

Chronic Administration of Hesperidin improves working, but not reference memory in Rat Model of Alzheimer's disease

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Short Report

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Abstract

Objective

Despite extensive research on Alzheimer's disease (AD), still there is no appropriate and reliable treatment for it. Therefore, finding novel therapeutic interventions has great health care importance. The aim of the present study was to evaluate the effectiveness of Hesperidin (Hsp) on cognitive decline and BDNF level in the hippocampus in AD.

Results

Streptozocin significantly increased the latency to platform, and reduced BDNF level in the hippocampus ($P < 0.001$) in the acquisition phase of spatial memory. Then, chronic administration of HSP significantly reduced latency to the platform, and increased the total time spent (TTS) in target quadrant in the healthy control group ($p < 0.05$). No significant changes in hippocampal BDNF and TTS was detected in AD animals receiving HSP in probe test. However, HSP successfully reduced latency to platform in the acquisition part ($p < 0.05$).

Conclusion

Our data suggest that chronic administration of hesperidin significantly improves both working and reference memories in the control healthy subjects, while facilitates only working, but not reference memory in AD groups.

Highlights

- I.C.V. injection of STZ significantly decreased BDNF levels in the hippocampus.
- Hsp significantly improved both working and reference memories in the control healthy group.
- Hsp significantly changed the hippocampal level of BDNF in the control group.
- Hsp significantly improved working, but not reference memory in the AD model, with no change in

Hippocampal BDNF.

Introduction

Alzheimer's disease is characterized by memory loss, extracellular accumulation of amyloid β peptides and intraneuronal accumulation of the hyperphosphorylated tau in the brain (1,2). Beside these abnormal proteins, neuroinflammation and oxidative stress represent another hallmark of AD brains. Despite

significant attempts, still there is no appropriate and reliable treatment for this progressive neurologic disease.

There is no doubt that, AD is associated with aging, and aging is accompanied by elevation in reactive oxygen and nitrogen species [1, 2], mitochondrial dysfunction [3], and cognitive dysfunctions [4]. Therefore, one important strategy to combat with AD, could be rebalancing antioxidants-oxidants in the brain by particular antioxidant components, including natural flavonoids, which represent free radical scavenger activity [5, 6], to inhibit lipid peroxidation [7], and neuroinflammation [8].

Hesperidin (Hsp) (C₁₆H₁₄O₆), with chemical formula of 3', 5, 7-trihydroxy-4'-methoxy flavanone [9], is a natural bioflavonoid compound, with active medicinal properties found in citrus fruits such as lemons, oranges, and grapefruits. There is no doubt on citrus fruits benefits providing a combination of vitamins, minerals, and plant compounds that contribute to overall health. Hesperidin has been reported to remove hydrogen radicals and exert anti-inflammatory, antioxidant, antihypertensive and anti-cancer properties [10].

Thus, in the present study we speculated that Hsp treatment, might improve cognitive functions in animal model of AD. To approach this, we induced AD, by i.c.v injection of streptozocin (STZ), and treated animals by Hsp and finally assessed spatial memory by Morris water maze and the level of BDNF and glutathione in the hippocampus by western blotting and ELISA. The STZ produces pathological and behavioral features of sporadic AD, including oxidative stress, cognitive deficits, insulin resistance and accumulation of amyloid beta in brain [11].

Materials and Methods

Study Population

Animals

Overall, 32 male Wistar rats, aged three months and weight 200–250 g, were obtained from the School of Pharmacy Laboratory Animals breeding house and were transferred and adapted to the new environment in the Learning & memory laboratory. The animals were maintained in groups of four per cage under the controlled environmental conditions (22 ± 2°C; 12-hour light/dark cycle commencing at 7:00 a.m.) with free access to standard pellet diet and tap water. Environmental enrichment consisted of nesting material and PVC pipe tubes. The sample size was determined based on our previous study [12]. Inclusion criteria included having normal appetite, locomotion, and visual activities. Neither of animals showed low visual function, weight loss, or locomotion disturbance in our study.

Ethics statement

All experimental procedures were conducted in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee of Guilan University of Medical Sciences, Rasht, Iran (Approval ID: IR.GUMS.AEC.1403.001). All efforts were made to minimize animals stress and pain by providing daily handling and usage of appropriate anesthesia.

Stereotaxic surgery

Animals were anesthetized with a mixture of 90 mg/kg ketamine (TRITTAU, Germany) and 10 mg/kg of xylazine (SciENCelab, Houston) [20]. They were bilaterally cannulated under deep anesthesia, using a stereotactic apparatus (Stoelting, Chicago, IL, USA) according to the lateral ventricular coordination of anterior-posterior = - 0.8; medio-lateral = 1.6; and dorsoventral = - 3.4 (Paxinos and Watson) [13], and based on our previous protocol for preparing the guidewire cannula and injection with the Hamilton micro syringe pump [14]. After complete recovery, rats were randomly divided into four groups (n = 8 per group): Saline + Saline, Saline + Hsp, STZ+ saline, STZ + Hsp, and underwent the treatments for 21 days. Animals were acclimatized for 5 days, in the experiment room before starting the behavioral tests.

Drug administration

To induce AD model of rats, i.c.v injections of STZ (3 mg/kg, 1.5 mg/5 µl/side; Sigma-Aldrich, St. Louis, MO, USA) on days 1 and 3 following surgery were applied [15]. Then, STZ + Hsp group received a daily injection of Hsp (50 mg/kg) intraperitoneally (i.p), and the control group received the same volume of saline (0.9%) for 21 consecutive days, starting from the day after STZ injection. Dose of Hsp was chosen according to the study carried out by Donato et al [16].

Learning and memory tests

The Morris water maze (MWM) test was used to evaluate the animals' working and reference memory. The protocol included four blocks of 16 trials, and one probe test lasting 90 seconds. The device was a circular water tank (148 cm in diameter and 60 cm in height) with a rectangular platform (10 cm) positioned in the target quadrant, 1.5 cm below the water's surface. The temperature of the water was maintained at 26°C.

To assess the animal's eyesight, a visual test was also conducted after the end of probe test. Total time spent in the target quadrant (TTS), escape latency time to reach the platform, distance traveled, and swimming speed were recorded using a camera and quantified using “Ethovision 11 Noldus” tracking software (Netherlands), as previously described by our group [12, 17]. Parameters of latency and distance to reach the hidden platform, and time spent at target zone were measured digitally by software.

Hippocampus dissection

At the end of the experiment, animals were euthanized by controlled CO₂ inhalation in a sealed chamber individually, then they were decapitated using a rodent guillotine, in order to remove brains. Then, hippocampi were dissected and transferred to cooled cryotubes and moved to a nitrogen-containing flask. Ultimately, they were kept in the freezer at – 80°C for subsequent molecular assessments.

Biochemical analyses

BDNF Assessment

First, the hippocampal tissues were homogenized in a lysis buffer, and the homogenates were centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatants were separated on 15% SDS–PAGE gels and transferred to PVDF membranes using transfer buffer [18]. After that, membranes were blocked with 2% skim milk in TBS-T for 75 min, and incubated for 16–18 h at room temperature with diluted (1:300) primary antibodies against β -actin (Santa Cruz Biotechnology, USA, sc-517582), and BDNF (Abcam, Cambridge, UK, [EPR1292] ab108319). After three washes in TBS-T, membranes were incubated with secondary antibodies [m-IgGk BP-HRP (sc-516,102) and mouse anti-rabbit IgG-HRP (sc-2357), Santa Cruz Biotechnology, USA], diluted 1:1000, for 75 min at room temperature. Following last washing step, membranes were exposed to substrate solution for 1–2 h at room temperature to visualize protein bands [19]. Digital images obtained from a document scanner were analyzed with Image J software to quantify protein levels. β -actin and saline-treated groups served as the loading and experimental controls, respectively.

Glutathione Assessment

Intracellular glutathione (GSH) levels were determined using the DTNB (Ellman's reagent; 5,5'-dithiobis-(2-nitrobenzoic acid)) colorimetric method. Tissue samples were homogenized in mannitol buffer containing sucrose, mannitol, and EDTA to preserve cellular integrity and prevent oxidation of thiol groups. A Tris–ammonium buffer (pH 8.9) was used as the reaction buffer, and trichloroacetic acid (TCA) solution was used for protein precipitation.

For the assay, tissue lysates were mixed with TCA to precipitate proteins and centrifuged to obtain a clear supernatant. An aliquot of the supernatant was then combined with Tris–ammonium buffer (pH 8.9) followed by addition of DTNB reagent. DTNB reacts with free sulfhydryl groups of glutathione to produce the yellow-colored 5-thio-2-nitrobenzoic acid (TNB). After incubation, absorbance was measured using a 96-well microplate reader (Epoch, BioTek, USA), and glutathione levels were calculated from the absorbance values.

Statistical analysis

Normality of data distribution was evaluated using the Kolmogorov–Smirnov test, and then One-way ANOVA with Tukey's post hoc tests were used for statistical analysis. Results were presented as mean $\pm$ standard error, and statistical significance was defined as $P < 0.05$. SPSS version 16 (IBM Corporation, Chicago, IL, USA). GraphPad Prism (Version 10.1.0, Boston, Massachusetts, USA) was used for drawing figures.

Results

Behavioral assessments

Effect of Hesperidin on working memory in MWM

Two-way ANOVA analysis demonstrated significant changes between groups ($F(3, 512) = 18.30, p < 0.0001$), and blocks ($F(3, 512) = 17.70, p < 0.0001$, Fig. 2). However, the interaction between blocks $\times$ groups was not statistically significant ($F(9, 512) = 0.3125, P = 0.9709$).

STZ + Sal group showed significantly higher latency in all blocks compared with Sal + Sal ($p < 0.05$).

Administration of Hsp for 21 days to the STZ-receiving group, led to a significant decrease in latency time in blocks 2 and 4 ($p = 0.001$, Fig. 2). This indicates that Hsp improved working memory assessed by MWM.

Figure 2

Effect of Hesperidin on reference memory in MWM

Figure 3a demonstrates total time spent in the target quadrant (TTS) in the probe test of MWM, and Fig. 3b represents the related heat maps recorded by Ethovision. There was a significant difference between groups ($F(3, 29) = 5.762, P = 0.003$). The STZ group showed a significant reduction in TTS compared with the saline group ($p = 0.04$). However, AD rats receiving Hsp showed no significant difference compared to the STZ + saline group (Fig. 3a, b). A significant difference was found between Sal + Hsp and Sal + Sal ($p = 0.003$).

A significant difference in latency to platform was observed between groups ($F(3, 27) = 14.26, P < 0.0001$). One-way ANOVA test followed by Tukey post-hoc analysis, showed an increase in the average latency by the STZ group to reach the platform in comparison with the control group ($p = 0.0001$).

HSP + STZ, demonstrated lower latency to reach the platform compared with STZ + Sal, $P < 0.0001$.

Figure 4a, represents the swimming velocity in the MWM among groups during the reference memory. According to the One-way ANOVA results, there was no significant difference in the average swimming speed among groups ($p = 0.09$). The results of the visual test and velocity confirmed that, animals had normal locomotion and visual capacity throughout the study; thus, all performances in MWM were

related to memory tracking. Visual test was performed by placing individual animal in pool with apparent platform and recording the track to the platform.

Figure 3, 3a

Figure 4

Effects of Hesperidin on BDNF protein level in the rat hippocampus

Western blotting assay was used to measure the level of BDNF in the hippocampi. Figure 5a,b demonstrates the ratio of the mean hippocampal level of BDNF to β -actin, in each group of the study. STZ + Sal had a significantly lower level of BDNF in comparison with the Sal + Sal group ($p = 0.001$). However, administration of Hsp to AD models caused an insignificant increase in BDNF levels ($p = 0.67$).

Figure 5

Effect of Hesperidin on intracellular glutathione level in the rat hippocampus

One-way ANOVA revealed a significant difference in the glutathione levels among the experimental groups ($F(3, 28) = 20.09$, $P < 0.0001$). Post hoc analysis using Tukey's multiple comparisons test showed that STZ administration significantly reduced hippocampal GSH levels compared with the Sal + Sal control group ($p = 0.003$, Fig. 6), and treatment with Hesperidin significantly restored intracellular GSH levels in STZ-treated animals ($P = 0.002$).

Figure 6

Discussion

In the present study, we investigated the effect of Hsp, on STZ-induced memory impairment, and expression of BDNF in the hippocampus. The obtained data clearly demonstrated that 1) I.C.V. injection of STZ, significantly impaired spatial memory and decreased glutathione and BDNF levels in the hippocampus. 2) chronic i.p injection of Hsp in control healthy animals, caused significant improvement in both working and reference memories, while it failed to improve reference memory in the sporadic model of AD. 3) Significant increase in BDNF was found in the group of control, but not in AD animals. 4) Hsp significantly increased the glutathione levels in all groups.

The finding of significant improvement in both working and reference memories in the control healthy, but not AD group receiving Hsp, indicates the facilitatory effects of Hsp on both of these spatial memories in healthy animal, but only improved working memory in AD group. As it has been previously established, the AD model induced by STZ, manifests several complex pathological disturbances, including elevated hyperphosphorylated Tau [20], and amyloid beta [21], increased reactive oxygen

species [22], glucose metabolism disturbance [23], hippocampal choline acetyltransferase reduction, and memory dysfunction [24], which is unlikely could be alleviated by three weeks of Hsp administration. Although, we found improvement in working memory in AD model of animals, but Hsp treatment failed to restore reference memory. This negative result, partly is in line with the study of Wang et al., on the novel object recognition test in a mouse model of AD [25], and also Paneer Selvam et al. on spatial memory in Sprague–Dawley rats after treatment with Hsp [26].

In contrast, beneficial cognitive effects of prolonged Hsp treatment in the model AD model of 5xFAD mice has been reported [27]. Differences in the experiment design in our study, compared with their reports, including AD model (genetic vs. toxin-based), and duration of treatment, likely stand as reasons for different outcomes.

To explain why Hsp improved working, but not reference memory, it should be noticed that, the mechanisms underlying working memory are different from those of reference memory. For instance, Working memory involves short-term synaptic plasticity and rapid modulation of NMDA/AMPA receptor signaling [28], and possibly, HSP may affect neurotransmitters, including glutamate, dopamine, and acetylcholine, and consequently enhances short term memory [29].

In addition, working memory depends on hippocampal– prefrontal cortex (PFC) interactions, early- long-term potentiation (E- LTP), and catecholamines [30], while, reference memory relies strongly on CA1 place cells of the hippocampus [31] late -LTP, cholinergic signaling [32], and neurotrophic factors, particularly BDNF elevation [33].

According to our study, Hsp-induced antioxidant activity, as assessed by glutathione. Glutathione is responsible for maintaining the homeostasis of cells, through detoxification mechanism [34], and reducing the pro-inflammatory factors of TNF- α , IL-6 and nitrite ions [35].

Besides, Hsp is capable to enhance cerebral blood flow [36], and considering high metabolic demands of PFC during working memory tasks, this region, may benefit more than other areas like hippocampus.

Moreover, Hsp can upregulate brain-derived neurotrophic factor (BDNF) expression, but regional differences in BDNF induction may be critical. Lack of BDNF elevation in the hippocampus supports the insignificant effect of Hsp on reference memory. Future work including region-specific assays, measuring of mature vs proBDNF, and TrkB phosphorylation, will clarify the exact pattern of flavonoid - induced BDNF signaling pathways.

Meanwhile, the potent antioxidant and anti-inflammatory properties of flavonoids could provide selective benefits of PFC circuitry for working memory compared with hippocampal reference memory related mechanisms.

Taken all together, our data in agreement with some robust studies using other flavonoids such as quercetin [7] resveratrol [37], and also *Citrus* peel extract in subjects with cognitive decline [38], confirms

that restoring antioxidant capacity, solely, is not sufficient to normalize complex cognitive deficits in a multifactorial neurodegenerative context such as AD.

For future studies it is suggested to evaluate the other molecular pathways, and also the brain regions after more longer duration treatment and in a dose response, and time course study.

In conclusion, the present results indicate that, Hsp facilitates measurable short and long-term synaptic plasticity in the healthy subjects through increasing antioxidant glutathione and neurotrophic BDNF level in the hippocampus, but fails to induce long-term changes required for reference memory in AD status.

Limitations

There are some limitations for our study which should be noticed in the future studies:

- **Timing:** One of the limitations of this study, is probably short duration of Hsp treatment in a complex AD model, which is recommended to be extended at-least to two months in rat.
- 1. **Dosage:** Other limitation might be the dose used here (50 mg/Kg), may not be enough for synaptic changes, triggering neurotrophins, and cognitive functions. However, it should be cautious, that supraphysiologic doses, is not achievable in humans because of side effects, and stand as translational barriers.
- **Bioavailability and brain penetration:** Many flavonoids, including Hsp, are poorly absorbed, rapidly metabolized, or fail to cross the blood–brain barrier efficiently, and only a fraction reaches the brain in active form. Therefore, for future studies nano formulation of Hsp, might be more logic to reach an appropriate delivery of flavanol to the brain regions.

• Different brain regions

Other limitation of this study is lack of data from various region-specific BDNF level, including the prefrontal cortex, which could help to clarify its potential role in reference memory. Besides, it is worth to measure both pro and mature BDNF ratio.

Declarations

Ethics approval and consent to participate

The study was approved by the Institutional Animal Ethics Committee of Guilan University of Medical Sciences (Approval ID: IR.GUMS.AEC.1403.001). Since the study was based animal research, not human samples, informed consent was not applicable.

Consent to publication

Not applicable.

Institutional review board statement

NA.

Competing interests

The authors declare no competing interests.

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Author Contribution

PB designed this study, wrote, and revised the paper for important intellectual content. HN performed experiments and statistical analysis and wrote the first draft. MSh performed molecular assessment, read and revised the manuscript. EZ conveyed cellular glutathione assay. All authors approved the final version of the paper.

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Data availability

All data is available in the manuscript. The datasets obtained during this study are available from the corresponding author upon reasonable request.

Abbreviations

AD: Alzheimer's disease, A β : amyloid-beta, Hsp: Hesperidin, BDNF: brain-derived neurotrophic factor, STZ: streptozotocin, MWM: Morris water maze, TTS: total time spent in target quadrant, ICV: intracerebroventricular; NFT: neurofibrillary tangles, Sal: Saline

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Figures

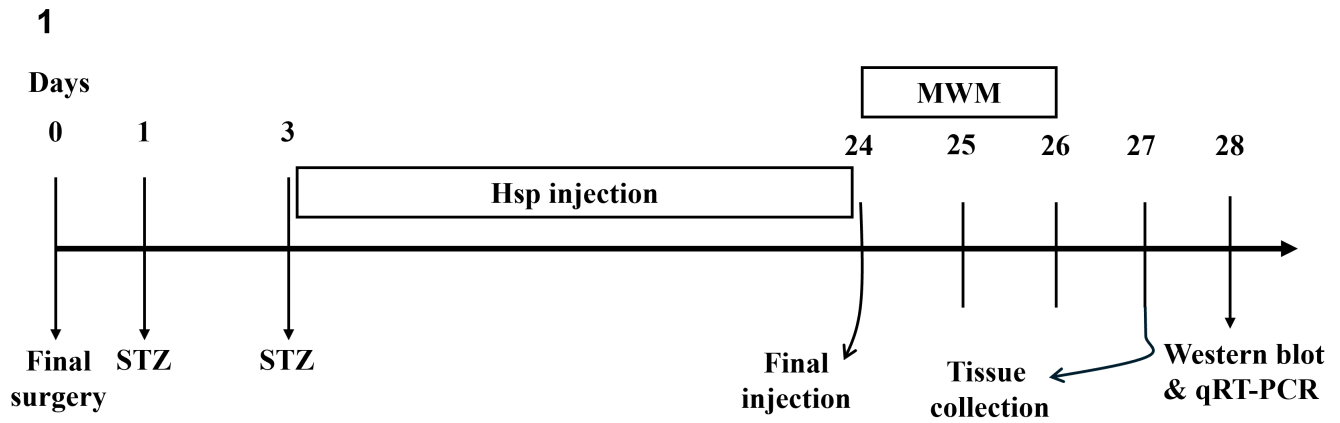


Figure 1

Figure1. Experimental procedure of intra-hippocampal injection of Streptozocin, treatment with Hsp, and biochemical assessments.

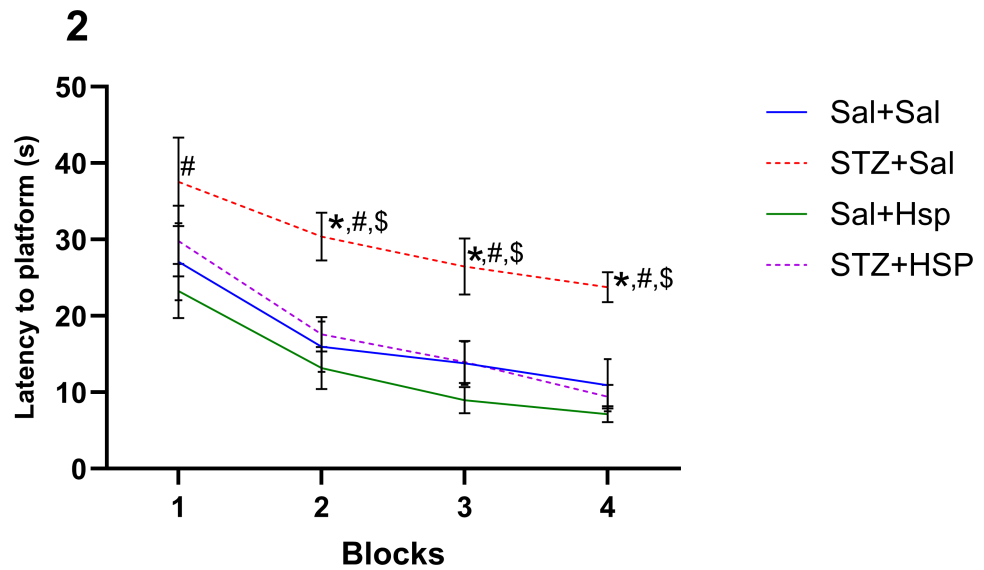


Figure 2

Figure 2. Comparison of the latency to reach the hidden platform in the working memory stage of the MWM. . * $p < 0.05$ in comparison with Sal+Sal, # $p < 0.05$ in comparison with Sal+Hsp, \$ $p < 0.05$ in comparison with STZ+Hsp. (n=8)

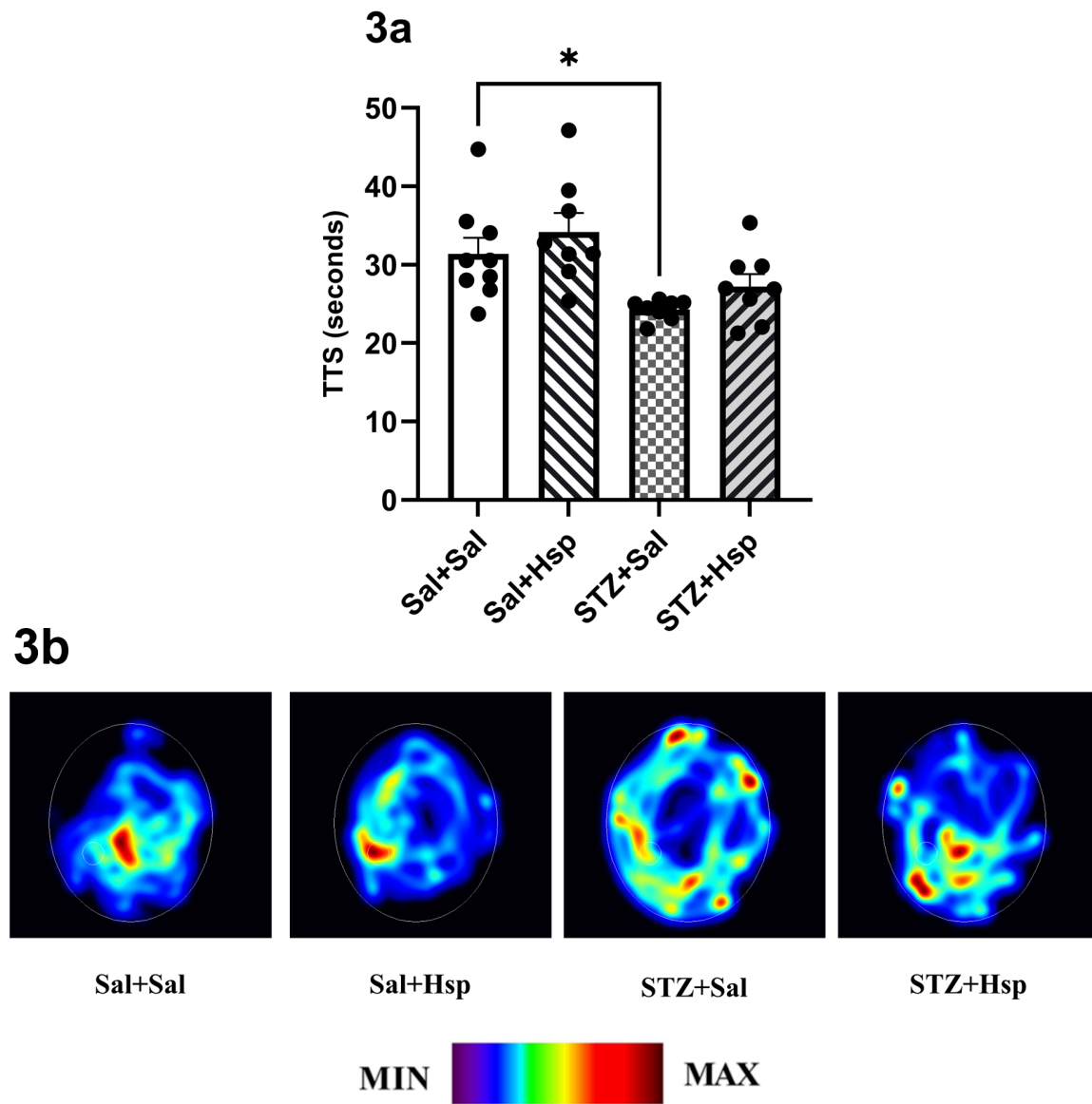


Figure 3

Figure 3 a, b) Comparison of the average total time spent in the goal quadrant of each group during the reference memory.

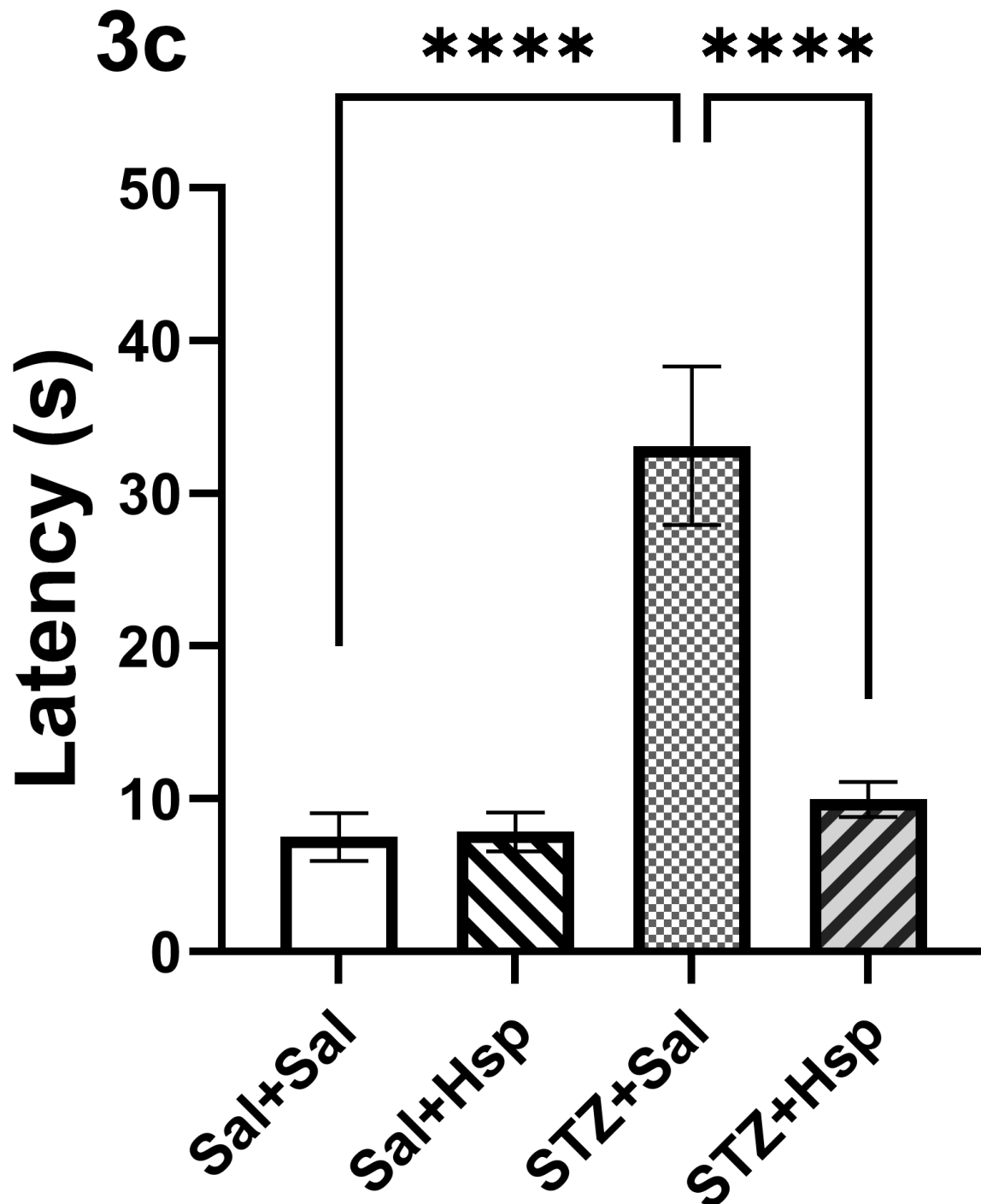


Figure 4

Figure 3 c) Comparing the average time elapsed by each group to reach the target quadrant in the reference memory. ((* $p < 0.05$, ** $P < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ $n=8$) b) Heat maps showing the representative MWM trajectory tracking of groups during the reference, increasing color intensity (arbitrary scale) shows increased time spent.

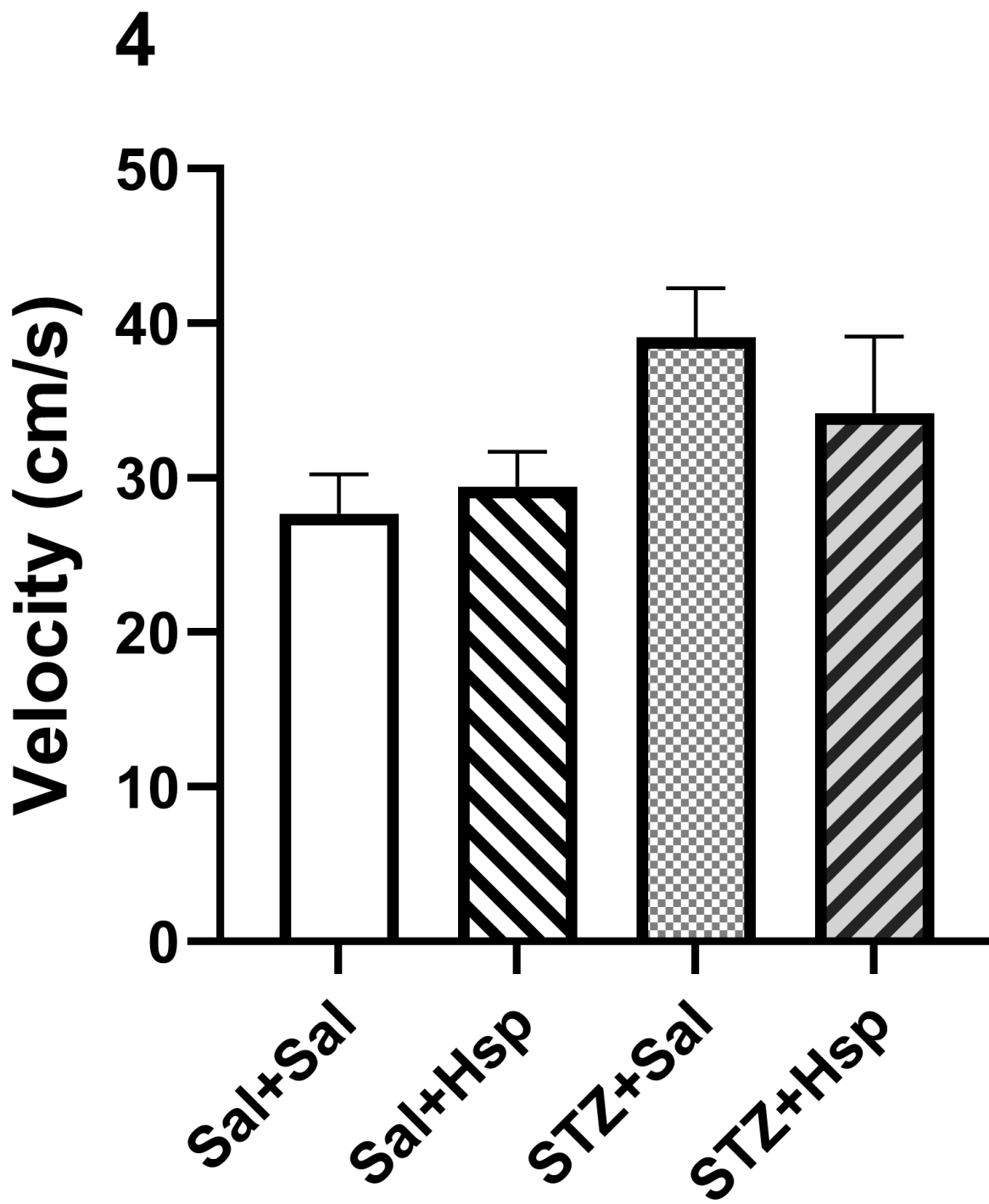


Figure 5

Figure 4. Comparing average swimming velocities among the studied groups in the probe phase.

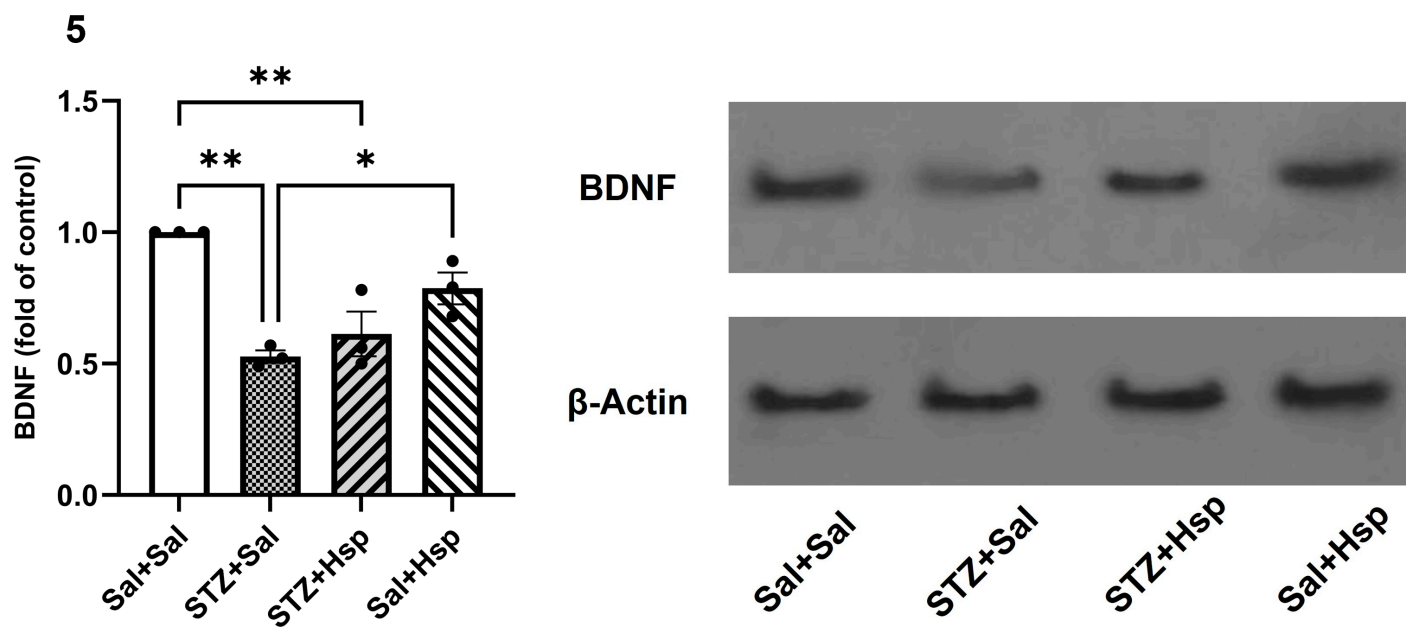


Figure 6

Figure 5a,b. Effect of intraperitoneal injection of Hsp on the expression level of hippocampal BDNF. Data are expressed as mean $\pm$ SEM (n=3 per group) (*p<0.05, **p<0.01).

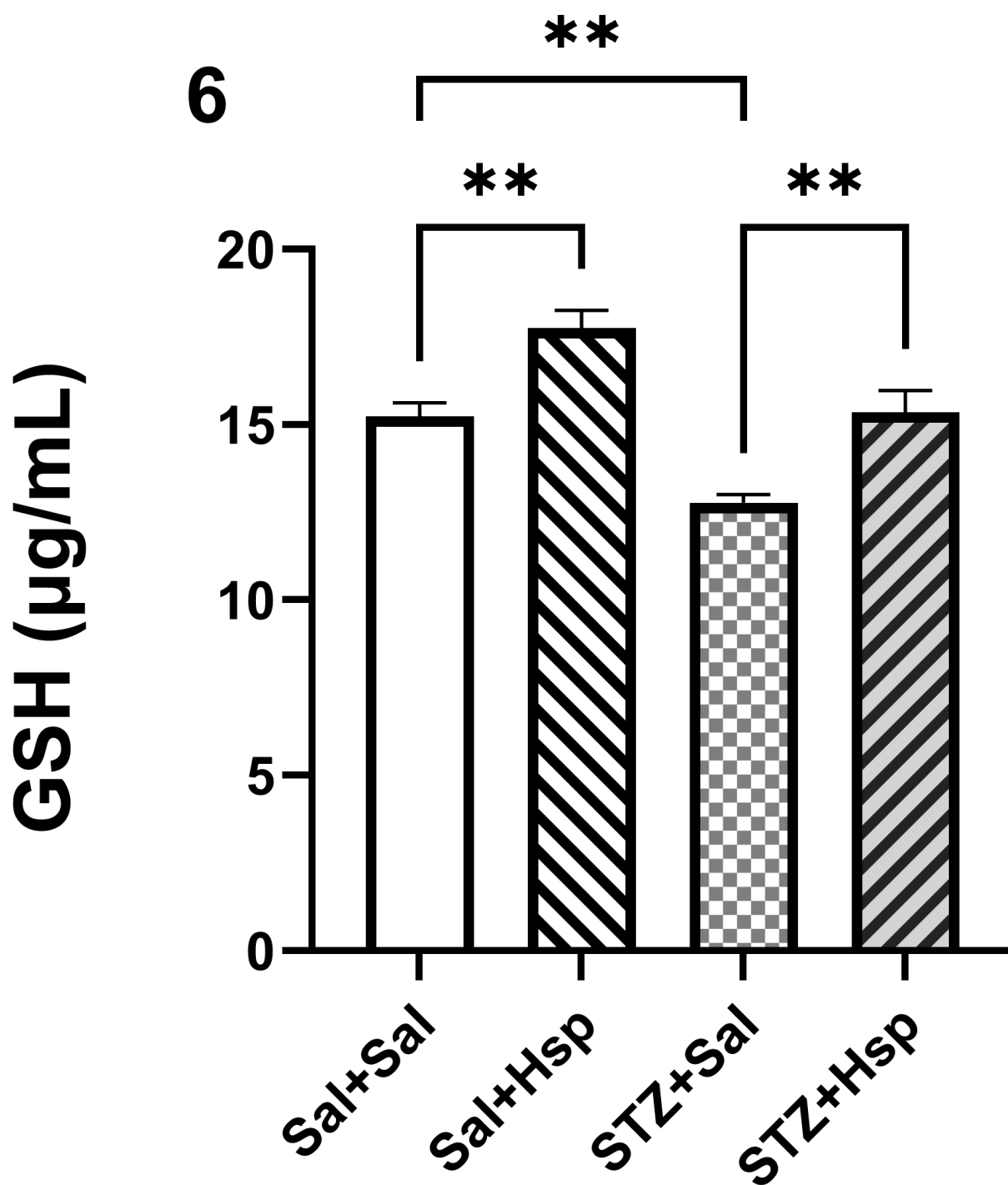


Figure 7

Figure 6. Intracellular glutathione levels measured using the DTNB (Ellman) colorimetric assay. Data are presented as mean $\pm$ SEM ($n = 8$). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. (** $p < 0.01$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [BDNFG1.N1F.jpg](#)
- [BDNFG2.N2F.jpg](#)
- [BDNFG3.N3F.jpg](#)
- [BactinG1.N1F1.jpg](#)
- [BactinG2.N2F1.jpg](#)
- [BactinG3.N3F1.jpg](#)