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PROTECTIVE ROLE OF MELATONIN AND TAURINE AGAINST TOXICITY INDUCED BY CAFFEINE IN BRAIN BY ABROGATION OF OXIDATIVE STRESS, DECREASE APOPTOSIS, AND ALTERS CEREBRAL MONOAMINE NEUROTRANSMITTERS IN MALE ALBINO RATS

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ABSTRACT: The current study aimed to investigate the protective effects of taurine and melatonin against caffeine-induced brain damage in rats. 36 male albino rats were divided into 6 groups: Group I: control, group II: Rats received 20 mg/kg melatonin for 28 days. Group III: Rats received 50 mg/kg taurine dissolved in distilled water daily for 28 days. Group IV: Rats received 50 mg/kg caffeine dissolved in distilled water for 28 days. Group V: Rats received melatonin as described in group II in concomitant with caffeine, as described in group IV. Group VI: Rats received taurine as described in group III in concomitant with caffeine, as described in group IV. Caffeine-induced rats showed significantly increased brain lipid peroxidation, Superoxide dismutase (SOD) and reduced glutathione (GSH) content were significantly decreased. Monoamine neurotransmitters, gene and protein expression levels of p53 and Bax were significantly increased in the brain of caffeine-induced rats. In contrast, caffeine administration down-regulated Bcl-2 both gene and protein expression in the brain of rats. DNA damage were detected in caffeine treated group compared with control. Gene and protein expression levels of p53 and Bax were significantly decreased in the brain of taurine and melatonin administrated groups. Taurine and melatonin significantly decreased MDA and DNA damage, levels of dopamine and norepinephrine, and enhanced activity of the antioxidant enzymes in the brain of rats. In conclusion, taurine and melatonin can impact caffeine-induced oxidative stress and apoptosis through their antioxidant activity.

INTRODUCTION: Caffeine (1,3,7-trimethyl-xanthine), a natural stimulatory compound, is probably the most consumed pharmacologically active compound in the world [1]. Adults consume caffeine in tea and coffee; both contain natural caffeine in their beans or leaves [2].

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Caffeine and other methylxanthines are used in clinical medicine as diuretics, analgesics, and muscle relaxants, and they can be used in the treatment of brain disorders such as headache [3]. Caffeine had been found to have a role in the inhibition of motor symptoms and dopaminergic neurons loss in Parkinson's disease [4]. Moreover, excessive amounts of caffeine can adversely affect the body through significant toxic effects [5] including anxiety, delirium, headache, insomnia, nervousness, dehydration, hyperglycemia, and arrhythmia [6]. In addition, high concentrations of caffeine induce cellular apoptosis [7], cell death [8].

Also, caffeine can cause indirect DNA damage as a result of the oxidative stress that it can cause [9]. It is obviously not a harmless compound and may cause significant toxicity and even lethality [10, 11].

Melatonin (N-acetyl-5-methoxytryptamine), an indole amine, acts as a hormone. It is secreted from the pineal gland during the night and also can be synthesized in the retina, bone marrow, gastro-intestinal tract, and skin [12]. The level of this hormone is controlled by dark-light cycle, gender, age, seasons, and physiological conditions [13]. Melatonin is an antioxidant, immune-modulator, anti-inflammatory agent, vaso-regulator, and oncostatic agent [14]. It was reported that melatonin affects the morphological features of nerve tissues and had a neuroprotective role through involvement in the regeneration of peripheral nerves. Moreover, melatonin exerts a positive effect on axon length and development after peripheral nerve stress [15].

Melatonin is able to cross the blood-brain barrier easily, enter the central nervous system and the cerebrospinal fluid through the choroid plexus, where it can protect against various neuro-degenerative diseases and brain injury. Exogenous melatonin had been shown to decrease the cerebral infarction area and promote the neuronal anti-lipid peroxidation reaction, thus playing a role in brain protection [16]. Many neurological disorders had been reported to be ameliorated by melatonin-administration, such as Alzheimer's disease [17], stroke, traumatic brain injury and hypoxia [18].

Taurine, 2-aminoethane sulfonic acid, is a simple sulfur-containing organic acid. It is found in all animal cells. In particular, the electrically excitable tissue such as the heart, retina, brain, and skeletal muscles and liver of mammals [19].

Taurine accounts for 0.1% of the whole-body weight of human and present in its free form in all organs [20]. It has antioxidant, anti-inflammatory, antiarrhythmic, and central nervous system neuromodulator effects.

Additionally, taurine stabilizes cell membranes, regulates levels of calcium ions in the blood and fatty tissues metabolism, regulates the development and function of skeletal muscle, the central nervous system, and the retina [21]. Taurine also plays an important role in innate immunity [22].

MATERIALS AND METHODS:

Chemicals: Caffeine, Melatonin, Taurine, tri-chloroacetic acid (TCA), thiobarbituric acid (TBA), 1,1,3,3 tetramethoxypropane, pyrogallol, and 5,5′-dithiobis-(2- nitrobenzoic acid) (DTNB) were purchased from Sigma- Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade and obtained from standard commercial supplies.

Experimental Animals: The experimental animals used in this work were 36 adult albino rats weighing about 120-150 g. They were obtained from the animal house of the National Research Center, El-Giza, Egypt. They were kept under observation for about 15 days before the onset of the experiment to exclude any intercurrent infection. The chosen animals were housed in plastic well-aerated cages (6 rats/cage) at normal atmospheric temperature and a normal 12-h light/dark cycle. The animals were not treated with antibiotics or insecticides, and they had free access to water and were supplied daily with a laboratory standard diet of known composition.

All animal procedures were in accordance with the recommendations of the Animal Ethics Committee of Beni-Suef University (Egypt), which conforms to the recommendations of the Canadian Committee for Care and Use of Animals [23].

Experimental Design: Experimental animals were divided into six equal groups:

Group I (Control): Rats received distilled water *via* oral gavage for 28 days.

Group II: Rats received 20 mg/kg melatonin dissolved in distilled water [24] *via* oral gavage daily for 28 days.

Group III: Rats received 50 mg/kg taurine dissolved in distilled water [25] *via* oral gavage daily for 28 days.

Group IV: Rats received 50 mg/kg caffeine dissolved in distilled water [26] *via* oral gavage daily for 28 days.

Group V: Rats received melatonin as described in group II in concomitant with caffeine, as described in group IV.

Group VI: Rats received taurine as described in group III in concomitant with caffeine, as described in group IV.

The doses of caffeine, melatonin, and taurine were balanced weekly as indicated by any change in body weight to keep up the comparable dosage for every kg body weight over the entire period of the experiment.

Blood and Tissue Sampling: At the end of the experimental period, animals were fasted overnight but allowed free access to water. Animals were sacrificed under mild anesthesia by diethyl ether, and blood samples were obtained from the carotid artery. Animals were decapitated and dissected, then brain tissues were rapidly excised and immediately perfused with ice-cold saline (0.9% sodium chloride). Blood samples were left for 15 min at a temperature of 25 °C to coagulate, then centrifuged at 5000 rpm for 10 min, and clear non-hemolyzed sera were collected and kept at -20 °C until used. Brain samples (10% w/v) were homogenized in chilled phosphate-buffered saline and the homogenates were centrifuged at 3000 rpm for 10 min at 4 °C by Centurion Scientific K3 cooling centrifuge (UK) to separate the nuclear debris. The clear homogenates were collected and stored at -20 °C until used. Also, brain specimens were preserved in -70 °C for gene expression analysis and western blot analysis.

Biochemical Assays:

Assay of Oxidative Stress and Antioxidant Defense System: Lipid peroxidation was estimated in brain homogenate by measuring malondi-aldehyde (MDA) levels following the method of Preuss *et al*. [27] Reduced glutathione (GSH) content and superoxide dismutase (SOD) activity were estimated following the methods of Beutler *et al*., [28] and Marklund and Marklund [29] respectively.

RNA Isolation and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR): Gene expression analysis of P53, BAX, and Bcl2 in brain samples was carried out by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was isolated from frozen brain samples using TRIzol reagent, treated with DNase I, and quantified at 260 nm. cDNAs were synthesized from 2 mg RNA and were amplified using SYBR Green master mix (Thermo Fisher Scientific, USA) with the primer sets outlined in **Table 1**. The obtained amplification data were analyzed by the $2^{-\Delta\Delta Ct}$ method [30,] and the values were normalized to β-actin.

TABLE 1: PRIMER PAIRS USED FOR PCR

Gene	Gene Bank accession number	Sequence 5′–3′
BAX	NM_007527	F: 5′ CGAGCTGATCAGAACCATC3'
		R: 5′ GAAAAATGCCTTTCCCCTTC3'
Bcl2	NM_009741	F: 5′ TAAGCTGTCACAGAGGGGCT3'
		R: 5′ TGAAGAGTTCCTCCACCACC3'
P53	NM_022112	F: 5′ GCTGCCCTCCCTTCTCCTAG3'
		R: 5′CCCCGACTTTGGAGTAGTCTGA3'

Western Blotting Analysis: Brain samples kept at −70 °C were used to investigate the effect of caffeine on the expression level of P53, BAX and Bcl2 using β-actin as a loading control using chemiluminescence kit (BIORAD, USA) [31].

Detection of DNA Single Strand Breaks (Comet Assay): The alkaline comet assay was performed as described by Singh *et al.* [32] To obtain single cells from brain, sample must be finely minced using sterile scissors or scalpels, cell dispersion can be achieved by enzymatic digestion of the sample using collagenase. A freshly prepared 10µL of single liver cells (10,000-50,000) in cold Hank's Balanced Salt Solution (HBSS) was mixed with 65µL of 0.7 low melting point agarose (LMA) at 37 °C and spread onto microscope slide pre -coated with 0.5 % normal melting point agarose (NMA) and the slide was covered with a third layer of LMA and a cover slip was applied to spread the sample.

The cells then lysed in lysis buffer consisting of 1% sodium sarcosinate, 2.5 M NaCl, 10 mM Na_2 EDTA, 1% Triton x -100, 10% DMSO and 10mM Tris, pH10 for 1 h at 4 °C. After the lysis, the slides were placed in an electrophoresis unit, the DNA was allowed to unwind for 20 min in the electrophoretic solution consisting of 300 mM NaOH, 1 mM EDTA, pH>13. Electrophoresis was

conducted at ambient temperature of 4°C for 20 min at electric field strength of 300 mA. The slides were then neutralized with 0.4 M Tris, pH7.5, stained with 2 µg/ml ethidium bromide and covered with cover slips.

To prevent additional DNA damage, all the steps described above were conducted under dimmed light or in the dark. The slides were viewed under a LeitzOrthoplan epifluorescence microscope (magnification 200x) equipped with an excitation filter of 515-550 nm and a barrier filter of 590 nm. The microscope was connected through a camera to a computer-based image analysis system (Comet Assay IV software). For each sample, 100 isolated comets (single-strand breaks of DNA migrate from nucleus to anode) were randomly selected and measured for comet tail length, % DNA in tail, and tail moment according to the definition by Olive and Banath (1993) [33].

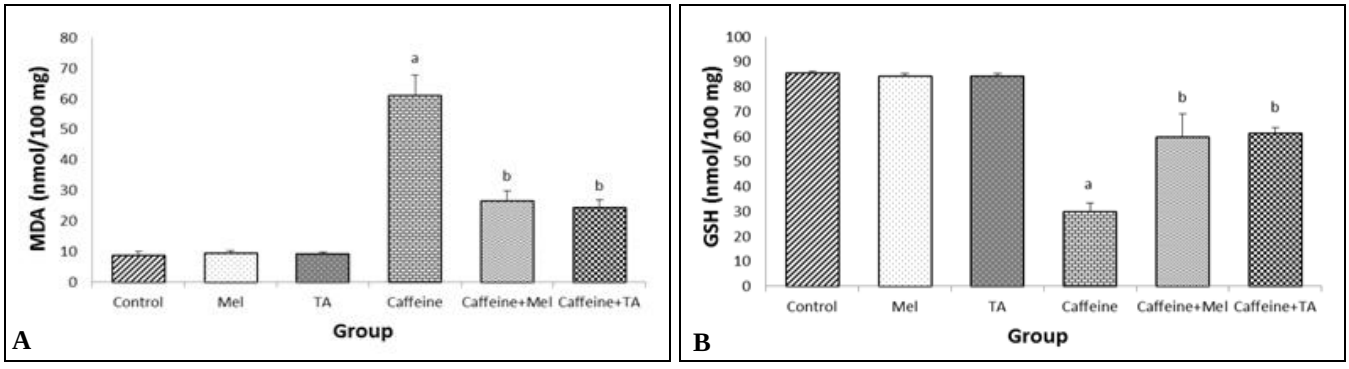
Tail moment = Tail length X % DNA in tail / 100

Estimation of Monoamines in Different Brain Tissue Regions: The determination of norepinephrine (NE) and dopamine (DA) content was carried out according to Ciarolone [34]. In this fluorometric assay, the monoamines are first oxidized to their "adrenochromes", and then re-arranged to their "adrenolutins", which are then detected by specific fluorescence at particular wave lengths of excitation and emission. Each brain region was separately weighed then homogenized in ice-cold solution of n-butanol (10ml/g tissue) then centrifuged at 4,000 rpm for 10 min at 4 ºC in a Heraeus Sepatech centrifuge. A volume of 2.5ml of each supernatant was transferred to a tube containing 1ml of 0.2N acetic acid and 5ml n-heptane. The tubes were then placed on a vortex mixer for 30 sec and centrifuged at 1000 rpm for 5 min. The organic supernatant was discarded from the aqueous phase, and 1 ml of the aqueous phase was transferred to another tube for the determination of NE and DA.

Determination of Norepinephrine (NE) and Dopamine Contents (DA): A volume of 0.2ml of EDTA was mixed with 1ml of supernatant, and then 0.1ml of 0.1N iodine was added and shook well for 2 min followed by 0.2ml of alkaline sulphite added with shaking for further 2 min. Finally, 0.2ml of 5N acetic acid was added and shook well while blank tube was prepared by adding 0.2 ml of 0.2N acetic acid instead of supernatant. For determination of norepinephrine the tubes were placed in boiling water bath for 2 min then cooled under tap water and the fluorescence was read at excitation 380 nm and emission 480 nm using Hitachi (F3010 model) spectrophotofluorometer. For the determination of dopamine, the tubes were placed in boiling water bath for 40 min then were cooled under tap water, and the fluorescence was read at excitation 320 nm and emission 480 nm using Hitachi (F3010 model) spectrophotofluorometer.

RESULTS:

Melatonin and Taurine Attenuate Oxidative Stress in Brain of Caffeine-Administered Rats: Caffeine-administered rats showed a significant ($P < 0.05$) increase in MDA, a marker of lipid peroxidation, when compared to the respective normal rats. Concurrent treatment with either melatonin or taurine for 28 days significantly ($P < 0.05$) decreased brain MDA content. In contrast, GSH content and activity of the antioxidant enzyme SOD in the brain of caffeine-administered rats were significantly ($P < 0.05$) declined. Rats treated with melatonin and taurine significantly ($P < 0.05$) prevented GSH decline and ameliorated SOD activity in rats brain **Fig. 1**.



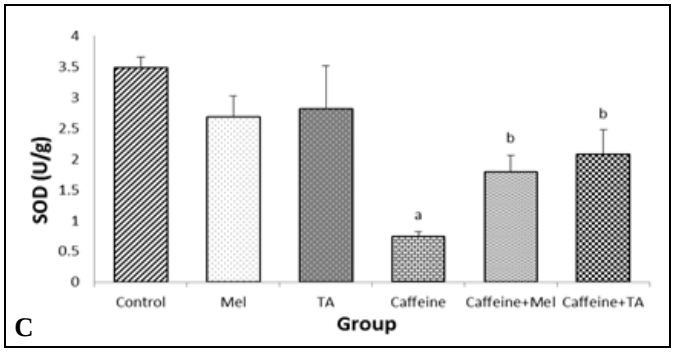


FIG. 1: MELATONIN AND TAURINE AMELIORATED THE OXIDATIVE STRESS EFFECT INDUCED BY CAFFEINE IN BRAIN OF CAFFEINE-ADMINISTERED RATS. (A) MDA, MALONDIALDEHYDE; (B) GSH, GLUTATHIONE; (C) SOD, SUPEROXIDE DISMUTASE. Data are expressed as Mean ± SD (N = 6). [a]Significantly different from control group, [b]Signifficantly different from caffeine group at $p < 0.05$

Melatonin and Taurine Prevent DNA Damage in Brain of Caffeine-Administered Rats: To further explore the protective effects of melatonin and taurine against caffeine toxicity, oxidative DNA damage was determined using comet assay. The data represented in **Fig. 2** and **3** show the effect of melatonin and taurine on DNA fragmentation in brain of control and caffeine-administered rats. Caffeine significantly ($P < 0.05$) increased DNA fragmentation as showed from the tail length and DNA% in comet tail. Oral supplementation of either melatonin or taurine for 28 days significantly ($P < 0.05$) attenuated DNA fragmentation in brain of caffeine-administered rats.

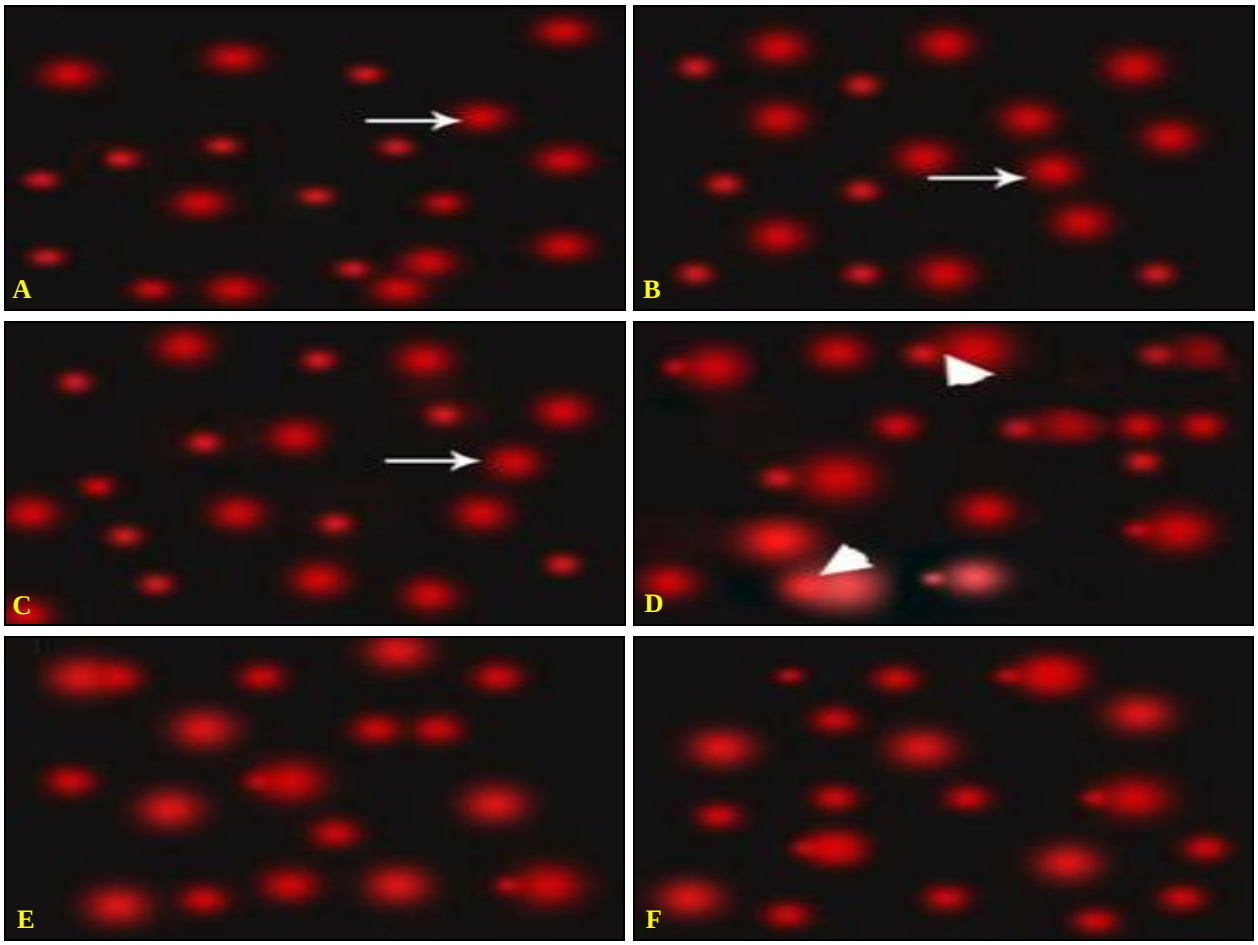


FIG. 2: MELATONIN AND TAURINE AMELIORATED THE INDUCED DNA DAMAGE IN BRAIN OF CAFFEINE-ADMINISTERED RATS. PHOTOMICROGRAPHS OF COMET ASSAY SHOWING DNA MIGRATION PATTERN IN BRAIN TISSUE FROM (A) CONTROL RATS, (B & C) NORMAL RATS TREATED WITH MELATONIN AND TAURINE, RESPECTIVELY. ALL OF WHICH SHOWING NORMAL SPOTS AND ROUND UNTAILED SHAPE (ARROWS), (D) CAFFEINE ADMINISTERED RATS SHOWING INCREASED NUMBER OF DAMAGED SPOTS WITH TAILED SHAPES (HEAD ARROWS) , (E&F) CAFFEINE ADMINISTERED RATS TREATED WITH MELATONIN AND TAURINE, RESPECTIVELY SHOWING DECREASED NUMBER OF DAMAGED SPOTS

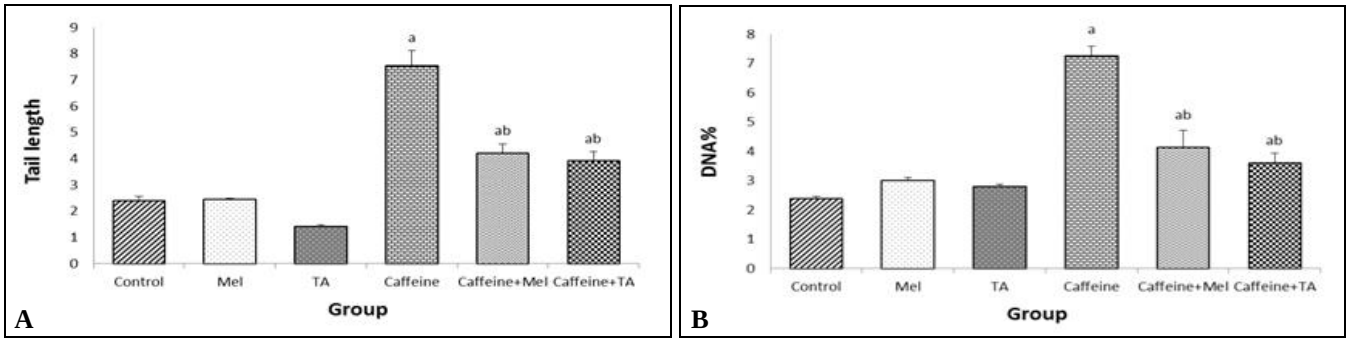


FIG. 3: MELATONIN AND TAURINE AMELIORATED THE DNA DAMAGE INDUCED BY CAFFEINE IN BRAIN OF CAFFEINE-ADMINISTERED RATS. PHOTOMICROGRAPHS OF COMET ASSAY SHOWING DNA MIGRATION PATTERN IN BRAIN TISSUE FROM (A) CONTROL, (B) CONTROL + MELATONIN (C) CONTROL + TAURINE, (D) CAFFEINE ADMINISTERED RATS, (E) CAFFEINE + MELATONIN AND (F) CAFFEINE + TAURINE

Melatonin and Taurine Exacerbate the Caffeine Effect on Neurotransmitters in Brain of Caffeine-Administered Rats: Dopamine and norepinephrine level **Table 2** in brain of caffeine-administered rats showed a significant ($P < 0.05$) elevation as compared to their respective normal controls. Melatonin and taurine supplementation produced a significant ($P < 0.05$) amelioration in both dopamine and norepinephrine levels as compared to caffeine administered rats.

TABLE 2: AMELIORATIVE EFFECT OF MELATONIN AND TAURINE ON NEUROTRANSMITTERS IN BRAIN OF CAFFEINE-ADMINISTERED RATS. (A) DA, DOPAMINE IN PICOGRAM/MILLILITER; (B) NE, NOREPI-NEPHRINE IN PICOGRAM/MILLILITER

Group / Parameter	Control	Melatonin	Taurine	Caffeine	Caffeine + Melatonin	Caffeine+ Taurine
Dopamine (Pg/ml)	33.90 ± 1.10	39.33 ± 2.17	38.28 ± 1.08	98.87 ± 8.81[a]	67.77 ± 3.26[b]	58.00 ± 3.27[b]
Norepinephrine (Pg/ml)	69.68 ± 4.48	79.28 ± 3.83	69.92 ± 2.12	136.70 ± 2.60[a]	93.80 ± 8.77[b]	87.77 ± 8.73[b]

Data are expressed as Mean ± SD (N = 6). [a]Significantly different from control group, [b] Significantly different from caffeine group at $p < 0.05$

Melatonin and Taurine Prevent Apoptosis in Brain of Caffeine-Administered Rats: Caffeine significantly ($P < 0.05$) increased the level of P53 and BAX gene and decreased Bcl2 gene expression in the brain of caffeine-administered rats as compared to their respective normal controls.

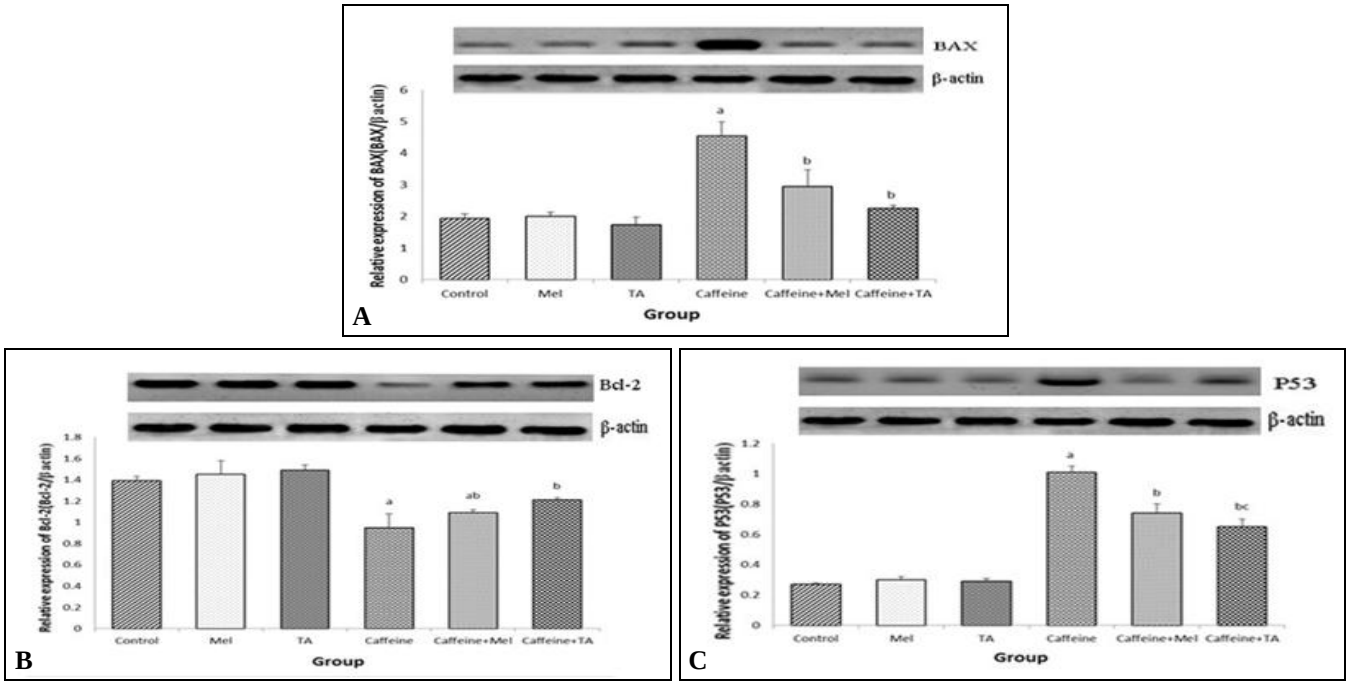


FIG. 4: AMELIORATIVE EFFECT OF MELATONIN AND TAURINE ON APOPTOSIS INDUCED BY CAFFEINE IN BRAIN OF CAFFEINE-ADMINISTERED RATS. (A) BAX, APOPTOSIS REGULATOR; (B) Bcl-2, (B-CELL LYMPHOMA-2), ANTI-APOPTOTIC MARKER; (C) P53, TUMOR SUPPRESSOR GENE. Data are expressed as Mean ± SD (N = 6). [a]Significantly different from control group, [b] Significantly different from caffeine group at $p < 0.05$

Treatment with either melatonin or taurine for 28 days markedly ($P < 0.05$) decreased brain level of P53 and BAX gene and increased Bcl2 gene in the brain of caffeine-administered rats. In the same trend, the protein expression level of P53 and BAX significantly ($P < 0.05$) increased in the brain of caffeine-administered rats, and Bcl2 protein expression level decreased. Co-treatment with either melatonin or taurine for 28 days markedly ($P<0.05$) decreased brain expression level of P53 and BAX protein and increased Bcl2 protein expression in the brain of caffeine-administered rats **Fig. 4**.

DISCUSSION: Caffeine is present in energy drinks, drugs, several food and beverage products, such as coffee, tea, and carbonated. It is probably the most commonly consumed pharmacologically active compound in the world [1, 35]. Some of the effects of caffeine could favor the generation of free radicals and lead to a subsequent increase in oxidative stress by increasing lipid peroxidation [36]. Although the wide use of caffeine in productions, its increasing consumption, and the conflicting results that had been generated by using of variable forms of caffeine and experimental methods little attention has been paid towards the study of its possible toxicity.

The present investigation evaluates the possible protective role of melatonin and taurine against caffeine toxic effects on the brain of rats, focusing on oxidative stress, inflammation, oxidative DNA damage, apoptosis, and monoamine neuro-transmitters.

The total antioxidant capacity is determined by measuring the markers of oxidative stress. MDA has been reviewed as a primary biomarker of free radical mediated oxidative stress and lipid damage [37]. The results showed the prooxidant effect of caffeine administration, evidenced by the significantly increased levels of MDA. Caffeine has been reported to induce the generation of ROS and eventually to result in oxidative stress as indicated by increased lipid peroxidation activity [38]. Choi *et al*., [39] supposed that coffee or its metabolites can be pro-oxidant and can increase lipid peroxidation. Similarly, Leelarungrayub *et al.* [40] reported a significant increase in MDA level in men consuming caffeinated coffee, when compared to decaffeinated coffee or control, followed by a submaximal exercise test. In the same trend, Metro *et al*., [41] demonstrated an increased intramuscular fat oxidation after caffeine-rich food consumptions.

In contrast, caffeine-induced rats showed a significant decline in brain GSH content. GSH has been considered as a biomarker of redox imbalance at the cellular level and the most abundant non protein thiol that defends against oxidative stress [37]. Intracellular GSH depletion significantly promotes mitochondrial ROS production and triggers mitochondrial membrane depolarization [42]. The declined GSH might be due to nicotinamide adenine dinucleotide phosphate (NADPH) depletion or increased consumption of GSH in non-enzymatic removal of oxygen-radicals [43].

In addition, reduced activity of the antioxidant enzyme SOD was observed in the brain of the caffeine-induced rats. SOD has been reported as an important defense enzyme that catalyzes the partitioning of superoxide radical, which produces hydrogen peroxide (H_2O_2) that is turned into H_2O and molecular oxygen by catalase [44]. In agreement with our findings, Cruz *et al*., [45,] and Ekaluo *et al*. [46] concluded that caffeine could cause lipid peroxidation and decrease the GSH content and SOD activity in molluscs (*Ruditapes philippinarum*) and rats, respectively.

These changes were significantly ameliorated by melatonin and taurine administration, which reduced the MDA level and increased GSH content and SOD activity. The ameliorative effect of melatonin could be attributed to its free radical scavenging ability. It prevents peroxidation, which is a common feature of other antioxidants. Also, it had been found to be more potent than glutathione and mannitol in hydroxyl radical scavenging activity and decrease the nitric oxide synthase (NOS) activity that is a pro-oxidative enzyme [47]. In addition to its highly free radical scavenging capacity, its metabolites are in a series of reactions that is known as the melatonin antioxidative cascade [48, 49]. This function relies only on melatonin's chemical structure, it reacts directly with free radicals and neutralizes their oxidative effects, and the same is true for its metabolites. No cell receptor is required to achieve this effect.

Hence, this function cannot be lost through evolution [50]. It has been reported that natural antioxidants such as vitamin E and melatonin might decrease MDA level and increase the activities of GSH levels in pathological conditions induced by oxidative stress [51]. It ameliorates oxidative tissue and DNA damage resulting from formaldehyde-induced toxicity, decreases MDA level, and increases GSH level in lung, liver, and kidney [52].

According to Wang *et al.,* [53] melatonin up-regulated the relative expression of the antioxidant enzyme SOD during *in-vitro* embryo development. Moreover, in an experimental model of carbon tetrachloride (CCl4)-induced liver injury, melatonin treatment attenuated liver injuries and diseases by inhibiting oxidative damage and other mechanisms. It increased SOD and GSH activity and decreased MDA level [54].

The ameliorative effect of taurine could be attributed to be a free radical scavenger, membrane stabilizer and hypolipidemic agent [55]. Some investigations indicated that taurine's antioxidant actions are related to the up-regulation of the activity of the antioxidant enzymes and reducing the amount of damaging ROS. Thus, taurine is able to indirectly elevate the activity of the antioxidant defenses. Also, taurine acts as an important anti-inflammatory agent, which includes a myeloperoxidase-catalyzed reaction between taurine and hypochlorous acid to generate an anti-inflammatory product, taurine chloramine. However, through the myeloperoxidase reaction, taurine also decreases the levels of the neutrophil-generated ROS, hypochlorous acid. Furthermore, its intra-mitochondrial depletion is connected to up-regulation in mitochondrial superoxide generation, leading to the suggestion that the mitochondria are the primary source of ROS generated by taurine deficient tissues [56, 57]. Also, taurine advances the synthesis of GSH and raises the action of GPx, and this could be the mechanism of enzymatic antioxidant defense [58].

In the other hand, taurine increases SOD activity in a dose-dependent manner [59]. Additionally, NIU *et al*. [60] reported that taurine supplementation was reported to be effective against oxidative stress, apoptosis, and inflammation in injured brain cells, it significantly decreased MDA content and increased GSH and SOD content in injured brain cells.

Reactive oxygen species can react with DNA, carbohydrates, proteins, and lipids in a destructive manner as a result of their high levels of chemical reactivity. Therefore, ROS are considered as DNA-damaging agents that promote oncogenic transformation, increase mutation rates, and function as cellular messengers in redox signaling, causing disruptions in normal mechanisms of cellular signaling [61]. The increase of oxidative stress results in double-strand DNA breaks [62]. The present study revealed that caffeine significantly increased DNA fragmentation as showed from the tail length and DNA% in the comet tail. As shown in the comet assay results, oral supplementation of melatonin and taurine significantly attenuated DNA fragmentation in the brain of caffeine-administered rats. These findings are consistent with Schmid *et al*. [63], who found that men with high caffeine consumption had significantly higher frequencies of sperm comet values with DNA damage compared to men with less caffeine consumption. Genotoxicity of caffeine was also reported by Selby and Sancar [64], who concluded that caffeine can intercalate into the DNA molecule of bacteria and block repair enzymes, and Aguirre-Martínez *et al*., [9] who found damage in the DNA of hemocytes of the Asian clam *Corbicula fluminea* after a 21-day exposure to caffeine.

Reiter *et al*., [65] reported that the first primary function of melatonin is to protect cells from oxidative stress, avoiding DNA, RNA, proteins, and membrane cell damage through its free radical scavenger capacity. Melatonin protects oocytes from DNA damage during prophase arrest by enhancing DNA repair via non-homologous end-joining (NHEJ) pathway and subsequently prevents the deterioration of oocyte quality during meiotic maturation [66]. In the same trend, it had been found that melatonin enhances the repair of oxidized DNA. This is maybe due to the ability of melatonin to transform guanosine radical to guanosine by electron transfer [67]. Moreover, it was reported that melatonin diminished the formation of 8-hydroxy-2′-deoxyguanosine (8-OH-dG), a damaged DNA product, 60–70 times more effective than some classic antioxidants (ascorbate and α-tocopherol) [68].

In the same trend, the ameliorative effect of taurine agreed with Abd El-Twab *et al*., [69], who found that oral supplementation of taurine for 6 weeks ameliorated ROS-induced DNA damage in testicular tissue of the diabetic rats. Another study reported that taurine treatment prior to $KBrO_3$ significantly attenuated DNA damage and DNA-protein cross-linking caused by $KBrO_3$ to the rat intestine [70].

Biochemically, the main action of caffeine is antagonism of adenosine A_1 and A_2 receptors. Neuropsychiatric effects are mediated largely by blockade of A_1 and A_2 receptors in the CNS. Adenosine A_1 receptors are present in almost all brain areas, but particularly in the cerebral cortex, hippocampus, thalamus, and cerebellar cortex [71]. Caffeine acting as an antagonist of A_2AR may inhibit an important A_2 AR-mediated tissue-protecting mechanism. Also, this suggested that caffeine might trigger tissue damage if consumed during an acute inflammation episode [72]. Caffeine has been indicated to occupy adenosine receptors and then block the neurotransmitter action. Adenosine receptors relate to interplay of release, reuptake, metabolism, and excretion of neurotransmitters [73].

We found that caffeine markedly elevated dopamine and norepinephrine levels in brain, while melatonin and taurine significantly down-regulated the level of dopamine and norepinephrine in the brain of caffeine-administered rats. Our observations were in a line with Volkow *et al*. [74], who interpreted that caffeine's DA-enhancing effects in the human brain are indirect and mediated by an increase in D2/D3R levels and/or changes in D2/D3R affinity. Also, norepinephrine level increased after caffeine treatment. These observations agree with Smith *et al*., [75] who concluded that caffeine opposes the reduction in the turnover of central noradrenaline.

There are links between adenosine A_2A receptors and the dopaminergic system in the brain. As adenosine inhibits dopaminergic neurotransmission, blockade of A_2A receptors by caffeine may increase dopaminergic activity and exacerbate psychotic symptoms [76]. The inhibitory action of melatonin on enhanced dopamine release was first demonstrated in excised female rat hypothalamic tissue in vitro, and it appears to be mediated by membranal, low-affinity melatonin binding sites by suppression of calcium influx into the stimulated nerve endings [77]. Melatonin inhibits dopamine release in the retina and mesencephalic dopamine areas [78]. It was reported that melatonin enhanced norepinephrine content in the adrenal medulla of chronically stressed rats [79].

The function of the neurotransmitter implies the existence of specific taurine receptors and the neuromodulator role of interference with the functions of other transmitter systems [80]. Taurine injection within the *Substantia nigra* reduces extracellular dopamine [81] and modulates striatal dopaminergic transmission [82], but another study reported that direct injection of taurine into the striatum had significantly increased extracellular dopamine [83].

Also, Chen *et al*., [84] report a significantly lower dopamine uptake was detected in the striatal synaptosomes of SHR rats that were fed with high-dose taurine than those of the controls. It also affects norepinephrine uptake and releases in rat cerebral cortical slices [85]. Pretreatment of taurine reduced the levels of dopamine, noradrenaline, and 5-hydroxytryptamine. Moreover, taurine triggers the elevation of striatal dopamine is dependent on impulse flow [86].

Increased ROS are related to excess cell loss and mediate the induction of apoptosis in various cell types [87, 88]. Our findings showed that caffeine increased P53 and BAX gene and protein expression levels but decreased Bcl-2 gene and protein expression levels in the brain. Treatment with melatonin and taurine significantly ameliorated these changes.

Several reports showed that high concentrations of caffeine induce cellular apoptosis [7]. Our observations were in line with Lu *et al*., [7], who found that treatment of osteoblasts with more than 0.5 mM caffeine triggered an increase in Bax and a decrease in Bcl-2 protein levels. Bax and Bcl-2 regulate changes in the mitochondrial membrane potential (MMP) and permeability, which play important roles in apoptotic processes [89]. Another study was carried out by He *et al*., [8] who showed that the mechanism of induction of apoptosis in JB6 Cl41

cells by caffeine involved activated p53, Bax, and caspase 3. It has been reported that caffeine had a mechanistic effect on cell cycle function, triggered apoptosis, and perturb key regulatory proteins, including the tumor suppressor protein p53 [90].

Melatonin supplementation upregulated the antiapoptotic Bcl-2 level and decreased the proapoptotic Bax level. The ability of melatonin to enhance the Bcl-2 level has been shown in rat brain and has an antiapoptotic role [91]. Furthermore, an in vitro study by Wang *et al*., [53] proved the anti-apoptotic effect of melatonin by increasing Bcl-2 level and down-regulating the pro-apoptotic gene p53. Our results are also consistent with Juknat *et al*. [92,] who found a decrease in Bax expression after pre-incubation of cultured rat astrocytes with 10 nm melatonin. Melatonin significantly decreased the mRNA and protein expression of BAX, while it enhanced the mRNA and protein expression of Bcl-2 of mouse Leydig cells, melatonin at concentrations of 10 and 100 ng/mL for 36 hours [93]. Melatonin protective effects appear to be associated with its antioxidant ability, which limits intra-mitochondrial glutathione loss and reduces mitochondrial protein damage and improves the electron transport chain activity [94, 95].

Under the condition of apoptosis, apoptotic cells undergo an exaggerated activation of the regulatory volume decrease in which taurine effluxes the cell. If the regulatory volume decrease is disrupted by preloading the cells with taurine several apoptotic steps, such as apoptotic cell shrinkage and DNA fragmentation, are blocked [96]. Although taurine loading did not inhibit early apoptotic events, such as caspase activation, it blocked the progression of the apoptotic cascade beyond the cell shrinkage step [96]. NIU *et al*., [60] found that taurine supplementation significantly reduced P53, caspases-3, and BAX mRNA expression and increased Bcl-2 mRNA expression in injured brain cells. Also, taurine significantly inhibited myocardial H/R-induced apoptosis, and the mechanism may be related to a down-regulated expression of PUMA [97].

CONCLUSION: Our study showed the protective effect of melatonin and taurine against caffeine toxicity in rat brain. The results revealed that caffeine increased oxidative stress by increasing MDA and decreasing GSH content and SOD activity. From comet assay results, caffeine caused DNA damage as shown from DNA fragmentation and tail %. Caffeine-administered rat showed markedly high levels of dopamine and norepi-nephrine, as well as significantly high levels of apoptotic markers, BAX and P53, and lower antiapoptotic Bcl-2. Melatonin and taurine ameliorated all of caffeine toxic effects.

As shown from our results, taurine appears to be more potent against caffeine toxicity than melatonin. From this point of view, taurine and melatonin can impact upon caffeine-induced oxidative stress and apoptosis through their antioxidant activity.

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