# Effect of Biocanin A in the Acute Phase of Diffuse Traumatic Brain Injury

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## ABSTRACT

Introduction: In various studies, administration of Biochanin A (BCA), aneuroprotective, anticancer, anti-oxidant, anti-inflammatory, anti-allergic, anti-hyperglycemic and anti-hyperlipidemic effects significantly decrease the injury in the brain associated with cell death and enhanced neurotransmitter release and activity. Biochanin A, the predominant phytoestrogens in the plant, mainly include isoflavones. Reports about the effect of phytoestrogens on the central nervous system, especially behavioral assessments, have been controversial. Thus, the aim of this study was to determine the neuroprotective and behavioral effects of Biochanin A in the acute phase following diffuse traumatic brain injury (DTBI) in male rats. Material & method: Male rats were given either Biochanin A or an equal volume of vehicle (DMSO; 50 mg/kg) at 1h after DTBI. Non-injured controls (three groups: with dimethyl sulfoxide (DMSO), and Biochanin A and one group that was untreated (sham group) and DTBI (with DTBI and without treatment) animals were also included. The animals at 24h post trauma were evaluated daily over 12 days for motor and cognitive outcomes. After two weeks, the animals were perfused with 0.9% saline followed by 10% phosphate-buffered formalin. The whole brain was dissected and removed, sliced, and stained with Cresyl Fast Violet (dark cell) and immunohistochemistry (GFAP and caspase-3 positive cells). Result: Biochanin A decreased dark cell numbers ($P<0.01$) and reduced cerebral cortical levels of GFAP and caspase-3 positive staining (respectively, $P<0.0001$ and $P<0.0001$). The researchers investigated the levels of brain edema in the injured animals and found that Biochanin A significantly increased the wet weight/dry weight ratios after brain injury ($P<0.01$). This increase was associated with an increase in permeability of the blood–brain barrier and was present up to 4 h post-injury, assessed using Evans blue dye ($P<0.01$). Conclusion: Biochanin A given acutely after injury decreased apoptotic-mediated cell death, dark cells and secondary injury with reduced astrogliosis. These results suggested that phytoestrogens such as Biochanin A might afford protection to those suffering from DTBI.

**Key Words:** Phytoestrogens, Biochanin A, Diffuse Traumatic Brain Injury (DTBI), Astrogliosis.

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## INTRODUCTION

Traumatic brain injury (TBI) is the most common cause of high morbidity and disability in people under the age of 45 years [1]. Despite advances in basic research and clinical trials of TBI therapies, such as progesterone, melatonin, stem cells therapy and neurosurgical interventions; many of the survivors do not quite recover and have remaining signs of permanent disability [2, 3]. The pathogenesis of TBI is primary induced by mechanical damage occurring at the time of trauma, which is associated with a series of secondary brain damage injuries [4, 5]. Secondary injury may begin immediately after the primary injury, and includes oxidant injury, apoptosis, and inflammation [5, 6]. Full brain regions are affected by secondary injury; the pathological process diminishes cognitive memory and motor behavioral by disrupting the macromolecular synthesis and synaptic plasticity [7, 8]. Diffuse traumatic brain injury (DTBI) is characterized by diffuse axonal injury (DAI) and degeneration of cerebral white matter throughout the brain and brainstem. DTBI, similar to TBI, can be propagated through both primary and secondary mechanisms [9, 10].

In various studies, the administration of estrogen in the acute phase decreases the severity of injury in the brain caused by cell death [11]. Estrogen decreases the

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consequences of injury cascade by reducing excitotoxicity, and enhancing neurotrophic support, synaptogenesis and axonal remyelinization [12, 13]. As such, treatment with 17β-estradiol has been studied in several animal models of central nervous system injury [14, 15]. Another estrogen, phytoestrogen, has been shown to be anticancer, anti-oxidant, anti-inflammatory and neuroprotective [16, 17]. Biochanin-A (BCA) belongs to the group of phytoestrogens which may be effective against L-glutamate-induced cytotoxicity [18].

BCA is a natural compound having a structural similarity to mammalian 17-β-estradiol, and potent in binding to estrogen receptor subtypes of ERα and ERβ and may produce estrogenic effects [19, 20]. This novel compound is found in soy, cabbage, alfalfa, chickpeas, and many other plants, and has many pharmacological effects such as antioxidant, anti-allergic, anti-inflammatory, anti-cancer, and anti-hyperlipidemic [21, 22]. BCA is effective for inhibiting activation of microglia and pro-inflammatory factors production and protection of dopaminergic neurons and other neuron cells in neurodegenerative disorders such as Parkinson's and Alzheimer's disease [23, 24]. However, the neuroprotective capacity of this phytoestrogen has not been well known. In the present study, the neurologic effects of BCA on brain edema formation and blood-brain barrier permeability were evaluated following an acute phase of DTBI in male rats. It was hypothesized that BCA reduces apoptosis and promotes neural repair by decreasing gliosis and promoting neurogenesis.

## MATERIAL AND METHODS

### a) Rat DTBI model

Male Sprague-Dawley rats (N=78; 250 to 270g; Ilam University) were kept under the standard conditions in the laboratory animal facility at Ilam University of Medical Sciences. All procedures listed in this article were approved by the guidelines of the Ethical Committee of the Faculty of Medical Sciences at Ilam University of Medical Sciences. In the vivo studies, 78 male rats were divided randomly into six groups (13 animals each; 5 animals for histology and motor behavioral assessment, 4 animals for edema study, and 4 animals for blood-brain barrier study) and were then assigned to:

Group I (n = 13): Lesion group (DTBI only) =DTBI

Group II (n = 13): received BCA+DMSO with DTBI =DTBI (BCA+DMSO)

Group III (n = 13): received vehicle (DMSO) with DTBI =DTBI (DMSO)

Group IV (n = 13): Noninjured control group received BCA+DMSO = N (BCA+DMSO)

Group V (n = 13): Noninjured control group received vehicle (DMSO) =N (DMSO)

Group VI (n = 13): Noninjured control group (sham)=Sham

The rats were anesthetized with an intraperitoneal injection of 80 mg/kg ketamine and 10 mg/kg xylazine. DTBI was induced according to the weight drop model of DTBI as previously described in Marmarou et al. (1994) with some changes [25]. Briefly, the DTBI was carried out between the lambda and bregma to expose the dorsal surface of the skull midway. The animals were placed on a 10 cm foam bed for deceleration following impact. The DTBI was induced by dropping a 450g weight from a height of 2 m onto a 10-mm diameter by 3 mm thick steel disc that had been fixed to the dorsal surface of the skull. The steel disc was removed and the skin was sutured after the injury. The animal was assessed daily over two weeks for motor and memory cognitive outcomes. There was no evidence of skull fracture following injury in any groups. 1h following brain injury, the animals were treated intraperitoneally with BCA (30 µg/kg) in solution to vehicle (DMSO) (according to the protocol, BCA was dissolved in a DMSO solution). The animals were monitored for pain and temperature for the first 24 h after the injury.

### b) Blood-brain barrier permeability

The permeability of the blood-brain barrier (BBB) was evaluated using Evans blue (EB) according to Barzo et al. (1996) [26]. 2h after the injury, Evans blue dye (4 ml/kg of 2% solution in phosphate-buffered saline) was injected intravenously in 4 animals from each group. After 1h, the animals were re-anesthetized as mentioned above, and perfused with saline. The meninges and vessels were removed, and the whole brain was removed and weighed. Brain samples were homogenized in 50% trichloroacetic acid solution, and centrifuged at 15,000 rpm/min for 20 min. The absorbance of the extracted dye in supernatant at 620 nm was determined using a spectrophotometer system. Amounts of the dye were reported as µg/g of brain tissue.

### c) Water content

Edema was assessed using the wet/dry method as previously described [27], where % water content (WC) = [(wet weight (WW) - dry weight (DW) /wet weight] × 100. Briefly, the brains were removed from the skull and weighed (WW) 24h after DTBI. The brains were then placed in an oven at 100ºC for 72 h. The brain samples were re-weighed to obtain dry weight (DW).

### d) Neurological assessment

Footfault test: Sensorimotor scoring was performed before DTBI and at days 1, 4, 7, 14, 21 and 28 after the surgery using the footfault test. The animals were evaluated in walking on a grid. The test was the ability to bear weight on the grid, which recorded the paw fall or

slip between the wires for each right forelimb and hindlimb [28].

Neurological severity score (NSS): To assess neurological function measurement, an NSS test was carried out before the injury and on days 1, 4, 7, 14, 21 and 28 after DTBI. The ability of each rat was evaluated with 10 different procedures to show their general status. In order to decrease the possibility of bias in experiment, the tests were carried out by two investigators. The NSS test protocol has been shown in Table 1 [29].

**Table 1. Neurological severity score (NSS)**

| Tasks | Detail of task | points | |
|---|---|---|---|
| | | success | failure |
| Exit circle | Failure to exit a 30cm diameter circle (for 2min) | 0 | 1 |
| Seeking behavioral | Seeking or loss of Seeking behavioral | 0 | 1 |
| Mono/ hemiparesis | Presence of mono or hemiparesis | 0 | 1 |
| Straight walk | Ability or inability to walk Straight | 0 | 1 |
| Startle reflex | Startle reflex or loss of Startle behavior | 0 | 1 |
| Beam walk | Inability to walk on a 3 cm wide beam<br>Inability to walk on a 2 cm wide beam<br>Inability to walk on a 1 cm wide beam<br>Inability to balance on a 1 cm wide beam | 0<br>0<br>0<br>0 | 1<br>1<br>1<br>1 |
| Round stick balancing | Ability or inability to balance on a round stick | 0 | 1 |
| Total score | 10 | 10 | |

### e) Histological assessment

### Nissl Staining

In order to evaluate morphological changes following DTBI, Nissl staining was used. Briefly, 3 weeks following the injury (DTBI), the rats were anesthetized by using an overdose of ketamine/xylazine, and then perfused with normal saline followed by 4% paraformaldehyde (Merck, Darmstadt, Germany) in PBS. The tissues were dehydrated for 45 min in 70%, 95% and 100% ethanol and in the next step, were cleared with xylene. The brain samples were embedded in paraffin and then stored at 37ºC. The 7µm paraffin sections were mounted on gelatin-coated slides. The slides were hydrated with ethanol to distilled water in different dilutions, and then stained using 0/1% Cresyl Violet for 10 min. Brain sections were investigated using an upright microscope (kern.German). Intact neurons were recognized as neurons with Nissl bodies, round nuclei and low basophilic neuron, while dark neurons were defined as abnormal neurons which were characterized by hyperbasophilic cytoplasm and shrunken morphology.

### Immunohistochemistry (IHC)

Tissue sections were permeated with triton x-100 (0.3%) blocked in goat serum 10%. Then, tissue samples were immunostained overnight with primary antibodies including mouse anti-GFAP monoclonal antibody (1:500; Abcam, Cambridge, UK) and anti-caspase-3 antibody (1:500; Abcam). The slides were incubated overnight at 4ºC, and after 24h were washed 3×10 min in PBS and incubated with the secondary antibody (rabbit anti-mouse antibody conjugated with FITC and Alexa Red) for 1 h at room temperature. Nuclei were counter-stained with 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes, 1:10000) for 3 min and after washing with PBS, then coverslipped with slide glue. Cross-sections were examined at 200X magnification under a fluorescent microscope (kern. German). The caspase-3 immunoreactive areas were randomly selected, and the number of caspase-3positive cells in 4 pre-selected areas (200 µm×200 µm) from each of 3 consecutive slices in all animals were counted. The fluorescent intensity of GFAP was estimated using Image J 1.43U (NIH, USA) software. Five sections from each group were used for the evaluation of fluorescence intensity, and the gray level value was measured using Image J software [30, 31].

### Statistical analysis

Data were shown as mean ± SEM and were determined by the analysis of variance using SPSS software. One-way analysis of variance (ANOVA) and Tukey's test post-hoc were used to evaluate the differences among and within the groups. A p-value of 0.05 was considered significant.

## RESULT

BCA-treated DTBI animals showed a significant increase in brain water content after the injury ($P<0.01$). Significant differences from sham levels occurred at 2h after DTBI (Figure 1A). This edema profile in the BCA-treated DTBI animals was similar to that observed in vehicle-treated DTBI animals ($P<0.01$) (Figure 1A). BCA administration in DTBI animals resulted in significantly less edema than in the vehicle-treated controls (DMSO+DTBI) at 2h after the injury ($P<0.05$). DMSO and BCA had similar effects on edema, and both treatments significantly reduced brain water content in injured animals at the acute phase of injury compared to the DTBI group.

Similar results were observed in the permeability of BBB in both BCA with DMSO and DMSO only administration in DTBI animals (Figure 1B). The schema of Evans blue extraction following DTBI has been demonstrated in Figure 2. BCA-treated DTBI animals showed a

significant decrease (P<0.05) in BBB permeability to Evans blue relative to the untreated group (DTBI only; Figure 1B). The administration of either BCA with DMSO or DMSO only significantly inhibited Evans blue extravasation. Despite decreased edema in BCA-treatment group, a significant increase in edema and BBB permeability was seen compared to shams (P<0.01).This increase in edema was significantly less than that seen in the DMSO-treated animals (P<0.05).

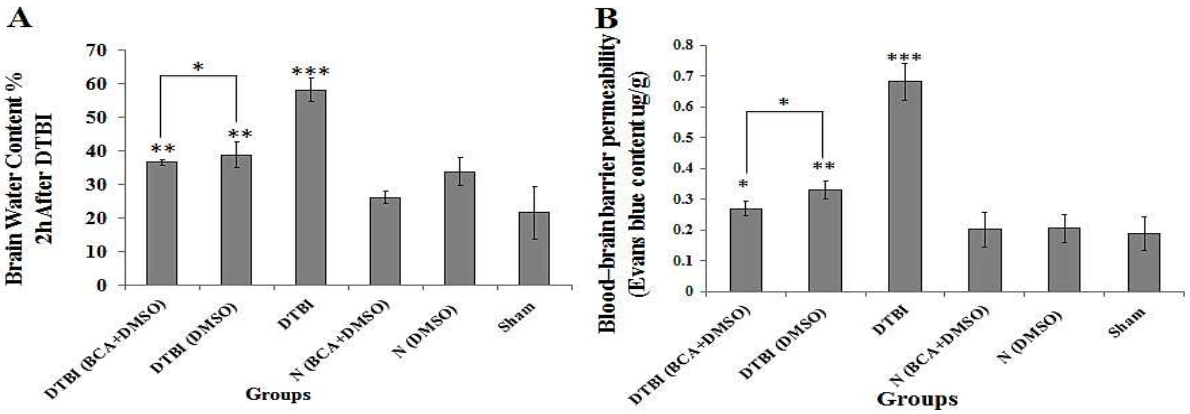


**Fig. 1. Alterations in brain water content (A) and Evans blue extravasation (B) in rat models of DTBI (*P<0.05,** P<0.01, *** P<0.001).**

Figure 2 shows the effect of BCA and DMSO on sensorimotor function (forelimb and hindlimb footfault) before and after DTBI. The findings showed that DTBI significantly increased the incidence of forelimb (P<0.05) and hindlimb (P<0.05) footfaults at 1–28 days after the brain injury compared with the pre injury baseline. Treatment with BCA significantly reduced the number of forelimb footfaults at 4–28 days after the injury compared to the treatment with DMSO and DTBI without the treatment (P<0.05). Non-injury groups (BCA and DMSO) did not show significant differences in the forelimb footfaults compared to the sham group (P>0.05). Similar results were found for the hindlimb; DTBI significantly raised the occurrence of contralateral hindlimb footfaults at 1–28 days after trauma. Treatment with BCA significantly decreased the number of hindlimb footfaults 4–28 days post-injury compared to the treatment with DMSO and DTBI alone (P<0.05). BCA treatment did not significantly change the hindlimb footfaults compared to the non-injury groups treated with BCA and DMSO.



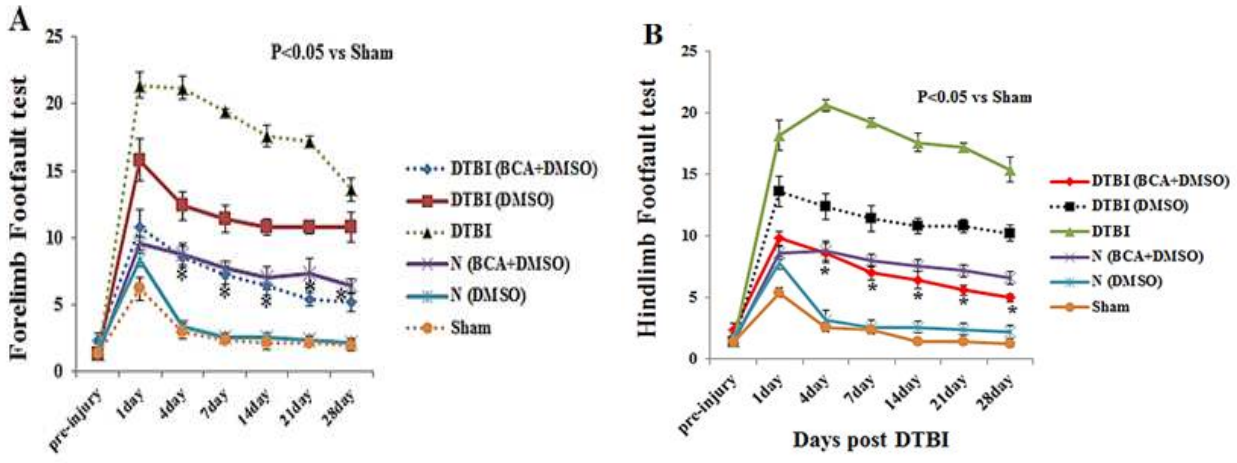


**Fig. 2. Time course of footfault sensorimotor scores, obtained starting from day 1 post injury until week 3 in DTBI rats treated with BCA and vehicle.** A and B graphs show scores for forelimb and hindlimb footfault tests, respectively. Early BCA treatment significantly reduced forelimb and hindlimb footfaults at days 4–28 compared with the sham group (*P<0.05)

Figure 3 demonstrates that the NSS score was significantly reduced in the BCA administration group at days 7–28 post-DTBI compared to sham and non-injury groups (P<0.05). In addition, DMSO alone did not significantly affect the NSS score compared to BCA dissolved in DMSO (P>0.05).

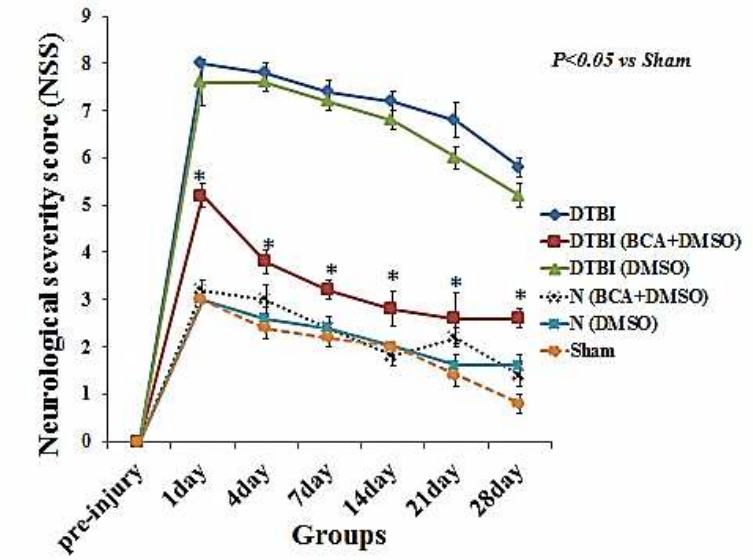


**Fig. 3. The linear graph shows the functional improvement detected in the neurological severity scores (NSS).** BCA treatment significantly lowered NSS scores at days 7–28 compared to sham group (P<0.05). DMSO did not affect NSS scores compared to the BCA administration group. Data were represented as mean ± SD (n= 5); * P<0.05.

Figure 4 shows intact neurons (IN) and dark neurons (DNs) in the coronal section of the brain in animals in different groups. The number of intact neurons in the DTBI group was significantly less than in other groups (P<0.01). The mean number of neurons in brain sections from the BCA administration group was significantly increased compared to DTBI with and without DMSO (P<0.05).



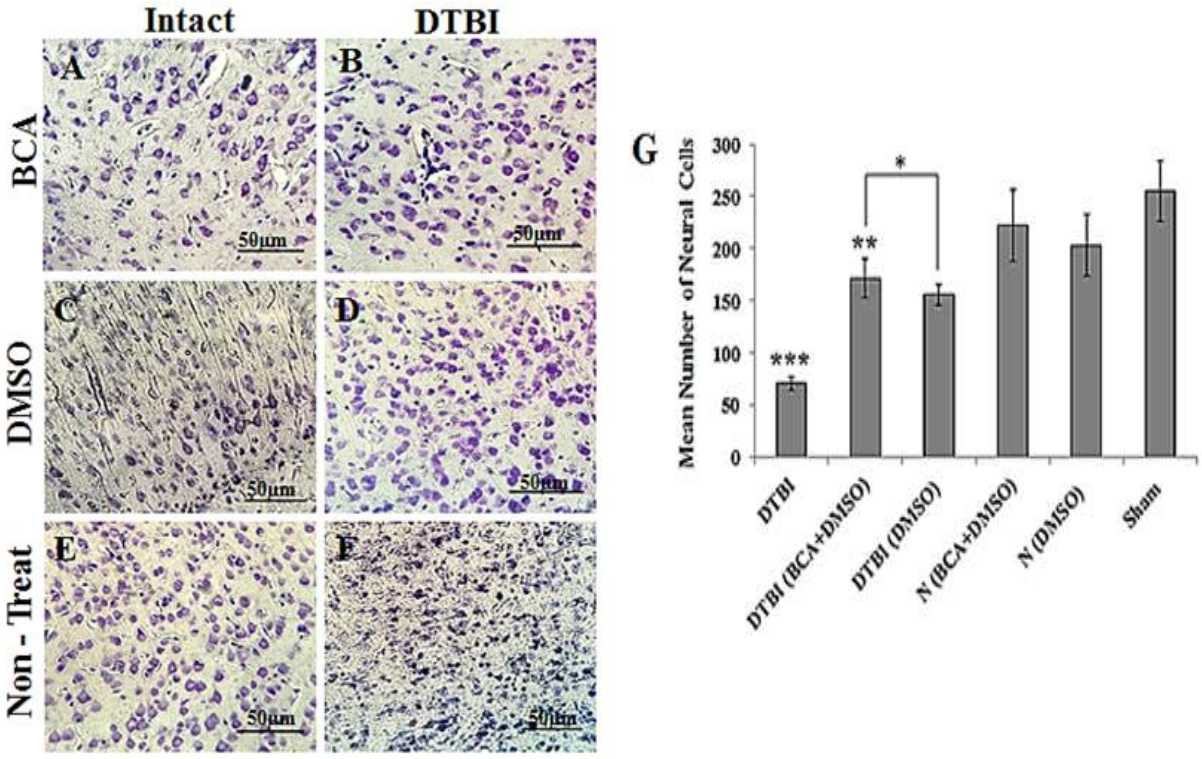


**Fig. 4. Demonstrates intact and dark neural cells stained by Cresyl Violet.** Bar graph of the number of normal-stained Nissl bodies counted per field from the brain section for experimental groups (mean±SEM; n=5 foreach group; *P<0.05,** P<0.01, *** P<0.001).

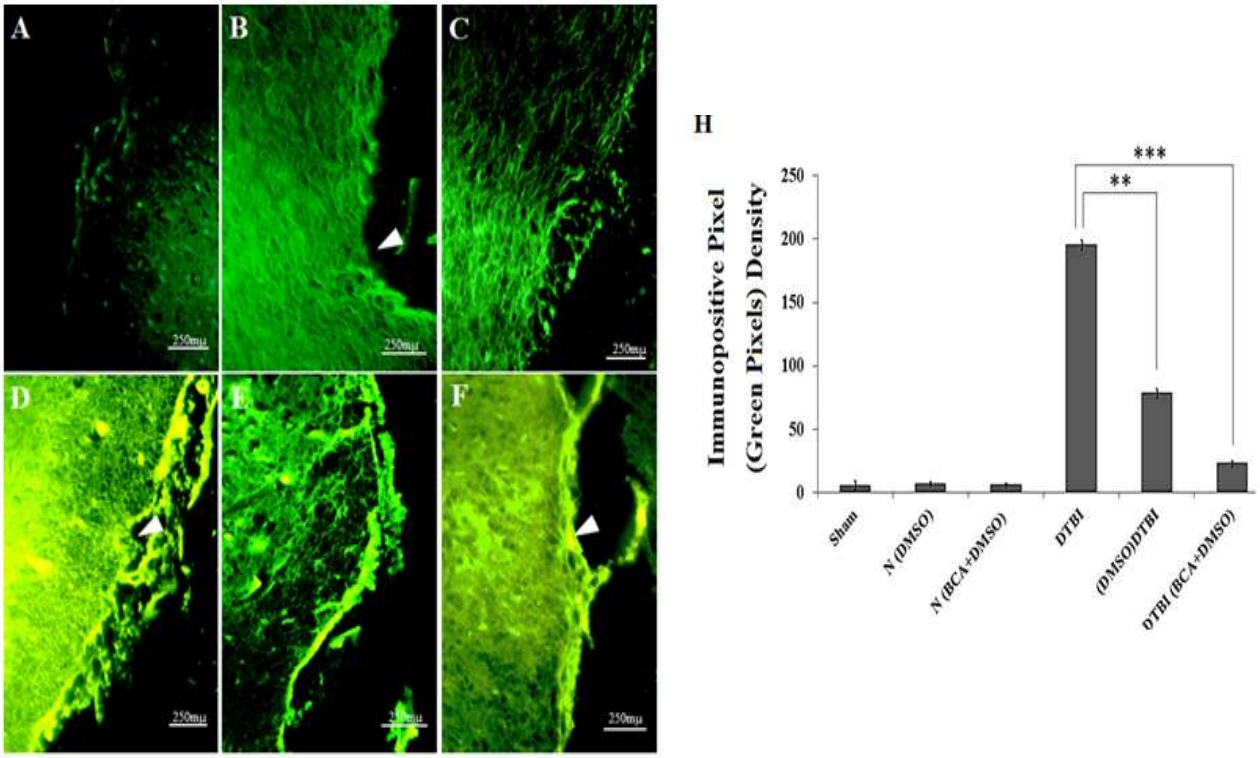


**Fig. 5: Effect of Biochanin-A (BCA) on astrogliosis with glial fibril acidic protein (GFAP) expression in brain tissue (28 days post injury).** A, B, and C represent the sham, non-injured control group with DMSO, and non-injured control group with BCA+DMSO, respectively. D, E, and F demonstrate the lesion group (DTBI only), DTBI with BCA+DMSO, and DTBI with vehicle (DMSO); the arrowhead indicates GFAP immunoreactivity. H The relative intensity of immunofluorescence (gray level values) of the GFAP (*P<0.05,** P<0.01).



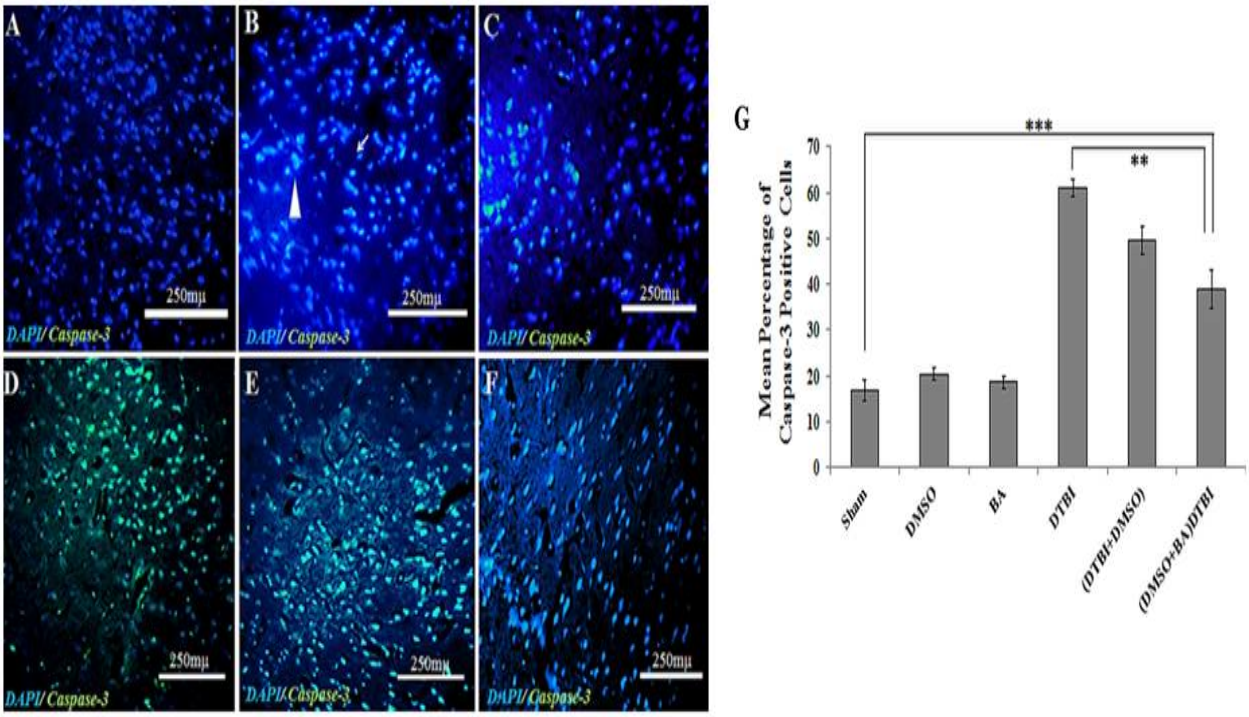


**Fig. 6. Effect of Biochanin-A (BCA) on apoptosis using immunofluorescent technique (28 days post injury).** A-F show the sham, non-injured control group with vehicle (DMSO), non-injured control group with BCA+DMSO, lesion group (DTBI only), DTBI with BCA+DMSO, and DTBI with vehicle (DMSO); respectively. The arrow indicates caspase-3 immunoreactivity, and the arrowhead demonstrates DAPI staining and no reactivity with antibodies.

## DISCUSSION

Currently, there are many restrictions to effective treatment of diffuse axonal injury in clinical applications. On the other hand, neuroprotective effects of phytoestrogen have drawn great attention to the treatment of traumatic brain injury, stroke and ischemia [32, 33]. However, the mechanisms related to these protective effects are still being researched. In many previous studies, the role of BCA was identified as anti-oxidative, anti-inflammatory and anti-allergic [22, 34]. According to the results of this study, it was found that the dosage at 30μg/kg BCA reduced apoptosis and gliosis, subsequently improving the blood-brain barrier, edema and behavioral functions in early stages of DTBI. One recent study found that BCA at a low dosage (20μg/kg) led to a more therapeutic effect than in a higher dosage (40μg/kg) in treating subarachnoid hemorrhages; these findings were consistent with the results of the current study. High-dose BCA appeared to cause a blood-brain barrier disorder, while BCA at a lower dose did not have an effect in sham rats in histological and neurological functions [35].

The data of this study clearly demonstrated that water content in acute BCA-treated animals was significantly decreased from that shown in the DTBI group without treatment. According to the data obtained from this study, treatment with 30μg/kg BCA resulted in decreased edema in male animals 2 h after DTBI. This was consistent with previous studies that have shown estrogen treatment in animals reduced posttraumatic edema in animal models [36, 37]. In addition, in injured animals, treatment with phytoestrogen (BCA) resulted in a significant inhibition in blood-brain barrier (BBB) permeability to Evans blue [38]. While treatment with DMSO caused upregulation of BBB permeability for drugs, controversial results have been shown [39, 40]; however, this finding was in agreement with the observations of this study. Some studies have shown the effects of exogenous estrogen and progesterone treatment on the progression of edema following traumatic brain injury [41, 42]. Additionally, estrogen protects cerebrovasculature by enhancing vasodilation and mitochondrial function and decreasing vascular inflammation [43, 44].

However, a number of studies have previously discerned that low doses of estrogen downregulate secondary injuries such as inflammation, cell death, and gliosis in acute spinal cord injury (SCI) [45]. Other literature indicated that gonadal hormones may reduce astrocyte proliferation and glial activation after a brain injury [46]. The present study has found that BCA as a phytoestrogenis was sufficient to produce such an effect in neuroglia cells and this finding was consistent with previous studies.

Caspase-3 is a mediator of programmed cell death via activation of the protease, release of cytochrome-c and suppression of DFF-45 (DNA fragmentation factor-45)/ICAD (inhibitor of caspase-activated DNase) [47, 48]. Estradiol indicated some protection of hippocampal neurons of CA1 against caspase-3 activity in a brain ischemic model [49]. The current study found that caspase-3 active peptide positive cells count were higher in untreated DTBI than in the group treated by BCA; this finding was in agreement with the previous study. In fact, BCA protected neurons against DTBI through caspase-3 inhibitor mechanisms.

The present data suggested that BCA may be effective in functional improvements, such as sensorimotor functions (decreased NSS and footfault scores). In addition, a previous study reported that BCA attenuated behavior disorders induced by rotenone in Parkinson's disease models [50]. Genistein, as a natural phytoestrogen and flavone, exhibited positive effects on motor function and neuropathic pain after peripheral nerve injury [51]. Several studies have detailed the efficacy of isoflavones, with soy as the main composition of BCA, in influencing cognition and aspects of memory whose effect may vary by dosage and types of isoflavones, gender, age and type of cognition test [32-34].

The therapeutic effects of isoflavones on such outcomes may be in the synthesis of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) or to influence the transforming growth factor beta1(TGF-b1), MAPK, tumor necrosis factor-alpha (TNF-α), or PPAR γ, which have been targets for therapeutic interventions [52-54]. Another study has explored the potential of BCA in a protective role in aluminum-induced neurodegeneration by suppressing the NF-κB signaling and anti-inflammatory effect [55].



## CONCLUSION

In conclusion, the data of this study indicated that BCA may attenuate BBB damage and edema formation through different mechanisms. In addition, this isoflavone induced neuroprotective effects by suppressing caspase-3 active peptide, astrocyte proliferation and synthesis of neurotrophic factors in a DTBI model in rats. These mechanisms ultimately led to improved sensory and motor functions after the brain injury. These findings suggested that BCA might be of use as a therapeutic agent for the treatment of traumatic brain injury and neurodegenerative disease. However, additional research is needed to investigate the details of the cellular and molecular pathways in mediating the protective action of BCA.

## Ethics

This study was approved by Ilam University of Medical Sciences Ethics Committee (IR.MEDILAM.REC.1395.109).

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