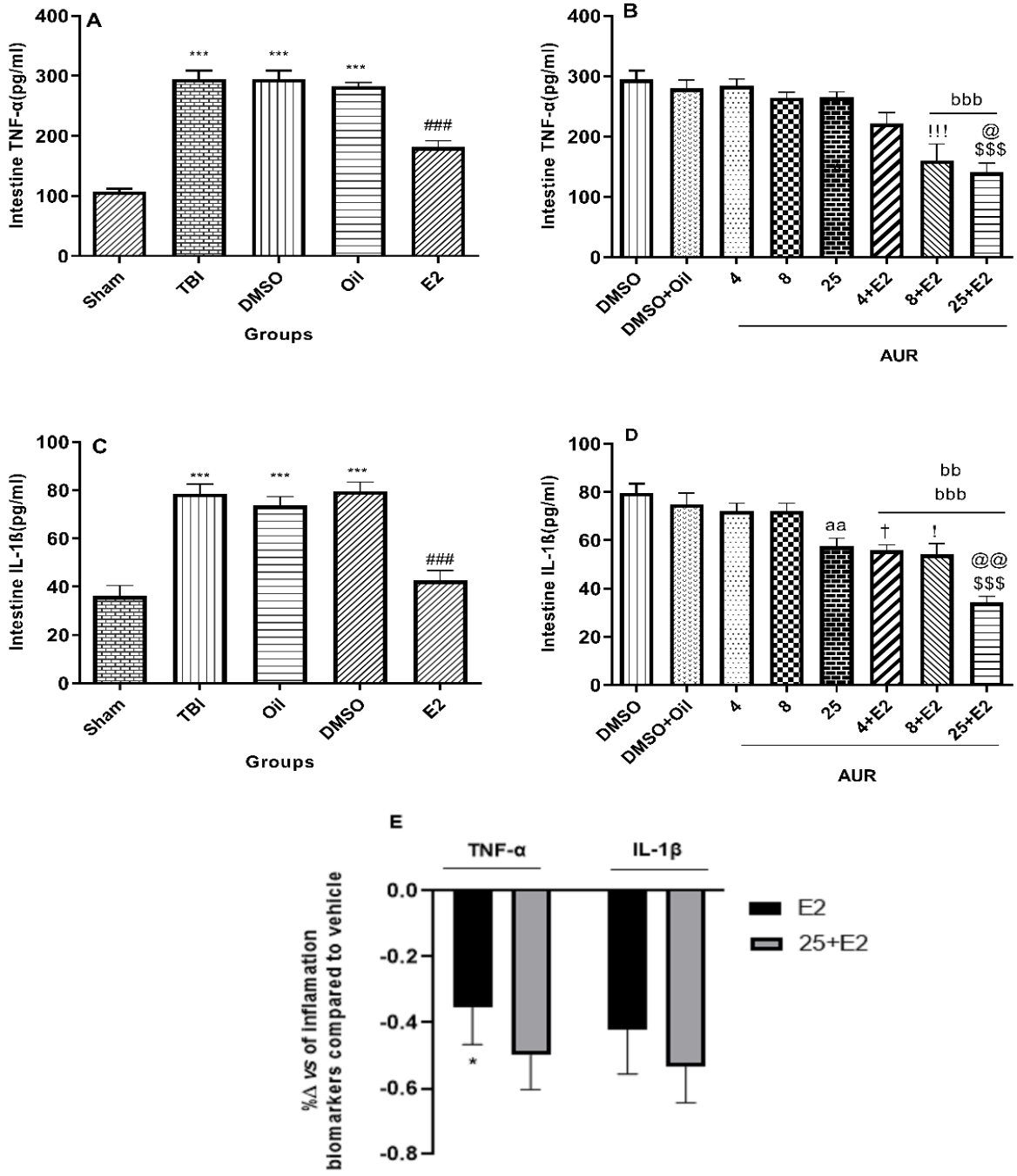

Figure 4. (A-B) Comparison of intestinal TNF-α (pg/ml) after TBI among the different groups. (n =7 in each group). The data are represented as mean ± SEM. (A), ***p<0.001, TBI, Oil and DMSO vs. Sham. $^{###}$p<0.01, E2 vs. Oil. (B), ^{bbb}p< 0.001, AUR 4 + E2, 8 + E2 and 25 + E2 vs. DMSO + Oil, $^{!!!}$p< 0.001, AUR 8 + E2 vs. AUR 8, $^{\$\$\$}$p< 0.001, AUR 25 + E2 vs. AUR 25. $^{@}$p<0.05, AUR 25 + E vs. AUR 4 + E2. (C-D) Comparison of intestine IL-1β (pg/ml) after TBI in the different groups. (n =7 in each group). The data are represented as mean ± SEM. (C), ***p<0.001, TBI, Oil and DMSO vs. Sham. $^{###}$p<0.01, E2 vs. Oil. (D), ^{aa}p<0.01, AUR 25 vs. DMSO, ^{bbb}p<0.001, AUR 8 + E2 and AUR 25 + E2 vs. DMSO + Oil. ^{bb}p<0.01, AUR 4 + E2 vs. DMSO + Oil, †p<0.05, AUR AUR 4 + E2 vs. AUR 4,! p< 0.05, AUR 8 + E2 vs. AUR 8, $^{\$\$\$}$p< 0.001, AUR 25 + E2 vs. AUR 25. $^{@@}$p< 0.01, 25 + E vs. AUR 4 + E2, and AUR 8 + E2. (E) Comparison of the effect of E2 and 25 + E2 on TNF-α and IL-1β (%) after TBI. *p<0.05, E2 vs. AUR 25 + E2.

The effects of AUR and E2 on the levels of intestinal IL-10

The average IL-10 levels in the intestines of various study groups are shown in Figure 5. Compared to the sham group 24 hr after TBI, IL-10 levels were significantly lower in the TBI, Oil, and DMSO groups ($p<0.001$). Figure 2A shows that IL-10 was significantly higher in the E2 group (163.2 ± 13.24 pg/ml) compared to the Oil group ($p<0.001$). Figure 5B indicates that IL-10 levels were increased in the AUR 25 group (147.5 ± 9.85 pg/ml) compared to the DMSO group. In the 4+E2 ($p<0.01$), AUR 8+E2, and AUR 25+E2 ($p<0.001$) groups, IL-10 was significantly higher compared to the DMSO+Oil group. Additionally, IL-10 was significantly increased in the AUR 4+E2 group (128.2 ± 9.85 pg/ml) compared to the AUR 4 group ($p<0.01$), in the AUR 8+E2 group (139.4 ± 9.85 pg/ml) compared to the AUR 8 group ($p<0.001$), and AUR in the AUR 25+E2 group (194.8 ± 9.85 pg/ml) AUR compared to the AUR 25 group ($p<0.001$). The IL-10 level in the AUR 25+E2 group was higher than the AUR 4+E2 group ($p<0.001$). The percentage changes in IL-10 compared to the vehicle in the E2 and AUR 25+E groups are shown in Figure 5C. The IL-10 level in the AUR 25+E group was reduced compared to the E2 group, although this reduction was not significant (Figure 5).

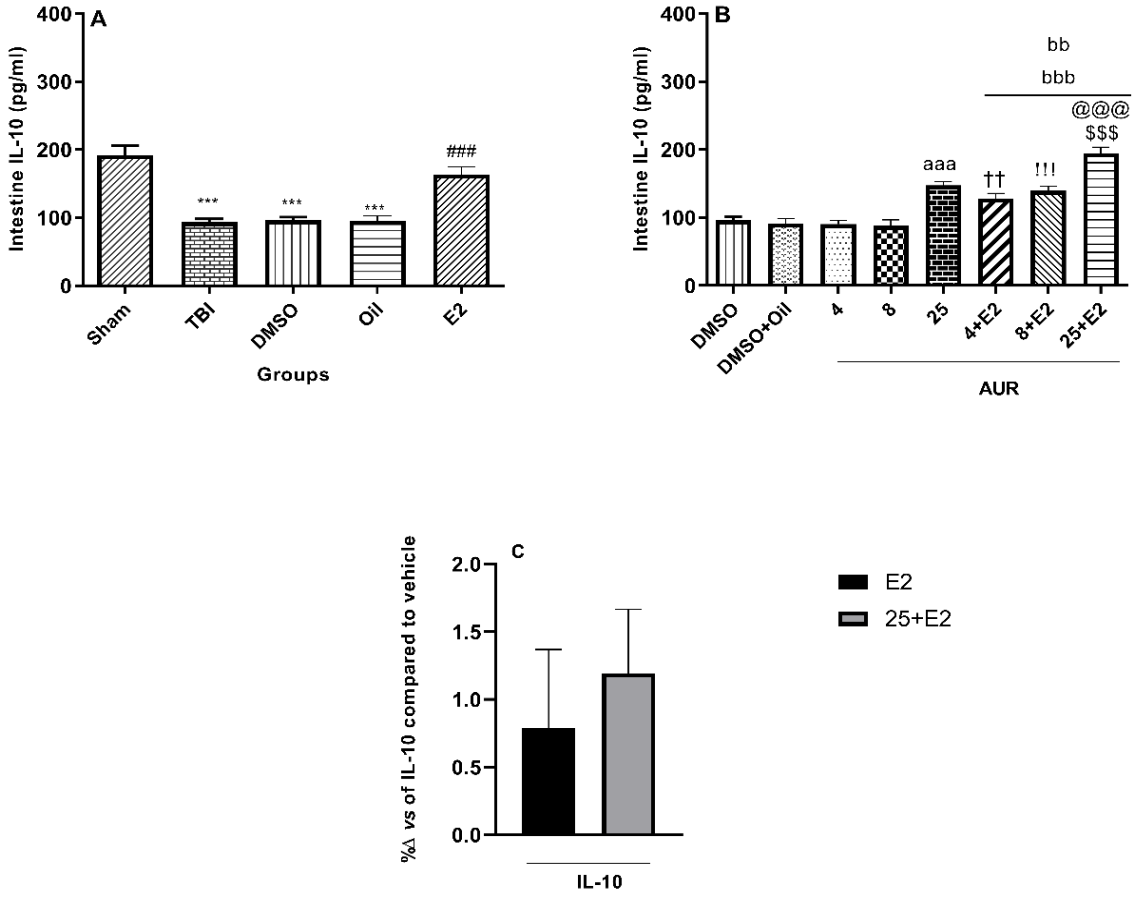

Figure 5. Comparison of intestinal IL-10 (pg/ml) after TBI among the different groups**.** (n =7 in each group). The data are presented as mean ± SEM. (A), $^{***}p<0.001$, TBI, Oil and DMSO vs. Sham. $^{###}p<0.01$, E2 vs. Oil. (B), $^{aaa}p<0.001$, AUR 25 vs. DMSO, $^{bbb}p< 0.001$, AUR 8 + E2 and AUR 25 + E2 vs. DMSO + Oil. $^{bb}p< 0.01$, AUR 4 + E2, vs. DMSO + Oil, $^{††}p<0.01$ AUR 4 + E2 vs.4, $^{!!!}p<0.001$, AUR 8 + E2 vs. AUR 8, $^{\$\$\$}p<0.001$, AUR 25 + E2 vs. AUR 25. $^{@@@}p< 0.001$, AUR 25 + E vs. AUR 4 + E2, and AUR 8 + E2. (C) Comparison of the effect of E2 and AUR 25 + E2 on IL-10 (%) after TBI.

Discussion

This investigation examined impact of AUR alone and in combination with E2 on brain edema, inflammation, and oxidative stress factors in rats' intestines following TBI. Key findings from the research include: High-dose AUR was found to effectively prevent intestine injury post-TBI, a novel discovery in this study. Co-treatment of AUR and E2 demonstrated the most efficient results, exhibited synergistic effects that may be attributed to the formulation's gradual drug release.

Due to its lipophilic nature, as well as its metabolism in the liver, AUR tends to have a slower systemic clearance (Li et al. 2013). Similarly, estradiol, when administered intraperitoneally, has a known profile of gradual release into the bloodstream (Egras and Umland, 2010). These factors together may contribute to a sustained therapeutic effect, particularly in the context of oxidative stress reduction and anti-inflammatory responses.

E2, high-dose AUR (25 mg/kg), and their combination were effective in reducing brain edema post-TBI, also, the compounds diminished oxidants markers (MDA and NO) and enhanced antioxidant capacity **(**to regulate oxidative stress levels). High doses of AUR (25 mg/kg), E2, and their co-administration led to a reduction in inflammation factors in rats' intestines after TBI. BWC, which increased after TBI, was reduced by AUR (25 mg/kg), E2, and their combination.

Studies have shown that E2 reduces BWC (Farahani et al. 2022). We recently reported that AUR has an anti-edema effect in the brain (Keshavarzi et al. 2021). E2 anti-edema mechanisms include free radical scavenging, modifying NO synthesis (Hayashi and Iguchi, 2010), reducing inflammatory cytokines and prostaglandins (Pedersen et al. 2017), lowering intracerebral pressure (ICP), and decreasing blood-brain barrier (BBB) permeability (Soltani and Khaksari, 2015). AUR protects by suppressing COX-2 mRNA, reducing pro-inflammatory cytokines, oxidative indicators, and microglial activation (Okuyama et al. 2015).

The research findings indicate that following TBI, oxidant agents such as MDA and NO increased, while antioxidant agents like GPX and CAT decreased in the intestine. The study demonstrated that E2 and AUR at a dose of 25 mg/kg individually modulated oxidative stress biomarkers in the intestine post-TBI. Moreover, the combined of E2 and AUR (25 mg/kg) was more effective in reducing MDA and NO levels while increasing GPX and CAT levels compared to administering each compound alone.

Oxidative stress is a devastating event after TBI, contributing to intestinal damage progression through the overproduction of ROS and insufficient antioxidants (Cruz-Haces et al. 2017; Khaksari et al. 2013b; Balmus et al. 2016). Proper cell protection requires balanced oxidative enzyme activity (Poljsak et al. 2013). TBI-induced oxidative stress in the intestine involves lipid peroxidation, mitochondrial failure, membrane loss, apoptosis, and inflammatory pathways like Nrf2 deficiency and increased TLR4/NF-κB/AP-1 signaling (Shandilya et al. 2022; Dumitrescu et al. 2018). Our research demonstrated that E2, as a strong antioxidant defense activator, protects against oxidative stress post-TBI (Farahani et al. 2022; Nilsen, 2008). While effective in reducing brain injury, E2 has potential side effects. AUR protects against free radicals (MDA and NO) by scavenging OH radicals, preventing peroxynitrite generation, and upregulating antioxidant enzymes (Pereira et al. 2018). Our results show that combining E2 with high-dose AUR is more effective at stabilizing oxidative stress than either alone.

Following TBI, TNF-α and IL-1β increased but effectively reduced by E2 and high-dose AUR. IL-10, which decreased after TBI, was increased with E2 and high-dose AUR treatment. These cytokines are linked to sustained intestinal injury and

increased permeability (Bansal et al. 2009). The TNF-α/IL-10 ratio is a critical biomarker of inflammatory status within the gut. Maintaining this balance is essential for intestinal homeostasis following TBI (Ma et al. 2017). Recent research suggests that TBI can cause intestinal damage, leading to elevated levels of inflammatory cytokines, particularly TNF-α, while simultaneously reducing the anti-inflammatory cytokine IL-10. This imbalance results in an increased TNF-α/IL-10 ratio (Cannon et al. 2023; Ma et al. 2017). A rise in this ratio promotes the transfer of cytokines into the bloodstream, amplifies neuroinflammatory responses, and contributes to further disruption of the blood-brain barrier (Banks, 2008).

E2 anti-inflammatory properties help to modulate immunity and protect against intestinal permeability post-brain injury (Khaksari et al. 2013b). Estrogen's effects are mediated through estrogen receptor α, reducing IL-6, IL-1β, and TNF-α (Bansal et al. 2009). Males produce higher TNF-α levels in response to intestinal injury, showing sex differences in inflammation (Homma et al. 2005). Estrogen inhibits cytokine formation via an NF-kB-dependent mechanism and reduces neutrophil leakage (Khaksari et al. 2013b). AUR also has proven anti-inflammatory effects on gastrointestinal inflammation (Genovese and Epifano, 2012). This study first reports AUR's anti-inflammatory effects on TBI-induced intestinal damage, with combined AUR and E2 treatment being most effective.

AUR protective actions include increasing IL-10/IL-4 ratio, inhibiting lymphocyte proliferation, suppressing matrix metalloproteinase(MMP) activity, and reducing prostaglandin E2 (PGE2), cyclooxygenase 2(COX-2), MCP-1, inducible nitric oxide synthase (iNOS), and TNF-α production (Bibak et al. 2019; Niu et al. 2015).

In conclusion, the study shows that high-dose AUR, like E2, can mitigate intestinal injury following TBI. The combination of AUR and E2 offers significant protective effects by reducing primary complications such as BWC, and secondary injuries such as oxidative stress (NO and MDA), and inflammation (IL-1β and TNF-α), while increasing antioxidants (GPX and CAT) and IL-10 in the intestine. This suggests that combination therapy could be essential in managing secondary damage post-TBI. Additionally, using AUR, may reduce potential secondary outcomes of E2 in TBI treatment.

Acknowledgment

The budget for this research work backed by the Physiology Research Center at North Khorasan University of Medical Sciences, Bojnurd, Iran (Bojnurd, Iran).

Conflicts of interest

The researchers stated no competing interests.

Funding

The funding for this research provided byNorth Khorasan University of Medical Sciences.

Ethical Considerations

This study was conducted in strict compliance with ethical guidelines for animal researches. All experimental protocols were approved by the [Norh Khorasan University of Medical Sciences Animal Care], and procedures followed the guidelines for the Care and Use of Laboratory Animals. Animals were housed under appropriate conditions with access to food, water, and environmental enrichment. Efforts were made to reduce the number of animals used while achieving scientific objectives, and anesthesia/analgesia was administered where necessary to alleviate pain. This research affirms the principles of Replacement, Reduction, and Refinement (3Rs) in animal experimentation.

Code of Ethics:

IR.NKUMS.REC.1400.108

Authors' Contributions

Sedigheh Amiresmaili: Supervision Conceptualization, Writing- Original draft preparation, Azadeh Seyedjoodaki, Ahmad Vosughi Motlagh: Data curation, Methodology, Amir Reza Afshari, Fatemeh Maghool; Software, Writing- Reviewing and Editing, Zakieh Keshavarzi: Supervision Conceptualization, Editing

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