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Original Article

THE THERAPEUTIC AND NEUROPROTECTIVE EFFECTS OF GREEN TEA IN A RAT MODEL OF TERLIPRESSIN-INDUCED CHRONIC HYPONATREMIA

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ABSTRACT

Objective: Hyponatremia (HN) is associated with mortality and morbidity risks due to the development of hyponatremic encephalopathy. Its rapid correction also carries a high risk of development of the serious cerebral disorder. This study investigated the possible therapeutic and neuroprotective effects of the green tea (GT) extract against HN and its complications in rats and compared those effects with the outcome of the rapid correction of chronic HN using hypertonic saline (HtNaCl).

Methods: Chronic HN was induced using terlipressin (TP; 0.2 mg/kg, s. c) and 2.5% d-glucose solution (equivalent to 5% initial bw/day, i. p) for 3 d. A stabilizing dose of TP (0.1 mg/kg) was used for the following 3 d, along with administration of either saline, GT (600 mg/kg/day, p. o), or HtNaCl (15 ml/kg/day, i. p). Serum sodium level, locomotor activity, pain reflex, and brain contents of iNOS and NO were assessed, together with a histopathological examination of brain tissues.

Results: TP-induced profound chronic HN that was corrected with administration of GT and HtNaCl. In a GT-treated group, correction of HN was coupled with improvement of TP-induced alteration of locomotor activity and brain histopathological picture. Elevation of brain iNOS and NO contents, along with detection of focal cellular necrosis and gliovascular proliferation changes in the HtNaCl-treated group indicated neuro pathological complications are accompanying the correction of HN with HtNaCl; a result that was not found in the GT-treated group.

Conclusion: Our findings revealed that GT corrected HN induced by TP in rats, and protected against the neuropathological features that characterized hyponatremic encephalopathy and accompanied with its rapid correction.

Keywords: Green tea, Terlipressin, Hyponatremia, Hyponatremic encephalopathy, iNOS, Rat

INTRODUCTION

Hyponatremia (HN) is defined as a decrease in serum sodium concentration to a level below 136 mmol/l [1]. It is the most common electrolyte disorder occurring in 15–30% of hospitalized patients [2]. Etiologies of HN include renal and extra-renal loss of sodium with water retention as in cases of mineralocorticoid deficiency, autoimmune diseases, adrenal hemorrhage, muscle trauma, burns, sweat losses, and gastrointestinal losses due to vomiting and diarrhea [3]. Moreover, various pharmacological agents are known to be associated with induction of HN, including, narcotics, sedatives, analgesics, hypoglycaemic agents, anti-neoplastic drugs, and antidepressants [4, 5]. HN can also arise in a variety of diseases such as congestive heart failure, liver cirrhosis, and renal failure whether acute or chronic [6]. The most common cases of HN include exercise-associated HN, particularly in athletes who participate in endurance events [7], ecstasy-associated HN [8], and postoperative HN [9].

HN is physiologically significant when it indicates a state of extracellular hypo-osmolality and a tendency for free water to shift from the vascular space to the intracellular space. However, although cellular edema is well tolerated by most tissues, it is not tolerated in the brain as its maximum swelling is limited to 8% secondary to the presence of the rigid calvarium [3]. In severe HN, this cytotoxic cerebral edema results in the development of hyponatremic encephalopathy, which is a medical emergency that can be lethal [10]. The rate of development of HN plays a critical role in its pathophysiology and subsequent treatment [11]. In acute HN that is developed rapidly over a period of hours (<48 h), a more severe degree of cerebral edema for a given serum sodium level results. Thus, it occurs with alarming hyponatremic encephalopathy findings, and the primary cause of morbidity and death is brainstem herniation and mechanical compression of vital midbrain structures [12]. In chronic HN, when serum sodium concentration falls slowly over a period of several days or weeks (≥ 48 h), a slower process of adaptation then occurs in which the brain cells extrude sodium and potassium as well as organic solutes including phosphocreatine, myoinositol, and amino acids, such as glutamine and taurine, from their cytoplasm to the extracellular space [13]. Compensatory extrusion of solutes allows intracellular osmolality to be equal to plasma osmolality and, in turn, reduces the flow of free water into the intracellular space [14]. Therefore, brain swelling is minimized with more modest symptoms and cases, almost, never die from brain herniation [14]. However, the principal causes of morbidity and death in chronic HN are status epilepticus, when chronic HN reaches levels of 110 mmol/l or less, and osmotic demyelination syndrome (ODS), which occurs in association with rapid correction of chronic HN [11]. ODS is characterized by focal destruction of myelin sheaths that cover axons in the brainstem, in the pontine and extrapontine areas, associated with serious neurologic sequelae [15]. Several lines of evidence have linked the pathogenesis of myelinolysis to the slow reuptake of organic osmolytes by the brain. [16, 17].

Treatment of HN consists of free water restriction and correction of the underlying condition. The daily fluid intake must be restricted to less than the urine output volume and the insensible losses of water during 24 h in order to cause a negative water balance and increase serum sodium concentration [3]. Some cases of chronic HN do not tolerate or comply with this degree of daily fluid restriction. In these cases, the use of diuretics that increase electrolyte-free water excretion may be added to the treatment protocol [18-20].

Green tea (GT) is a popular worldwide beverage that has been shown to possess several pharmacological activities [21-24]. It has been reported for its diuretic [25-28], anti-inflammatory [29], antiarthritic [30], antibacterial [31], antiviral [32], antiproliferative [33], antioxidant [34,35], neuroprotective [36, 37], and cholesterol-lowering effects [23, 38, 39]. Moreover, consuming green tea has

been found to protect against obesity [40, 41], cancer [42, 43], and cardiovascular diseases [44-46]. The chemical composition of green tea is complex; it contains xanthic bases (caffeine and theophylline), polyphenols commonly known as catechins, pigments, volatile compounds, proteins, amino acids, carbohydrates, vitamins (B, C, E), minerals, trace elements, and trace amounts of lipids and sterols [23, 24, 45]. Caffeine and theophylline account for the diuretic effect of GT [25-28]. It has been demonstrated that caffeine could increase urine production in patients with congestive heart failure and edema; however, theophylline has been found to induce even higher diuretic effect than caffeine [47, 48]. On the other hand, GT polyphenols are responsible for the neuroprotective and the antioxidant properties of GT. The present study aimed at testing the possible protective effects of GT extract against chronic HN and hyponatremic encephalopathy induced in rats using terlipressin (triglycyl–lysine vasopressin; TP), which is a long-acting analog of the antidiuretic hormone, vasopressin (VP) [49]. Moreover, the study compared the effects of GT with those resulted from the rapid correction of chronic HN using hypertonic saline (HtNaCl).

MATERIALS AND METHODS

Animals

Adult male albino Wister rats, weighing 180–200 g, was utilized in the present study. Standard food pellets and tap water were supplied ad libitum unless otherwise stated. Animals and food pellets were obtained from the animal house colony of the National Research Center (NRC, Egypt). Ethical considerations in handling laboratory animals that stated by NRC were followed throughout the study period.

Drugs

TP vials (Glypressin®; Ferring Pharmaceuticals, Germany) were used in the current study. GT water extract, in the form of powder, was obtained from Technomate for Chemicals and Pharmaceuticals (Egypt), and was used orally in a dose of 600 mg/kg/day [35]. All other chemicals were of the highest available commercial grade.

Experimental design

Chronic HN was induced in rats according to the method established in our previous study with colleagues [49]. The animals were randomly allocated into four groups; each group consisted of 10 rats. During the induction phase of this experiment (days 1–3), rats of the first group received s. c injections of saline and served as the normal group. In the remaining three groups, chronic HN was induced by injections of TP (0.2 mg/kg/day, s. c) and 2.5% d-glucose solution (equivalent to 5% initial bw/day, i. p) for 3 successive days. Rats of TP-induced HN group had free access to distilled water only during this phase of experiment with no access to food.

In the 2nd phase of the experiment (days 4–6), animals of all groups had free access to food and tap water. In this phase, the normal group received saline; the other three groups received TP (0.1 mg/kg/day, s. c.) as a stabilizing dose. Group 2 served as the HN-control group; groups 3 and 4 were treated with GT (600 mg/kg/day, p. o.) and 1 M sodium chloride solution (hypertonic saline, HtNaCl; 15 ml/kg/day, i. p.), respectively.

Estimation of serum sodium level

Blood samples were withdrawn from the retro-orbital plexus of each animal, under light diethyl ether anesthesia, following induction phase (on day 4), and one hour following the last drug administration in the treatment phase (on day 6). The blood was allowed to coagulate and then centrifuged, using a cooling centrifuge (Sigma and laborzentrifugen, 2k15, Germany), at 3000 rpm for 20 min for serum separation. Serum sodium level was estimated using specific reagent kit (Stanbio Laboratory, USA).

Measurement of locomotor activity

On days 0, 4 and 6 of the experiment, locomotor activity was assessed by detecting rat movements using grid floor activity cage (Model no. 7430, Ugo-Basile, Italy). Interruptions of infrared beams were automatically detected during a 10 min test session. Beam interruption information was processed in the activity cage software to provide an index of horizontal movements [50, 51]. Change of the basal locomotor activity was calculated for each rat.

Evaluation of pain reflex

The delay in pain reactivity, as a neuronal reflex, was evaluated on days 0, 4 and 6 of the experiment using the hot-plate test (7280 Ugo Basile, Italy) according to the method described by Laviola and Alleva [52]. Change of the basal response to the nociceptive stimulus was calculated for each rat.

Tissue sampling and estimation of brain inducible nitric oxide synthase (iNOS) and nitric oxide (NO) contents

Directly after collecting the last blood sample in the experiment, rats were decapitated under light diethyl ether-anesthesia, and their brain was carefully isolated and dissected through the midline into two hemispheres. One brain hemisphere from each rat was immediately weighed and homogenized in ice-cold potassium chloride (1.15%; pH 7.4) to yield a 20% (w/v) tissue homogenate (using MPW–120 homogenizers, Med instruments, Poland). The homogenates were centrifuged using a cooling centrifuge (Sigma and laborzentrifugen, 2k15, Germany) at 4000 r. p. m for 10 min; the supernatant was used for biochemical estimation of brain content of iNOS, using iNOS ELISA kit (MyBioSource, USA), and NOx (nitrite and nitrate, stable metabolites of NO) utilizing an NOx concentration assay kit (Cayman chemical company, Germany).

Histopathological examination of brain tissues

The other brain hemisphere of each rat was removed and immediately placed in 10% formalin. Afterward, brains were sectioned coronally at six levels. Sections were stained with hematoxylin and eosin (H&E), and processed for light microscopy evaluation of neuronal density, edema, and inflammation.

Statistical analysis

All the values are presented as means±standard error of the means (SE). Comparisons between different groups were carried out using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc tests. For behavioral parameters, square root transformed percent was calculated according to Jones *et al.* [53] then comparisons between different groups were carried out using the nonparametric one-way ANOVA followed by Dunn's multiple comparisons post hoc test. The difference was considered significant when $p<0.05$. GraphPad prismsoftware (version 6) was used to carry out these statistical tests.

RESULTS

Serum sodium level

Subcutaneous injection of TP produced stable HN demonstrated by decreased serum sodium level to 109.85±4.8 and 112.08±5.3 mmol/l on days 4and 6, respectively. Treatment of rats with GT or HtNaCl corrected TP-induced HN (fig. 1).

Saline, rats, treated with saline and represented the normal group; TP, rats treated with terlipressin and served as a hyponatremia (HN)-model group; TP-GT, rats with TP-induced HN treated with green tea; TP-HtNaCl, rats with TP-induced HN treated with hypertonic saline.

[a] Significantly different from the normal group at the corresponding day at $p<0.05$.

[b]Significantly different from TP group at the corresponding day at $p<0.05$.

Locomotor activity and Pain reflex

Induction of HN in rats using TP was coupled with a decrease in the basal locomotor activity that was observed on days 4 and 6 (table 1). A delay of the basal pain reflex was also observed following the induction phase (on day 4); however, no significant difference was observed in a TP-treated group on day 6 (table 2). Treatment of rats with GT improved that TP-induced decrease in locomotor activity; however, HtNaCl failed to do so (table 1). On the other hand, rats treated with GT showed a significant delay of the response to pain stimulus as compared to both normal and HN group (table 2).

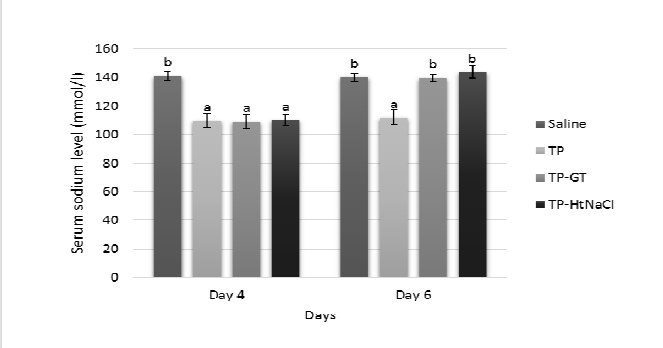

Fig. 1: Effects of TP-induced HN, and the co-administration of GT and HtNaCl on serum sodium level in rats

Saline, rats treated with saline and represented the normal group; TP, rats treated with terlipressin and served as hyponatremia (HN)-model group; TP-GT, rats with TP-induced HN treated with green tea; TP-HtNaCl, rats with TP-induced HN treated with hypertonic saline.

[a]Significantly different from normal group at the corresponding day at $p < 0.05$., [b]Significantly different from TP group at the corresponding day at $p < 0.05$.

Table 1: Effects of TP-induced HN, and the co-administration of GT and HtNaCl on the locomotor activity of rats

Groups	Locomotor activity				
	Count/10 min.			Square-root-transformed % of basal activity	
	Day 0	Day 4	Day 6	Day 4	Day 6
Saline	162.40±13.43	179.80±11.91	178.60±7.81	1.06[b]±0.04	1.06[b]±0.03
TP	118.60±25.24	55.20±16.70	80.40±15.36	0.69[a]±0.12	0.83[a]±0.02
TP-GT	161.20±26.84	96.00±21.19	155.60±25.08	0.78[a]±0.09	0.98[b]±0.01
TP-HtNaCl	123.40±10.09	73.80±14.35	70.20±8.50	0.76[a]±0.11	0.76[a]±0.08

Saline, rats treated with saline and represented the normal group; TP, rats treated with terlipressin and served as a hyponatremia (HN)-model group; TP-GT, rats with TP-induced HN treated with green tea; TP-HtNaCl, rats with TP-induced HN treated with hypertonic saline.

Data are presented as mean±SE., [a] Significantly different from the normal group at the corresponding day at $p<0.05$., [b]Significantly different from TP group at the corresponding day at $p<0.05$.

Table 2: Effects of TP-induced HN, and the co-administration of GT and HtNaCl on the response of rats to the nociceptive stimulus

Groups	Delay of pain reflex				
	Nociceptive response (sec)			Square-root-transformed % of basal response	
	Day 0	Day 4	Day 6	Day 4	Day 6
Saline	38.25±3.71	27.72±3.05	29.02±2.62	0.84[b]±0.07	0.86±0.06
TP	24.30±4.25	31.06±3.58	26.56±3.38	1.20[a]±0.08	0.98±0.13
TP-GT	26.58±2.60	35.38±4.51	38.38±5.61	1.13[a]±0.05	1.21[ab]±0.10
TP-HtNaCl	33.25±2.36	39.30±4.18	34.18±3.33	1.06[a]±0.04	0.99±0.03

Saline, rats treated with saline and represented the normal group; TP, rats treated with terlipressin and served as a hyponatremia (HN)-model group; TP-GT, rats with TP-induced HN treated with green tea; TP-HtNaCl, rats with TP-induced HN treated with hypertonic saline.

Data are presented as mean±SE., [a] Significantly different from the normal group at the corresponding day at $p<0.05$., [b]Significantly different from TP group at the corresponding day at $p<0.05$.

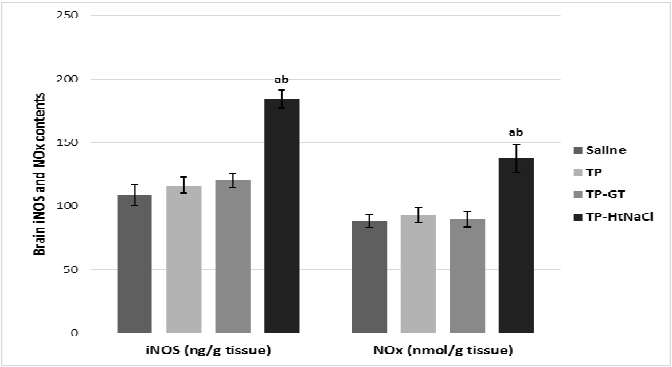

Fig. 2: Effects of TP-induced HN, and the co-administration of GT and HtNaCl on brain contents of iNOS and NOx in rats

Saline, rats treated with saline and represented the normal group; TP, rats treated with terlipressin and served as hyponatremia (HN)-model group; TP-GT, rats with TP-induced HN treated with green tea; TP-HtNaCl, rats with TP-induced HN treated with hypertonic saline., [a] Significantly different from normal group at $p<0.05$., [b]Significantly different from TP group at $p<0.05$.

Brain iNOS and NO contents

Treatment of rats with TP to induce HN had no effect on the brain contents of iNOS and NOx. Similarly, administration of GT to TP-treated rats showed normal iNOS and NOxcontent; whereas, administration of HtNaCl increased brain iNOS and NOx content in rats with TP-induced HN (fig. 2).

Histopathological examination of brain tissues

The brain sections prepared from normal rats showed normal brain tissue with normal astrocytes and fibrillary background (fig. 3a), while those prepared from rats with TP-induced chronic HN indicated a marked glial brain edema (fig. 3b). Treatment of rats with GT markedly ameliorated the brain edema observed in rats with TP-induced chronic HN and restored the normal histological structures of brain tissues (fig. 3c). The brain sections prepared from rats with TP-induced chronic HN treated by HtNaCl revealed marked amelioration of brain edema; however, neuronal cell necrosis associated with congestion of cerebral blood vessel was detected (fig. 3d).

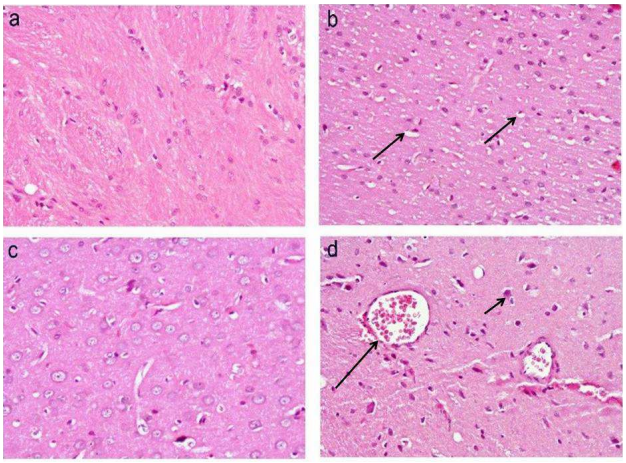

Fig. 3: Photomicrographs of brain sections prepared from (a) a normal rat, (b) a rat with TP-induced chronic HN, (c) a rat with TP-induced chronic HN treated by GT, and (d) a rat with TP-induced chronic HN treated by HtNaCl (H&E X 400)

(a) Showing normal brain tissue with normal astrocytes and fibrillary background. (b) Showing marked glial brain edema (arrow). (c) Showing marked amelioration of the glial astrocytes and fibrillary background brain edema observed in rats with TP-induced chronic HN. (d) Showing neuronal cell necrosis (short arrow) associated with congestion of cerebral blood vessel (long arrow).

DISCUSSION

In the present study, injection of TP (0.2 mg/kg/day, s. c.) along with 2.5% d-glucose solution (equivalent to 5% initial bw/day, i. p), for 3 successive days, induced profound (<115 mmol/l) chronic (>48 h) HN that was evidenced by decreased serum sodium level on days 4 and 6. A hypotonic dextrose solution was used as a source of electrolyte-free water to produce expansion of the extracellular fluid volume; while, TP was used as an antidiuretic agent to prevent the excretion of that electrolyte-free water for the entire period of the experiment. Both elements must be satisfied to induce HN [54]; since the administration of antidiuretic agent without water does not result in HN [55], and administration of electrolyte-free water in the absence of antidiuretic agent leads, only, to a large water diuresis [56]. TP is a long-acting analog of VP, the antidiuretic hormone [57]. Like VP, TP stimulates vascular vasopressin type 1 (V_1) receptor and renal tubular V_2 receptor, resulting in vasoconstriction and renal free water reabsorption, respectively. However, TP has a relatively higher affinity for the V_1 receptor and lower affinity to the V_2 receptor compared to VP [58]. Nevertheless, in agreement with the finding of the present study, Krag *et al.* [59] reported that TP induces V_2 receptor-mediated antidiuresis and induces a decrease in plasma sodium level. In the common experimental animal models of HN, VP has been used, either as multiple doses per day or as a continuous infusion via subcutaneously implanted osmotic minipump [60, 61]. Interestingly, a single daily dose of 0.2 mg/kg TP for three successive days induced chronic HN in the current model. The different pharmacokinetic properties of VP and TP explain this outcome. TP has a long duration of action and its effective half-life time is 6 h, whereas that of VP is only 6 min [58, 62]. For that, this TP-induced HN model that was established in our previous study with colleagues (unpublished data [49]), is less complicated than the common VP model. In addition, TP is available in the markets (glypressine; Ferring Company, Germany), while VP is not commercially available in all countries [62]. A stabilizing dose of TP (0.1 mg/kg/day, s. c.) was used for another three days following the induction phase. This regimen was carried out to prevent the spontaneous correction of chronic HN [48], and to simulate its clinical conditions [63]. The current TP-induced HN resulted in neurological complications in rats evidenced by the deterioration of the locomotor activity and pain reflex and confirmed by the histopathological findings revealed a marked brain edema. The chronic locomotor deficit that persisted for 6 d, and the delay of pain reflex detected on the 4th day, in rats with TP-induced HN are parallel to some of the typical signs of hyponatremic encephalopathy found in patients with HN [14]. However, the improvement of the pain reflex on day 6 may be due to the gradual adaptation of the brain to HN by extruding organic solutes from their cytoplasm, which decreases the degree of cerebral edema by allowing intracellular osmolality to be equal to plasma osmolality without a large increase in cell water [64]. This explanation may be supported by the study established that the impaired response to pain stimuli in patients with HN occurs in the more advanced state of cerebral edema rather than that associated with a reduction of locomotor activity [11].

Following the induction phase, administration of GT (600 mg/kg/day, p. o), for 3 successive days, corrected the serum sodium level in rats. This protective effect of GT against HN may be attributed to its diuretic effect [25, 26, 28], as previous studies showed that diuretics are useful in controlling edematous hyponatremic states [65, 66]. Improvement of locomotor activity was also observed in GT-treated rats; a finding that indicates a neuroprotective effect of GT against TP-induced hyponatremic encephalopathy. This conclusion was confirmed by the outcome of the histopathological investigation that revealed a marked improvement of TP-induced brain edema in the GT-treated group. In addition, Michna *et al.* [67] reported a direct stimulatory effect of oral administration of green tea and caffeine on locomotor activity in mice.

On the other hand, delayed response to the nociceptive stimulus was noticed in GT-treated rats; though it was normal in untreated rats with TP-HN. This finding may be explained by the stated analgesic effect of GT [68, 69].

Administration of HtNaCl in the present study corrected HN itself but not its effect on locomotor activity. Actually, administration of high concentrations of NaCl results in a rapid correction of serum sodium level, which causes a serious cerebral demyelinating disorder known as osmotic demyelination syndrome (ODS) [70]. The clinical manifestations of ODS usually develop after a couple of days (average 4–6 d) following changes in sodium levels. Hence, the non-corrected locomotor activity observed with HtNaCl administration in the current study may be due to the beginning of the pathological changes in the brain that finally led to ODS. Fortunately, these findings were supported by the present histopathological observations of focal cellular necrosis with neovascular proliferation changes in the cerebral tissue of rats treated with HtNaCl, though the marked amelioration of brain edema. These observations are in agreement with those of previous studies showing that rapid correction of experimental animal models of HN with HtNaCl resulting in demyelinative lesions associated with focal cellular necrosis, gliovascular proliferation changes, and accumulation of active microglia [71-73]. These microglia have been found to produce proinflammatory cytokines,

which are potent inducers of iNOS [74]. The induction of iNOS by proinflammatory cytokines results in NO production, which aggravates oligodendroglial injury and demyelination [73]. In the present study, significantly high levels of iNOS and NOx were observed in the brain tissues of hyponatremic rats treated with HtNaCl; this indicated the presence of inflammatory cerebral damage coupled with excessive iNOS activation and NO production. The presence of this inflammatory cerebral damage in HtNaCl-treated rats was also supported by the findings of the locomotor assessment and histopathological analysis. On the other hand, iNOS and NOx levels were normal in the brain tissues of rats with TP-induced HN, indicating that, iNOS activation and NO production are not involved in the neuropathological features of hyponatremic encephalopathy. In accordance, studies that assessed iNOS activity in experimental animals with HN showed that iNOS activity increases only with rapid correction of chronic HN [71-73]. GT successfully managed brain iNOS and NOx levels after the correction of HN in the present study. This result, together with the findings of the histopathological investigation, demonstrated the neuro-protective effect of GT against the pathological consequences of rapid correction of HN. The study of Nakagawa and Yokozawa [75] showed a direct scavenging of nitric oxide and superoxide by GT. On the other hand, Srivastava *et al.* [76] demonstrated that GT polyphenols act as potent inhibitors of nitric oxide generation independent of their antioxidant properties. Correspondingly, many studies reported the inhibition of iNOS expression by GT and GT polyphenols [77-80]; and considered it as an important mechanism underlying the neuroprotective effect of GT [81]. Choosing the 8 d duration for the experiment, before sacrificing the animals and isolation of brain for estimation of iNOS and NOx contents and histopathological examination, was guided by a previous study [60], to allow the development of the inflammatory cerebral damage that eventually leads to ODS, if any. In addition, another study [73] found that prominent accumulation of activated microglia was seen by 3 d after correction and that accumulation occurred parallel to the demyelinating changes.

CONCLUSION

In conclusion, the current study revealed that TP-induced HN rat model produced the main neuropathological features of human HN as manifested by the observed decrease of locomotor activity, delay of pain reflex, and development of the characteristic histopathological changes of the brain tissues. Administration of GT markedly improved TP-induced chronic HN in rats and protected against the neuropathological features that characterized hyponatremic encephalopathy and accompanied with its rapid correction. The study suggests GT as a therapeutic agent for protection against the neuropathological complications associated with chronic HN and its rapid correction.

CONFLICT OF INTERESTS

Declared none

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