

Human chorionic gonadotropin decreases the phosphorylated tau protein level in streptozotocin-Alzheimeric male rats' hippocampus

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Abstract

Introduction: The pharmacological suppression of luteinising hormone or human chorionic gonadotropin (hCG) can reduce A β plaques in the brains of rats and mice, but the effects of hCG on the phosphorylated tau protein level in the hippocampus have not been studied. Therefore, we investigated the effects of hCG on the phosphorylated tau protein level and its effect on hCG receptor-immunoreactive neuron density in the hippocampus of Alzheimer's disease (AD) model rats (streptozotocin [STZ] injected intracerebroventricularly).

Material and methods: The rats were administered hCG (50, 100, and 200 IU/200 μ l saline, intraperitoneally) or vehicle once/day for three days after injection of STZ. The passive avoidance memory test was performed 6 hours after the last hCG injection. The phosphorylated tau protein level in the hippocampus was measured by ELISA, and hCG receptor-immunoreactive neurons were shown by immunohistochemical technique in areas of hippocampus.

Results: Treatment with hCG attenuated memory deficiencies and reduced the level of phosphorylated tau protein in the hippocampus. hCG also improved the density of hCG receptor-immunoreactive neurons. The high dose of hCG hormone (200 IU/200 μ l saline) seemed to have a significant effect on passive avoidance memory, phosphorylated tau protein concentration, and accumulation of hCG receptor-immunoreactive neurons in Alzheimeric rats' hippocampus.

Conclusions: In conclusion, hCG can provide protection against memory deficits induced by STZ and it can inhibit accumulation of tau hyperphosphorylation in the hippocampus. Furthermore, hCG can increase the hCG receptor-ir neurons number in the rats hippocampus after ICV injection of STZ.

Key words: hCG, streptozotocin, passive avoidance memory, phosphorylated tau protein, hCG receptor, hippocampus.

Introduction

Alzheimer's disease (AD) is the most widespread type of dementia in the world, and an estimated

5.4 million Americans of all ages had AD in 2016 [7]. Alzheimer's disease, which has been considered as a neurodegenerative disorder, affects disproportionately individuals over 65 years old. Epidemiological

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data show that AD affects 11% of persons above 65 years of age and one-third of people over the age of 85 years (32%) [33]. In the developed world, AD is considered to be the sixth principal cause of death although estimates involving death from related health issues reflect the risk even greater [5,6]. Alzheimer's disease is pathologically characterised by the presence of intracellular hyper-phosphorylated neurofibrillary tau tangles (NFTs), extracellular deposits of plaques of amyloid β ($A\beta$), and neuronal cell death [49,50].

Tau protein belongs to the group of microtubule-associated proteins (MAPs) [36]; it is a neuronal protein that is phosphorylated in its proline-rich region. In fact, phosphorylation facilitates accumulation of tau in the formation of paired helical filaments (PHFs). Between the kinases known to phosphorylate tau, GSK3 plays a key main role [9]. Physiologically, tau plays an important role in microtubule stabilisation (tau has a vital role in stabilisation of microtubules), neurite outgrowth, and axonal transportation [66]. Tau is the main component of the PHFs that make up the NFTs, and it is abnormally phosphorylated in PHFs and NFTs [35]. In pathological conditions, such as AD, not only does abnormal phosphorylation of tau protein reduce its tubulin binding capacity leading to microtubule disorganisation, but also this protein self-polymerises and combines in the form of NFTs [36]. Furthermore, tau phosphorylation, which leads to tau dysfunction, results in declined cell viability. Actually, in neurodegenerative diseases in which tau pathology has been observed, the phosphorylation of tau is abnormal [35]. The spatiotemporal progression of tau combinations from the hippocampus and entorhinal cortex to cortical areas [18,28] has been shown to be related with cognitive deficits [30,32], supporting a critical role of tau pathology in AD-related memory deficiencies [37].

Human chorionic gonadotropin (hCG) is a heterodimeric glycoprotein hormone that is structurally and functionally similar to luteinising hormone (LH) [47]. Human chorionic gonadotropin has the ability to cross the BBB (blood-brain barrier), and its receptors are expressed by neurons in the brain [12]. While oestrogen is recognised as an important modulator of mood, anxiety, and memory, the roles of the gonadotropin hormones, LH, and hCG in the regulation of memory and behaviour are unclear. Most research on the effect of gonadotropin hormones

on behaviour have focused on the role of hCG in the modulation of behaviours experienced during pregnancy including nausea and vomiting, appetite, revulsion to food and smells, nervousness, anxiety, insomnia, and cognitive deficits including memory impairment [19,55]. In culture systems, hCG has been reported to support the survival and neurite outgrowth in primary neurons, and it induced differentiation of PC12 cells, probably through activation of LH/CG-R (LH/choriogonadotropin receptor) [3,47]. hCG treatment enhanced recovery of motor function in rats with complete spinal cord transection. These outcomes suggest a potential role for hCG/LH and LH/CG-R in the development and maturation of the mammalian nervous system [47]. However, recent associations between elevated LH levels and increased risk of AD have caused a re-evaluation of the role of the gonadotropin hormones in the modulation of neuropathology, AD-related behaviour, and memory [15,16,58,61].

Both hCG and LH hormones bind to the same LH/hCG receptor [53] and signal through a common G-protein-coupled receptor [46]. hCG has more potential than LH due to its higher receptor binding affinity and a longer circulatory half-life [53]. Although LH/hCG receptor has been thought to be limited to gonads, evidence shows its expression in non-gonadal tissue such as the CNS [3,4,21]. The presence of LH/hCG receptors was proven in the median preoptic area of hypothalamus, hippocampus, amygdala, cerebellum, cerebral cortex, and brain stem of the rat, with high density in hypothalamus and hippocampus [39]. Both neurons and glial cells were shown to express LH/hCG receptor [3,4,65]. The LH/hCG receptor may be involved in the development and differentiation of neurons, sleep-wake activity, and the controlling feedback mechanism of gonadotropin-releasing hormone and LH synthesis in the hypothalamus and pituitary gland, respectively [38,44,45,59].

Association of LH and hCG in AD progress was discussed [54]. For example, if the LH level is reduced, it can decrease Ab levels in mouse brain and can prevent memory loss in neurotoxin-induced AD model. High levels of LH or direct exposure to LH can increase Ab levels. Also, LH endorses amyloidogenic pathway of metabolism of amyloid precursor protein, secretion of Ab, and its deposition in the aged brain. Administration of hCG leads to an accumulation of Ab40, soluble Ab1-40 and Ab1-42 levels, and

cognitive deficits, as well as an increase in activity of b-secretase in a dose-dependent manner. Ablation of LH activities through suppression of LH/hCG receptor gene leads to a reduction Ab accumulation, plaque formation, and progress in neuropathological features in a mouse model of APP^{SW+}/LHR⁻.

Previous studies have shown that LH/hCG administration increased Ab accumulation in rat and mouse brains [14,17,25], but the effects of hCG on phosphorylated tau protein level in the hippocampus have not been studied. Hence, we investigated the effects of hCG on learning and memory impairments after ICV injection of streptozotocin (STZ) in rats. We also examined its effect on hCG receptor-immunoreactive (-ir) neuron density and phosphorylated tau protein level in the hippocampus of AD model rats.

Material and methods

Animals

Ten-week-old male Wistar rats with weighing 200 ± 20 g (Pasteur Institute, Tehran, Iran) were used. The animals were maintained at temperature of $22 \pm 3^\circ\text{C}$ on a light/dark (12-hour) cycle, with access to food and water *ad libitum*. The animals were acclimatised for one week prior to any experimentation. The experimental protocol was checked and approved by the Committee of Ethics in Golestan University of Medical Sciences, Gorgan, Iran. All surgery was performed under anaesthesia, and all efforts were made to reduce animal suffering.

Surgery and intracerebroventricular administration of streptozotocin

For intracerebroventricular (ICV) administration of STZ (Sigma, USA), the rats were anaesthetised with a mixture of ketamine/xylazine and located in a stereotaxic frame (David Kopf Instruments, USA) with flat-skull position. Under sterilised conditions and anaesthesia, 21-gauge guide cannulae were implanted in the right and left lateral ventricles in the rats' brains. The stereotaxic coordinates were -0.8 mm posterior; ± 1.5 mm lateral to the bregma; -4.2 mm deep from the dural surface [52]. Cannulas were secured to the skull with acrylic dental cement. Afterward, stainless steel stylets (27 gauge) were inserted into the guide cannulae to keep patency prior to microinfusions. After surgery, animals were allowed to recover for at least seven days [48].

Injected ICV solution was STZ in all groups (except the control group), where $5 \mu\text{l}$ /injection site was slowly injected by a 27-gauge Hamilton microsyringe ($10 \mu\text{l}$) during a one-minute period. To ensure diffusion, the microsyringe was left in place for one minute after each infusion. Rats received STZ (3 mg/kg in normal saline) on days 1 and 3 of the experiment [62].

Experimental protocol

One week after surgery, rats were trained using passive avoidance task. Then, the rats received ICV injections of STZ, and eight days after the first STZ injection, learning and memory impairment was tested using passive avoidance task. The rats that showed no learning and memory deficits after STZ-injection were excluded from the study. Six adult male rats were assigned to the control group – without cannula surgery, no drug treatment, and no behavioural test. Twenty-four rats, including STZ-injected rats, were then distributed in 4 groups (6 rats each), according to the treatment agent used; the untreated group (received vehicle: saline, $200 \mu\text{l}$, IP injection) and hCG treated groups received hCG (Darou Pakhsh Pharmaceutical Mfg. Co., Iran) with three doses of 50, 100, and $200 \text{ IU}/200 \mu\text{l}$ saline for three days, respectively. The IP injections of hCG and or saline were given at 9:00 a.m. On the last injection day, the memory investigations were carried out 6 hours later at 3:00 p.m. [45]. Doses of hCG were selected based on a previous study [45].

Passive avoidance task

The passive avoidance task (step-through inhibitory) has been used to study learning and memory in response to a stressful stimulus. This apparatus contained two equal light and dark boxes ($20 \times 20 \times 30 \text{ cm}^3$), connected to each other with a guillotine door ($7 \times 9 \text{ cm}^2$). The floor of the dark compartment was made up of stainless steel bars (3 mm in diameter and 1 cm intervals). All rats were allowed to adapt in the experimental room for at least one hour prior to the experiments. Then, each rat was gently placed in the light compartment; the guillotine door was opened after 5 s and the rat was allowed to enter the dark compartment. The latency with which the rat entered the dark box was recorded (acquisition trial). Rats that had

an initial latency of more than 120 s were excluded from further experiments. When the rat completely entered the dark compartment, the guillotine door was closed and an electric shock (1.5 mA intensity, 50 Hz) was sent to the floor grids for 3 s and then after 20 s, the rat was returned to its home cage. Two minutes later, the rat was retested in the similar way as in the previous trials; if the rat during 120 s did not enter the dark compartment, a successful acquisition of inhibitory avoidance response was recorded. Otherwise, when the rat entered the dark box before 120 s, the guillotine door was closed and the rat received the shock again. After retesting, if the rat learned inhibitory avoidance response successfully, it was relocated to the cage. On the test day each rat was gradually placed in the light compartment for the retention trial and the latency time to enter the dark compartment was recorded and described as step-through latency. The retention trial was set a limit of 300 s as cut-off time [10].

Tissue preparation

Forty-eight hours after the last test, all rats were anaesthetised with chloroform, and the brains were rapidly detached and rinsed with ice-cold PBS solution (pH 7.4) to remove any blood clots. The hippocampal slices of one hemisphere were homogenised in a certain amount of PBS solution and then centrifuged at 5000 rpm for 20 minutes. The supernatants were stored at -20°C until used for phosphorylated tau protein assay by ELISA technique. The brain tissue of another hemisphere was removed and used for an indirect immunohistochemistry technique.

Determination of phosphorylated tau protein concentration in hippocampus tissue by ELISA

The phosphorylated tau protein concentration in the hippocampus was determined using an ELISA kit (ZellBio GmbH, Germany) in hippocampus tissue. A kit was used based on the Biotin double antibody sandwich method. The colour change was measured with an ELISA reader at a wavelength of 450 nm. The phosphorylated tau protein concentration of hippocampus tissue was expressed as nanograms per litre of tissue homogenised.

Immunohistochemical staining

The brain tissue of another hemisphere was fixed in 4% paraformaldehyde for one week. Histological processing including dehydration, clarification with xylene, and then embedding in paraffin wax was performed [34]. The $6\text{-}\mu\text{m}$ sagittal serial sections were collected from the hippocampal formation at the following coordinates: lateral 1.40 mm to 3.90 mm [52]. An interval of $24\ \mu\text{m}$ was placed between each two serial sections. Five slices from each hippocampus were selected for immunohistochemistry staining. Then immunohistochemical staining was carried out as described previously [48]. Briefly, sections after deparaffinisation with xylene, rehydration in graded alcohol solutions, and washing with distilled water, were incubated at 60°C for 5 minutes. Then the sections were covered with epitope retrieval solution (Tashkis Baft, Iran) at $90\text{-}95^{\circ}\text{C}$ for 20 minutes, cooled at room temperature for 20 minutes, and washed with washing buffer (PBS/Tween 20 in 0.1% Triton X-100). Subsequently, the slides were placed in 3.5% hydrogen peroxide, washing buffer, blocked with avidin/biotin blocking solution (Dako, Denmark) for 20 minutes at room temperature and again rinsed with washing buffer. Subsequently, samples were incubated at 4°C overnight with Anti-hCG Receptor Rabbit monoclonal antibody (1 : 200, Abcam Inc., USA) and washed with washing buffer, anti-rabbit IgG antibody (Abcam Inc., USA) was applied for 60 minutes at 37°C , and then they were washed. Incubation with streptavidin HRP protein (1 : 5000, Abcam Inc., USA) for 30 minutes at room temperature and washing were the next processes. Chromogen DAB was used (Dako, Denmark), which was applied on the slices for 20 minutes at room temperature, followed by rinsing with distilled water. Meyer's Haematoxylin was used to stain the background by applying it lightly on the slides for 3-4 seconds. The sections were rinsed with distilled water. The slices were dehydrated, cleared, and finally mounted with Entellan (Merck, Germany) glue, which is suitable for visualisation under a light microscope.

Immunohistochemical evaluation

The stained hCG receptor-ir neurons were photographed by digital camera (DP72, Olympus, Japan) equipped with a (BX 51, Olympus, Japan) light microscope for hippocampal CA1 and CA3 areas and dental gyrus area. For CA1 and CA3 a field of $30,000\ \mu\text{m}^2$,

and because of the density in the DG area a field of $4800 \mu\text{m}^2$, was selected. Using Image J software the density of the hCG receptor-ir neurons was measured [48].

Statistical analysis

SPSS 16.0 (Armonk, NY, USA) for Windows for statistical data processing was used, and results were expressed as mean \pm SD. To examine the normal distribution, data was carried out by Shapiro-Wilk test. By one-way analysis of variance followed by post-hoc Tukey test, data were analysed for overall multiple comparisons. For all comparisons, $p < 0.05$ was considered to be statistically significant.

Results

Behavioural results

As shown in Figure 1 (after STZ injection), treatment of the rats with STZ decreased the step-through latency time. In the other words, STZ impaired their inhibitory passive avoidance memory. On the final test day, hCG injection with doses of 50, 100, and 200 IU/200 μl saline increased the step-through latency time compared to the STZ-saline group, confirming an increase in improvement of inhibitory passive avoidance memory. Tukey post-hoc analysis showed that there was a significant difference between 200 IU/200 μl saline dose of hCG and the STZ-saline groups' step-through latency time ($p < 0.001$, Fig. 1). Furthermore, we observed significant differences in the step-through latency time among the hCG-treated groups ($p < 0.01$, Fig. 1). These results demonstrated that hCG inhibited the effects of STZ treatment.

The phosphorylated tau concentration in the hippocampus

We observed a significant increase in the phosphorylated tau concentration of the hippocampus in treated rats with STZ in comparison to the control group ($p < 0.001$, Fig. 2). The phosphorylated tau concentration decreased slightly after treatment with hCG at 50 and 100 IU/200 μl saline doses (Fig. 2). Significantly, the phosphorylated tau concentration in the hippocampus was lower in the hCG group (200 IU/200 μl saline) than in the STZ-saline group ($p < 0.001$, Fig. 2), but we did not see differences in

the phosphorylated tau concentration among the hCG-treated groups (Fig. 2).

Hippocampal hCG receptor-ir neuron density

In CA1, CA3, and DG hippocampal areas after treatment with STZ, the number of hCG receptor-ir neurons

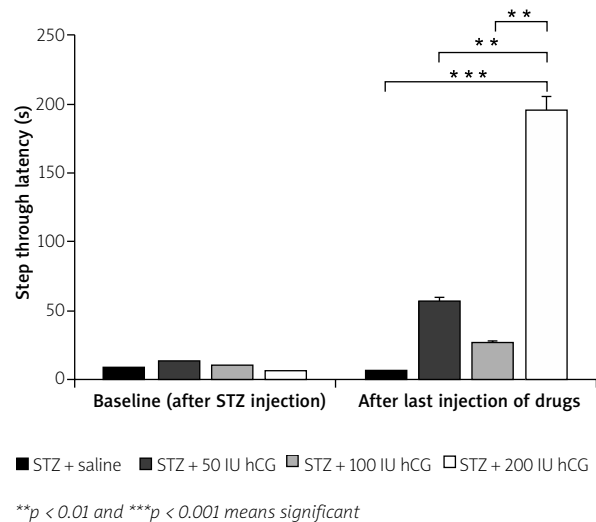


Fig. 1. The step-through latency time after treatment with human chorionic gonadotropin (hCG). Columns represent mean \pm SD.

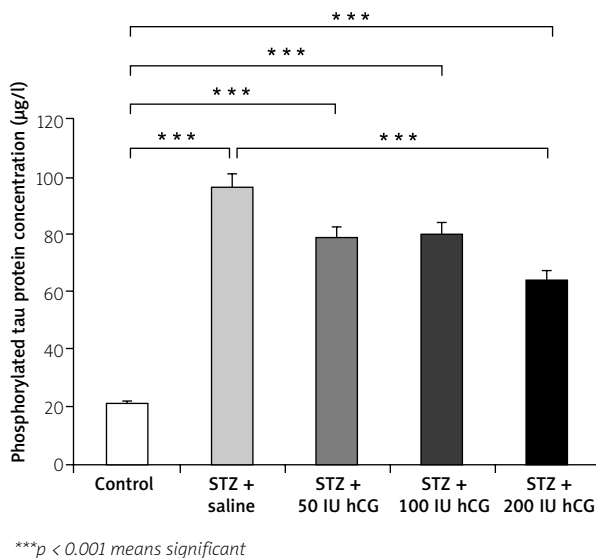


Fig. 2. Effect of human chorionic gonadotropin (hCG) on the phosphorylated tau protein concentration in the hippocampus. Columns represent mean \pm SD.

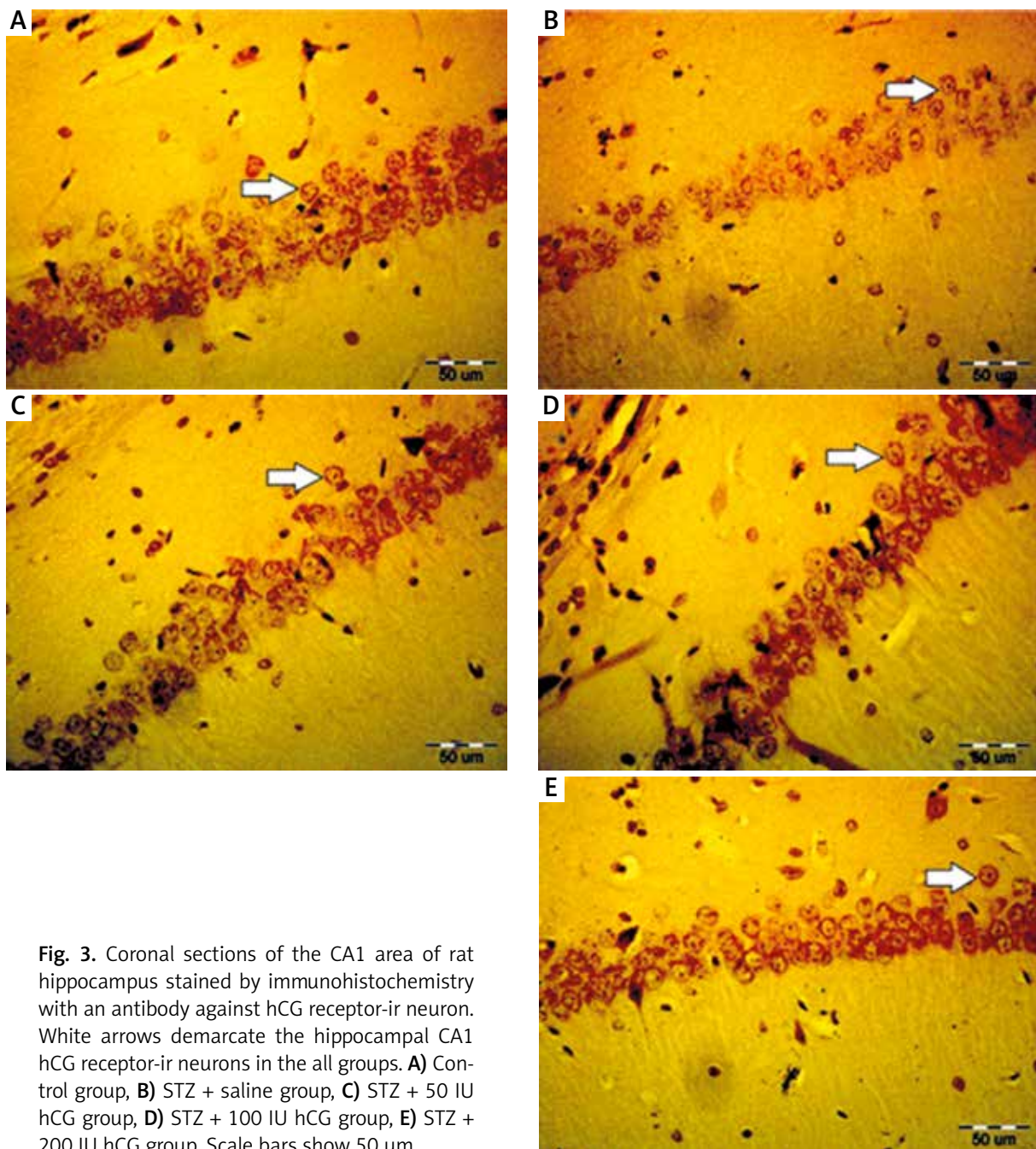


Fig. 3. Coronal sections of the CA1 area of rat hippocampus stained by immunohistochemistry with an antibody against hCG receptor-ir neuron. White arrows demarcate the hippocampal CA1 hCG receptor-ir neurons in the all groups. **A)** Control group, **B)** STZ + saline group, **C)** STZ + 50 IU hCG group, **D)** STZ + 100 IU hCG group, **E)** STZ + 200 IU hCG group. Scale bars show 50 μ m.

was decreased (Figs. 3 and 4). Furthermore, we observed a significant difference among control and STZ-saline groups in the DG area of the hippocampus ($p < 0.05$, Fig. 4). After treatment with hCG, we observed an apparent rise in the number of hCG receptor-ir neurons at CA1 and CA3 of the hippocampus and DG area (Figs. 3 and 4). Our results also showed a significant increase of the hCG receptor-ir neuron numbers in hCG-treated

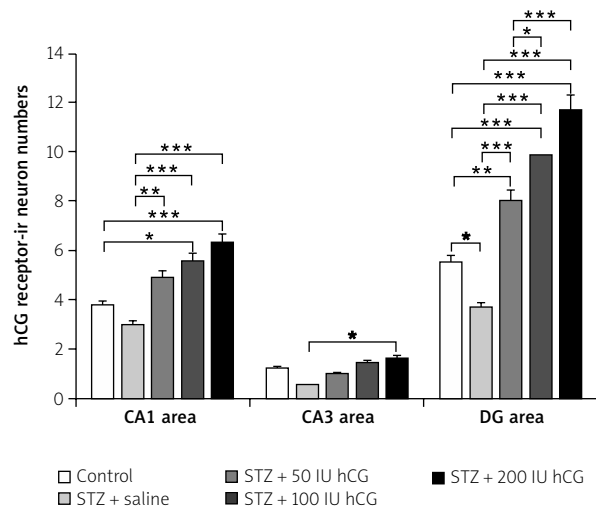
rats with 50, 100, and 200 IU/200 μ l saline doses at hippocampal CA1 and DG areas (Fig. 4). In the CA3 area of the hippocampus the density of hCG receptor-ir neurons was significantly higher in the hCG group (dose of 200 IU/200 μ l saline) than in the STZ-saline group ($p < 0.05$, Fig. 4). In the DG area of the hippocampus, we observed significant differences among hCG-treated rats with different doses of hCG (Fig. 4).

Discussion

We showed that a dose of 200 IU/200 μ l saline hCG can improve inhibitory passive avoidance memory deficiency and decrease hippocampal phosphorylated tau concentration in ICV-STZ injected rats. Furthermore, our data revealed that hCG at a high dose (200 IU/200 μ l saline) increased the number of hCG receptor-ir neurons in the hippocampus of ICV-STZ injected rats.

Streptozotocin injection effects numerous AD-like behavioural and pathological signs, such as memory deficit, increased acetylcholinesterase activity, long-term potentiation, and reduced hippocampal synaptic transmission [1,2,57]. It also caused tau hyperphosphorylation in the brain [26]. As expected, in our study we found that the injection of STZ caused a passive avoidance memory deficit. This result of STZ-induced memory deficit is in agreement with Agrawal *et al.* (2011) [2] and Zamani *et al.* (2011) [64].

After treatment with a high dose of hCG, passive avoidance memory deficit induced by STZ was attenuated. Similarly, Babahajian *et al.* [12] demonstrated that co-administration of vitamin E and hCG improved ischaemia-induced neurodegeneration and passive avoidance memory impairment. In contrast with our findings, Lucaks *et al.* [45] showed that a single high dose of hCG administered to ovariectomised rats has no effect on latency to enter the open field or on working memory in the T-maze, while Berry *et al.* [14] found that single or multiple injections of high hCG doses to ovariectomised rats negated the benefits of oestrogen replacement on working memory in the object location test and reference memory in the Barnes maze. Also, Berry *et al.* [14] reported that hCG treatment to oestradiol-implanted female rats significantly increased soluble A β 40 and A β 42 levels. Furthermore, Barron *et al.* [13] reported that hCG administration induces hyperactivity and anxiety (in open field Maze and Taste Neophobia Task) and working memory dysfunction, without altering reference memory (in Morris Water Maze). In this study, they also found that although treatment with hCG slightly increased Ab40 levels, levels of the longer, more toxic form (Ab42) were unaffected. Burnham *et al.* [23] also found that females that received hCG in the dorsal hippocampus had reduced spatial memory compared to vehicle infusions, showing that hCG/LH can act directly on the hippocampus to affect cognition in female rats. Also, they found



* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ mean significant

Fig. 4. The bar graphs represent the mean density of hCG receptor-ir neurons in control, STZ + saline, STZ + 50 IU hCG, STZ + 100 IU hCG, and STZ + 200 IU hCG groups in the hippocampal CA1, CA3, and DG areas. Columns represent mean \pm SD.

that administration of the LH/CG receptor antagonist, deglycosylated hCG, to the dorsal hippocampus of ovariectomised female rats reversed the deficit in spatial memory. Indeed, this shows that even when oestradiol levels are moderately high, hCG/LH can suppress spatial memory [14]. These studies implicate the gonadotropin hormones in complex and diverse actions in the brain and may have significance for understanding the effects of menopausal hormone changes on cognition and AD risk [13].

Abnormal hyperphosphorylated tau protein can cause NFTs in neuron cell bodies or in other cells of the brain [8,20,22]. In numerous neurodegenerative diseases known as “tauopathies”, these types of intraneuronal tau masses are observed. Alzheimer’s disease, frontotemporal dementia, progressive supranuclear palsy, Parkinson’s disease, and Pick’s disease are some of them [20,22]. In AD, the earliest histopathological alterations with the hallmarks of neurofibrillary masses and amyloid depositions are commonly identified within the medial temporal lobes, including the entorhinal cortex, the parahippocampus, the hippocampus, amygdala, and uncus, and spread throughout the frontal, parietal, and

temporal neocortices and the subcortical regions by the time of a full diagnosis of AD [63].

We found that STZ increased the phosphorylated tau concentration in the hippocampus. Our data are similar with those reported by Deng *et al.* (2009) [29] and Correia *et al.* (2013) [27], showing that STZ injection raises the expression of hyperphosphorylated tau. In tau transgenic mice, hippocampal tauopathy led to hippocampus-dependent learning and memory ability deficiencies [60].

Herein, we observed a decrease in phosphorylated tau concentration in high dose of hCG-treated rats. This result shows that hCG effectually protect the neurons against phosphorylated tau, which could explain the effect of hCG in reversing memory deficits. Most studies endorse a link between phosphorylation of tau and AD behavioural signs and suggest that decreasing tau phosphorylation may reduce cognitive deficits [41].

In the present study, STZ reduced the number of hCG receptor-ir neurons in all subareas of hippocampus and hCG therapy could increase these neurons in the hippocampus. Interestingly, LH receptor expression in glial cells increases with glial proliferation [4], and in primary neuronal cultures, activation of LH receptor with hCG increases the number of neurite-bearing cells [3]. Furthermore, cell viability was increased with treatment by hCG, as observed through increased total protein content and decreased DNA fragmentation [3]. The findings of Babahajian *et al.* indicated that the application of Trolox and hCG at the same time after ischaemia-reperfusion had a neuroprotective effect and improved the neuronal cell survival [11].

Because hCG and LH are similar structurally and functionally [47], LH has been shown to regulate phosphorylation of tau *in vitro* [24] and *in vivo* [40]. Genetic ablation of LH receptor in a bigenic mouse model of AD (APPsw⁺ mice) reduced tau phosphorylation (by ~50%) that was induced by A β precursor protein overexpression in these animals [40]. Luteinising hormone has been revealed to modulate the expression of many genes involved in cytoskeletal organisation [56]. In this respect, it has reported concurrent rises in both LH expression [43] and phosphorylation of tau [31, 42] by rat embryonic neurons during *in vitro* differentiation. Importantly, Palm *et al.* reported that the levels of LH mRNA are reduced in hippocampal formation of AD patients compared to controls,

and in ovariectomized 3 × Tg AD mice LH immunoreactivity in the superior colliculus was reduced [51].

Conclusions

In conclusion, hCG can provide effective protection against memory deficits induced by STZ, and it can inhibit accumulation of tau hyperphosphorylation in all areas of the hippocampus. Furthermore, hCG can increase the hCG receptor-ir neuron numbers in the rats hippocampus after injection of STZ.

Acknowledgments

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Conflicts of interest

The authors report no conflicts of interest.

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