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Research report

# Neuroprotective effects of estrogen treatment on ischemia-induced behavioural deficits in ovariectomized gerbils at different ages

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ABSTRACT

Although much is known about the protective effect of acute estrogen therapy in cerebral ischemia, relatively little is known about its effect on functional outcome at different ages. The impact of age is, however, important on the efficacy of steroids in the central nervous system. We investigated whether a single dose of estradiol pre-treatment would be neuroprotective in young (4 months), middle-aged (9 months) and old (18 months) female gerbils following 10 min global brain ischemia. Apoptotic and necrotic cells were labelled and quantified in the affected hippocampus; exploratory activity, attention and memory functions were tested using open field, spontaneous alternation, novel object recognition and hole-board test. Age effect and treatment effect were analysed. High single dose (4 mg/kg b.w.) of estradiol pre-treatment exposed a marked neuroprotective effect against hippocampal cell loss in all age groups. In behavioural tests, however, age-related differences could be observed. In middle-aged and old animals the worsening in memory function following ischemia was more prominent compared to that in the young ones. In the Y-maze and the novel object recognition tests the middle-aged, in the hole-board test (investigating working memory and total time) the old gerbils had the worst functional outcome. Only reference memory in hole-board test did not change by age. Estrogen improved memory performances in all the tests at every age. We can conclude that age of experimental animals is a factor worsening the outcome following brain ischemia. A single-dose estrogen therapy prevents the lesion-induced behavioural dysfunctions and the hippocampal cell loss.



## 1. Introduction

In the clinical practice usually the elderly subjects are affected by ischemic stroke, while preclinical animal data are collected mostly from different stroke models of young animals. Very few systemic studies do exist on ageing animals and their behavioural consequences in response to brain ischemia.

The ageing brain reacts in a different manner to ischemic brain injury compared to young's, as there are age-related structural and functional changes in the brain [3,6,18,24]. Increased neuronal loss [2,10,32,33,35], increased glial scar formation [33], early macrophage activation [33], decreased plasticity of the cerebrovascular wall [34] together with worst functional recovery [2,33,56] are associated with ageing.

Local neurosteroid production in the hippocampus together with the circulating gonadal hormones plays an essential role in brain functions, such as in synaptic plasticity and memory formation [14,36]. Changes in synaptic density during estrous cycle and following OVX are attributed to estradiol [36]. Exogenous estradiol also increases the synaptic density even following one single dose of estradiol [38], and enhances hippocampus dependent learning by modifying GABA-ergic and cholinergic release and receptors [11,17].

In cerebral ischemia estrogen moderates blood–brain barrier dysfunction [26], reduces excitotoxicity [7,22,52], and inflammation [22,44,46], functioning as antioxidant [7], increases cerebral blood flow [22,23,31], and increases the expression of cell-survival mediators (such as bcl-2) together with inhibiting death-promoting cascades (p75, caspase-3, caspase-12, TNF-α, IL-1 and IL-6, etc.) [28]. Estrogen therapy can lead to enhanced recovery following ischemic events of brain tissue in young animals [22,28,41,46] based on mainly chronic assessment that is a model of

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perimenopausal estrogen supplementation. The efficacy of a single-dose estrogen administration after experimental brain ischemia is not well documented. Further unsolved question is the estrogen effect on cerebral ischemia at different ages and its impact on functional outcome, as the ageing brain can react differently to any drug that had been protective in the young [12,16,47,49]. In case of estradiol therapy, as estrogen receptor expression is altered with reproductive senescence, its efficacy is also doubtful [22,27,43].

The aim of our study was to compare therapeutic effect of a single-dose estradiol (E2) pre-treatment on different behavioural performances after brain ischemia in young, middle-aged and elderly female gerbils. In our study we used an accepted method by Chen et al. [4]. We chose to examine a single-dose therapy as it is clinically more relevant than chronic treatment – in preventing or threatening global cerebral hypoperfusion and ischemia, such as related to cardiac and vascular surgery, etc.

## 2. Materials and methods

### 2.1. Animals

Female Mongolian gerbils of 4 (4 mo, young), 9 (9 mo, middle-aged) and 18 (18 mo, aged) months of age were used for these experiments. Animals were housed in an air-conditioned room at 22 ± 1 °C with a 12 h light/dark cycle (light on at 07.00 a.m.). Food and tap water were available ad libitum. In all animal groups bilateral ovariectomy was performed to eliminate endogenous estradiol production. For the experiment ovariectomized (OVX) female gerbils were randomly assigned into three experimental groups, 15 animals per group: 1. control animals (sham operation (carotid exposure without clipping the arteries) + vehicle treatment; abbreviated as sham-veh or sham), 2. ischemia affected animals (ischemia + vehicle; abbreviated as isch-veh or ischemic), 3. estrogen-treated gerbils (ischemia + estradiol treatment, abbreviated as isch-E2 or estrogen treated). We used five animals from each group for histological examinations, their brain samples were collected on post-operative day 4. The rest of gerbils from each group were designed for behavioural tests starting on post-operative day 7. General locomotor activity and hyperactivity were evaluated in novelty-induced exploration and in spontaneous alternation tests, effects of ageing and hippocampal damage were estimated by spontaneous alternation and novel object recognition tests. Both memory functions (working and reference memories) as well as spatial learning capacity of hippocampal and cortical structures were observed in hole-board spatial learning task. On post-operative day 12 these gerbils were sacrificed. All the experimental procedures carried out on animals had been approved by the Animal Examination Ethical Council of the Animal Protection Advisory Board at the Semmelweis University, Budapest.

### 2.2. Ovariectomy (OVX) and transient bilateral carotid clipping

Animals were anaesthetised initially with 4% halothane in a 30% $O_2$/70% $N_2O$ mixture and the anaesthesia was maintained during the course of experiment with 1.5–2.5% halothane breathing spontaneously via facemask. Bilateral OVX surgery was carried out through two small lateral abdominal incisions and both right and left horns of the uterus were exposed. A ligature of non-absorbable silk filament was placed around the two horns of the uterus and both ovaries to avoid bleeding, then ovaries were carefully removed, the uterus remained intact. Two weeks later using the same anaesthesia, brain ischemia was induced as follows. The common carotid arteries were exposed through a cervical incision at the ventral midline and were carefully separated from the surrounding tissue and vagal nerves. Both arteries were clipped with atraumatic aneurysm clips (Codman, Johnson and Johnson, Le Locle, NE, Switzerland) for 10 min. Following the occlusion clips were removed to restore blood flow. The same surgical procedure was performed on the sham-operated group, but without the actual ligation. Thirty minutes before the surgery sham-operated and untreated ischemic animals were injected intraperitoneally with vehicle solution (50% alcohol and 50% normal saline) in a dose of 0.4 ml/100 g body weight, while estradiol treated ischemic animals subjected to ischemia were injected by 0.4 mg/100 g body weight 17β-estradiol (4 mg/kg body weight, Sigma Chemical Co. St Louis, MO, USA) dissolved in the vehicle solution (0.1% estradiol solution).

### 2.3. Brain histology, TUNEL and caspase-3 double labelling

On the 4th post-operative day animals (five from each group) were sacrificed (decapitation was performed under deep Halothane/$O_2$/$N_2O$ anaesthesia) and brains were removed and immersion fixed in buffered paraformaldehyde (in 10% buffered paraformaldehyde for 2 days and in 4% buffered paraformaldehyde for another 5 days) then embedded into paraffin. From the dorsal hippocampus region (starting at the level −2.2 mm to bregma) three paralleled coronal 10-μm-thick sections were collected 0.1 mm from each other. The apoptotic and necrotic cells were labelled by TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labelling) and caspase-3 immunostaining [42,48,51].

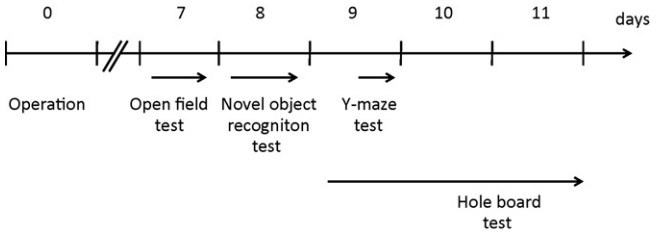


**Fig. 1.** Time line of behavioural tests.

Briefly, TUNEL reaction-mixture (In Situ Cell Death Detection Kit, Roche, Germany; 60 min) was used for immunostaining according to the manufacturer's protocol. After rinsing with phosphate buffered saline (PBS), sections were incubated in caspase-3 primary antibody solution (diluted 1:100, RnD Systems, Germany; 60 min) followed by rinsing with phosphate buffered saline and incubation with fluorescence conjugated goat anti-rabbit secondary antibody (diluted 1:100, Alexa 568, Molecular Probes; 40 min).

For quantitative analysis of apoptotic and necrotic cells, randomly selected non-overlapping areas (0.15 $mm^2$) of the hippocampal CA1 and CA2 zones were selected, TUNEL and TUNEL–caspase-3 double-labelled cells were counted using 60× objective, and an average number was calculated based on all sections in each individual. We took five hippocampal images within each brain hemispheres for a total of 10 images per coronal slice using three parallel sections from each animal. The TUNEL and caspase-3 positive cells were counted automatically with Image J 1.37 software (NIH, USA). Data were analysed blind to exclude the operator bias. All images were taken with the same settings of confocal microscopy (Bio-Rad MRC 1024 confocal system, Bio-Rad Corp., Hertfordshire, England on a Nikon Optiphot inverted microscope, Donsanto Corp., Nattick, Massachusetts, USA).

### 2.4. Behavioural tests

All animals were previously habituated to experimental manipulations by 1 week long daily handling before behavioural tests. For age-related behavioural differences gerbils were tested for general and exploratory activity, attention, short- and long-term memory functions. The adaptation of these behavioural tests to gerbils was described previously [51]. Briefly, novelty-induced exploration was examined on post-operative day 7, followed by novel object recognition on day 8, and by spontaneous alternation on day 9. Accommodation to the hole-board spatial learning task was started on day 8, while the spatial learning test itself was carried out on days 9–11. Animals were food deprived from post-operative day 8 (after the novel object recognition test) to the 11th post-operative day, receiving restricted food supply, while their body weights were maintained on the 95–98% of their original weights (Y-maze test was made after the hole-board test, when animals were fed already). In this way the animals were motivated enough to collect the reward, i.e. sunflower seed in the hole-board arena. On post-operative day 12, animals were sacrificed the same way as for brain histology. For the time line of the behavioural tests see Fig. 1.

*Novelty-induced exploratory activity* was carried out in a cylindrical open field (diameter 80 cm) surrounded by a wall of 35 cm high. Animals were placed in the centre of arena and during a 3 min period the following behaviours were recorded: latency time to start exploration (s), horizontal activity (crossing) – number (scores) of lines crossed between sectors in the outer and inner circles were recorded separately, vertical activity (number of rearing) – number and duration of standing up into an upright position, frequency and duration of face washing and body grooming. In addition activity was calculated by the following equation:

$$\text{activity} = \text{crossing inner} + \text{crossing outer} + (\text{number of rearing} \times 2).$$

*Spontaneous alternation* [51] is mainly a hippocampus-dependent behaviour serving for assessing attention toward novelty and working memory. This test was estimated in a black plastic Y-maze with sawdust on the floor. The arms were 50 cm long, 30 cm high and 10 cm wide and converged at 120° angle. Each animal was placed at the centre of the maze and allowed to move freely (3 min). Alternation was defined if the animal entered the arm different from the two previously entered arms; an error was recorded if the animal went back to either of the two arms just previously visited. The percentage of relative alternation was calculated from the ratio of the number of alternations divided by the number of total arm enters – 2. The value was multiplied by 100.

*Novel object recognition* [51] was tested in a habituated open field arena. During the first trial (5 min) two identical objects were placed into the arena and gerbils were allowed to freely explore them. After a 90 min delay the animals were tested for another 5 min. During this second trial one object from the first trial (familiar object) was replaced by a novel object. Frequency (score) and duration (second) of visiting the objects were registered. The total number and duration of visits towards both objects served for general exploratory activity. The percentage of *recognition*

*index* was calculated from the ratio of duration of visits to the novel object *divided by* the duration of visits to novel plus familiar objects and the ratio was multiplied by 100.

*Spatial learning ability* [51] was studied in a hole-board apparatus using food reward as positive motivation. The test apparatus had a rectangular arena with 16 holes (4 × 4 arrays) on the floor. After 3 days accommodation to the test box (30 min every day) gerbils were started to be food-deprived. On the next 2 days a sunflower seed was put into each hole and the animals were placed in the hole-board apparatus 5 times a day until most of the rewards were found. During the next three test days (days 9–11) rewards were placed only into four holes applying a different pattern for each animal, but constant for the same animal. Each gerbil received three trials per day, each trial lasting 5 min or until all four rewards were collected. Average time for collecting rewards was calculated each day. The percentage of *working memory* (WM) was calculated from the ratio of the number of food rewarded visits *divided by* the number of visits and revisits to the baited set of holes. The percentage of *reference memory* (RM) was calculated from the ratio of the number of visits and revisits to the baited set of holes *divided by* the number of visits and revisits to all holes. Results of working and reference memory were multiplied by 100. The means of three trials conducted on each test day were taken for statistical analysis.

### 2.5. Data analysis

*Histopathology*: results are presented as means ± SEMs in different hippocampal cornu ammonis (CA) regions. For statistical analysis a two-way ANOVA with age and treatment as independent factors was used followed by Tukey's post hoc test.

*Behavioural experiments*: comparing treatments at different ages two factorial ANOVA was used followed by Tukey's post hoc test. If the interaction between age effect and treatment effect gave significance with ANOVA, the interaction was separately analysed comparing sham-operated and ischemic animals, as well as comparing ischemic and E2 pre-treated ischemic animals between 4- and 9-month-old and between 4- and 18-month-old ages. Results of ANOVA ($F$ values, degrees of freedom and $p$ values) as well as results of interaction between age effect and treatment effect are indicated in the text. Results of post hoc test comparing two independent groups are indicated on figures as followings: $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001 vs. sham-operated females; $^{\#}p$ <0.05; $^{\#\#}p$ <0.01; $^{\#\#\#}p$ <0.001 vs. ischemic females. In the spontaneous alternation test the difference of relative alternation from chance level (33.3%) and in novel object recognition test the difference of recognition index from chance level (50%) were estimated by Student's $t$-test. For statistical significance $p$ <0.05 was accepted as criterion.

## 3. Results

### 3.1. Histopathology

In all the ischemic groups in hippocampal CA1 and CA2 regions significantly higher number of TUNEL-positive necrotic (CA1: $F$-treatment [2, 36] = 2184.23, $p$ < 0.001; post hoc between sham control and ischemic: $p$ < 0.001; CA2: $F$[2, 36] = 1865.39, post hoc between sham control and ischemic: $p$ < 0.001) and TUNEL–caspase-3 double-labelled apoptotic cells were counted, than in the sham-operated animals (CA1: $F$-treatment [2, 36] = 1414.92, $p$ < 0.001; post hoc between sham control and ischemic: $p$ < 0.001; CA2: $F$[2, 36] = 1074.64, post hoc between sham control and ischemic: $p$ < 0.001). E2 pre-treatment preserved hippocampal cells in all age groups (post hoc between ischemic and isch-E2: $p$ < 0.005 both in CA1 and CA2) (Table 1). Statistically no age effect was observed. A representative picture of the hippocampal CA1 region of an ischemic and an estrogen-treated ischemic animal is shown in Fig. 2.

### 3.2. Behavioural tests

#### 3.2.1. Novelty-induced exploration in open field

Activity in the open field significantly decreased with age (Fig. 3A, two-way ANOVA, overall age effect: $F$[2, 48] = 17. 57, $p$ < 0.001) and the ischemia enhanced activity (treatment effect $F$[2, 48] = 7.01, $p$ < 0.01). Within the main effect of treatment significant differences could be found between sham controls and ischemic females ($p$ < 0.05), likewise E2-treated ischemic and vehicle-treated ischemic groups ($p$ < 0.001). There were no significant interactions between the age effect and treatment effect. Multiple comparisons with Tukey's post hoc test between two independent groups were

**Table 1**
Number of TUNEL-positive and double-labelled cells in hippocampal CA1 and CA2 areas induced by transient ischemia on OVX gerbils. Means ± SEMs are shown.

| | 4-month old | | | 9-month old | | | 18-month old | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sham operation + vehicle | Ischemia + vehicle | Ischemia + 17β-estradiol | Sham operation + vehicle | Ischemia + vehicle | Ischemia + 17β-estradiol | Sham operation + vehicle | Ischemia + vehicle | Ischemia + 17β-estradiol |
| TUNEL CA1 | 1.2 ± 1.3 | 251.4 ± 34.55$^{**}$ | 3 ± 1.87$^{\#\#}$ | 0.4 ± 1.28 | 259.3 ± 28.63$^{**}$ | 2 ± 1.62$^{\#\#}$ | 0.9 ± 0.31 | 261.7 ± 35.1$^{**}$ | 3 ± 1.43$^{\#\#}$ |
| TUNEL and caspase-3 CA1 | 0.6 ± 0.89 | 181.2 ± 20.6$^{**}$ | 2.3 ± 2.28$^{\#\#}$ | 1.2 ± 0.62 | 179.3 ± 24.3$^{**}$ | 0.7 ± 1.63$^{\#\#}$ | 0.72 ± 0.7 | 178 ± 25.4$^{**}$ | 4.2 ± 2.4$^{\#\#}$ |
| TUNEL CA2 | 0.6 ± 0.89 | 189.1 ± 21.35$^{**}$ | 2.2 ± 2.28$^{\#\#}$ | 0.97 ± 0.4 | 193.7 ± 20.42$^{**}$ | 2.9 ± 0.7$^{\#\#}$ | 1.4 ± 0.83 | 196 ± 23.6$^{**}$ | 3.81 ± 1.3$^{\#\#}$ |
| TUNEL and caspase-3 CA2 | 0.4 ± 0.54 | 148.2 ± 19.13$^{**}$ | 1.2 ± 1.3$^{\#\#}$ | 0.9 ± 0.63 | 158 ± 28.2$^{**}$ | 1.89 ± 0.9$^{\#\#}$ | 1.8 ± 0.53 | 163 ± 27.5$^{**}$ | 2.8 ± 1.1$^{\#\#}$ |

$^{**}$ $p$ < 0.001 vs. age matched sham-operated animals.
$^{\#\#}$ $p$ < 0.001 vs. age matched ischemic animals.

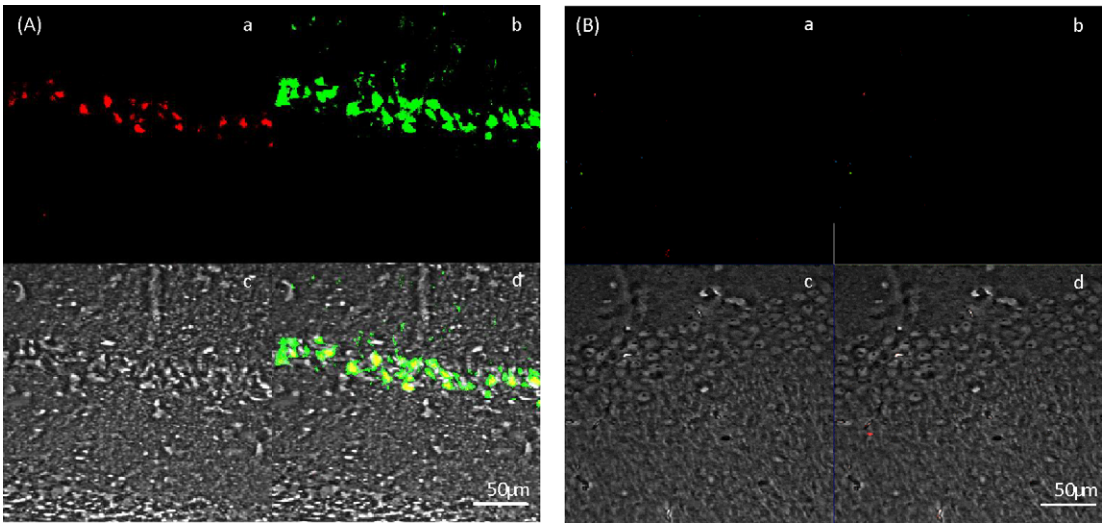


**Fig. 2.** Representative confocal microscopic pictures of hippocampal CA1 regions in an ischemic (Panel A) and an estrogen-treated ischemic (Panel B) animal. Panel A: ischemic animals; caspase-3 labelled cells (a), TUNEL-labelled cells (b), phase contrast (c), merge (d). Panel B: estrogen-treated ischemic animals; caspase-3 labelled cells (a), TUNEL-labelled cells (b), phase contrast (c), merge (d).

significant only at 4 months of age between sham-control and ischemic animals.

Latency to start exploration, frequency and duration of grooming and rearing did not show remarkable changes by age or among any of the examined groups, therefore data are not shown.

### 3.2.2. Spontaneous alternation

Spontaneous alternation did not show remarkable fluctuation in the course of ageing (Fig. 3B). Ischemia evoked a marked decrement in alternation, which was prevented by E2 pre-treatment, i.e. the treatment effect proved to be significant with two-way ANOVA: $F[2, 43] = 43.07$, $p < 0.001$. Furthermore, there was a significant interaction between the age effect and treatment effect ($F[4, 43] = 4.21$, $p < 0.01$). In details, ischemia disturbed the performance in all ages ($F[2, 34] = 2.47$, $p = 0.098$). Assessing age-related differences in the effect of ischemia showed a difference only between 9 mo vs. 4 mo old animals ($F[1, 22] = 7.02$, $p < 0.05$). E2 was most effective also in 9 mo old animals, i.e. interaction between 4 and 9 mo was significant: $F[1, 18] = 23.76$, $p < 0.001$; and between 9 and 18 mo as well: $F[1, 21] = 7.72$, $p < 0.05$, while no significant interaction could be obtained between 4 and 18 mo old groups. It may be mentioned that each group alternated significantly above the 33% chance level.

### 3.2.3. Novel object recognition

In this test two-way ANOVA revealed a significant age effect: $F[2, 42] = 3.50$, $p < 0.05$ (Fig. 3C), and also a significant treatment effect ($F[2, 42] = 24.53$, $p < 0.001$). Interaction between these two factors did not reach significance ($F[4, 42] = 2.35$, $p = 0.074$), but among the ischemic groups, with and without E2 treatment, there was a significant interaction with age ($F[2, 26] = 4.85$, $p < 0.05$). Statistical analysis obtained between the corresponding two groups by post hoc multiple comparisons with Tukey showed that ischemia itself resulted in a decrement in visiting novel object at 9 mo ($p < 0.05$) and 18 mo of ages ($p < 0.05$), while E2 elicited a significant increase in object recognition compared to age-matched ischemic animals at these ages ($p < 0.01$ at 9 mo, $p < 0.05$ at 18 mo). It should be mentioned that in the 4 mo group there were only tendencies without significant differences. Similarly to the findings in the spontaneous alternation test there were age-dependent E2 effects between 4 mo vs. 9 mo and 9 mo vs. 18 mo old gerbils (comparisons between ischemic and E2 groups, $F[1, 15] = 6.25$, $p < 0.05$ and $F[1, 20] = 4.31$, $p = 0.05$), but no interaction could be found between the 4 mo vs. 18 mo old groups. E2 pre-treatment was more effective at 9 mo than either 4 or 18 mo, but the E2 effect was not different at 18 mo compared to 4 mo. Finally, control and E2-treated animals visited novel object significantly above chance level, but ischemic animals failed to do so in all the three age groups based on the paired *t*-test.

### 3.2.4. Spatial learning in hole-board

Behavioural findings obtained in this learning test are summarized in Fig. 4. Time collecting reward (4A), working memory (WM, 4B) and reference memory (RM, 4C) were evaluated. The time needed for collecting rewards and the working memory performance significantly altered during ageing indicated by a significant effect of age with two-way ANOVA ($F[2, 47] = 13.25$, $p < 0.001$; $F[2, 47] = 4.37$, $p < 0.05$, respectively). No age effect could be found in the reference memory performance. Treatment effect was significant in all measures of hole-board learning (time: $F[2, 47] = 15.02$, $p < 0.001$; WM: $F[2, 47] = 17.23$, $p < 0.001$; RM: $F[2, 47] = 5.36$, $p < 0.01$).

To evaluate possible differences among the three age groups interactions were computed between age and treatment factors: time to collect reward showed borderline significance: $F[4, 47] = 2.42$, $p = 0.062$; WM was significant: $F[4, 47] = 2.61$, $p < 0.05$; and RM was not significant: $F[4, 47] = 0.82$, $p = 0.48$. In case of time spent to collect all seeds (Fig. 4A) only ischemia, not E2 interacted with age, and only between 4 and 18 mo of ages ($F[1, 23] = 5.18$, $p < 0.05$; $F[1, 23] = 3.04$, $p = 0.096$). In the oldest age studied, ischemia was more effective to suppress spatial learning than in the young animals.

Age of gerbils interacted with ischemia treatment in assessing working memory performance. Interaction was significant between ages 4 and 18 mo ($F[1, 23] = 9.07$, $p < 0.01$) and was almost significant between 9 and 18 mo ($F[1, 24] = 3.70$, $p = 0.066$). A significant drop in WM ($p < 0.01$) following ischemia was observed but only in the 18 mo old animals (Fig. 4B). Furthermore, there was a significant interaction between the effect of E2 on ischemia and ages considering 4 and 18 mo animals (comparison between isch-E2 and isch-veh groups: $F[1, 23] = 5.74$, $p < 0.05$) indicating that E2 completely prevented the effect of ischemia in the oldest animals. In contrast, reference memory did not show significant alteration during ageing, and the effect of ischemia and E2 pre-treatment were not significant according to post hoc multiple comparisons (Fig. 4C).

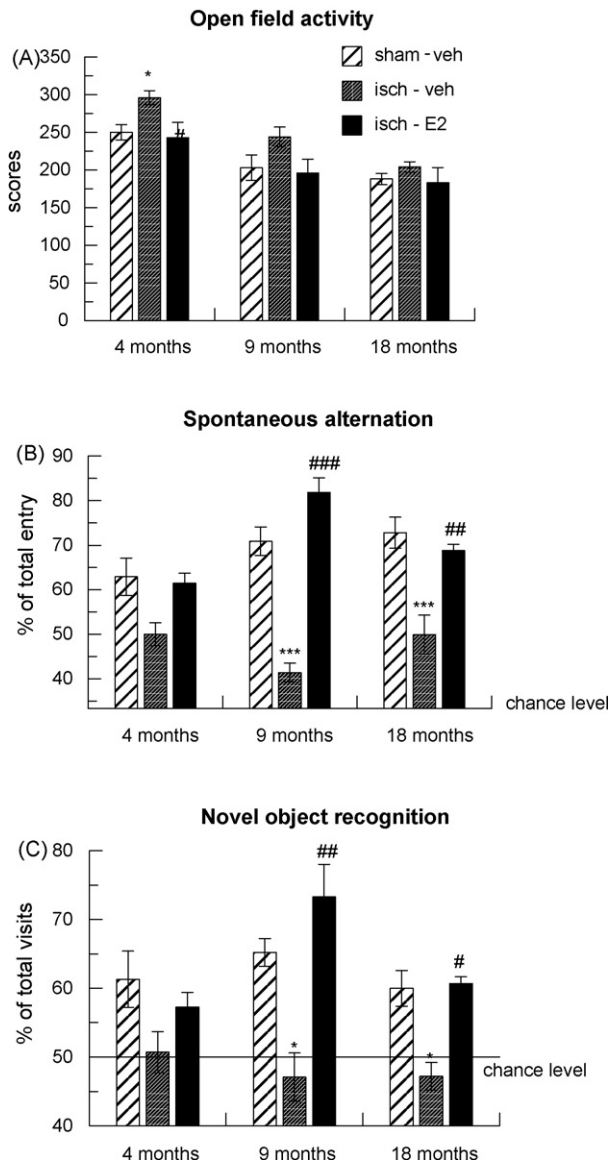


**Fig. 3.** Panel A: effect of transient forebrain ischemia and estrogen pre-treatment on novelty-induced exploration of gerbils at different ages in open field arena. Exploration is calculated from scored value of horizontal and vertical activities according to equation in the text. The activity of 4, 9, and 18 months old animals was measured for 3 min. Figures show mean ± SEM. $^{*}p$ <0.05 vs. sham-operated female using Tukey's post hoc test following two-way ANOVA. Panel B: spontaneous alternation of 4, 9 and 18 months old gerbils following acute forebrain ischemia and estrogen pre-treatment. $^{***}p$ <0.001 vs. sham-operated female; $^{\#\#}p$ <0.01, $^{\#\#\#}p$ <0.001 vs. ischemic female using Tukey's post hoc test following two-way ANOVA. Panel C: recognition index measured during novel object recognition of 4, 9 and 18 months old gerbils following acute forebrain ischemia and estrogen pre-treatment. $^{*}p$ <0.05 vs. sham-operated female; $^{\#}p$ <0.05; $^{\#\#}p$ <0.01 vs. ischemic female using Tukey's post hoc test following two-way ANOVA.

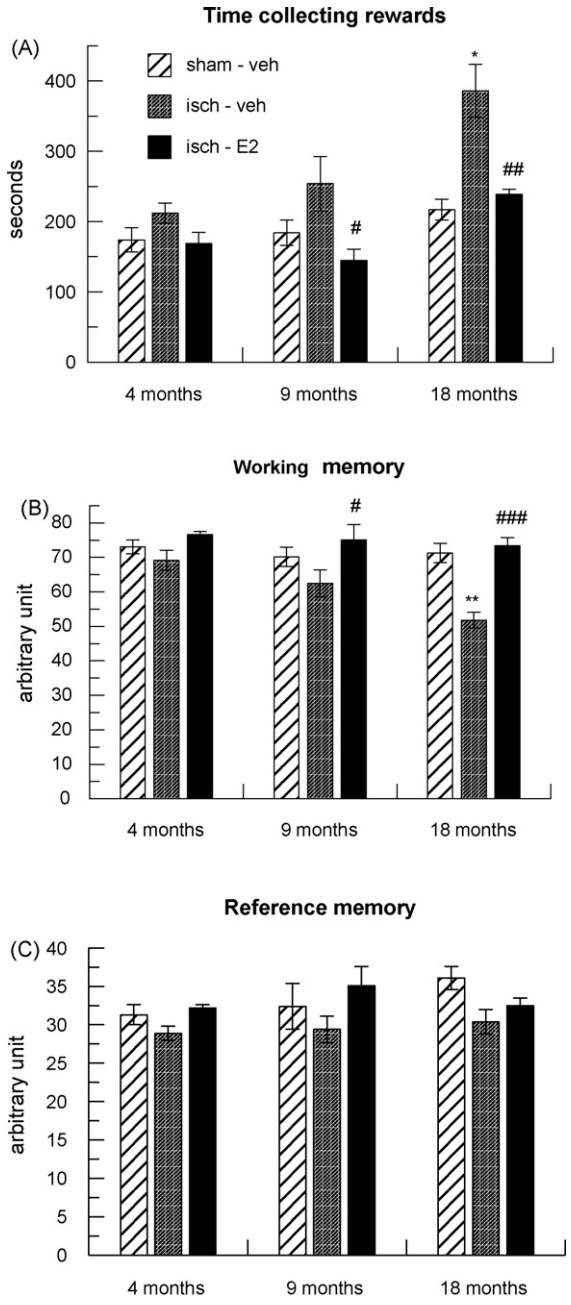


**Fig. 4.** Panel A: the time for collecting rewards in hole-board spatial learning task of 4, 9 and 18 months old gerbils following acute forebrain ischemia and estrogen pre-treatment. Means show the total time calculated during 3 days from the averages of three daily trials. $^{*}p$ <0.05 vs. sham-operated female; $^{\#}p$ <0.05; $^{\#\#}p$ <0.01 vs. ischemic female using Tukey's post hoc test following two-way ANOVA. Panel B: working memory in hole-board spatial learning task of 4, 9 and 18 months old gerbils following acute forebrain ischemia and estrogen pre-treatment. Columns show the average of mean working memory of daily trials. $^{**}p$ <0.01 vs. sham-operated female; $^{\#}p$ <0.05; $^{\#\#\#}p$ <0.001 vs. ischemic female using Tukey's post hoc test following two-way ANOVA. Panel C: reference memory in hole-board spatial learning task of 4, 9 and 18 months old gerbils following acute forebrain ischemia and estrogen pre-treatment. Columns show the average of mean reference memory of daily trials using Tukey's post hoc test following two-way ANOVA.

## 4. Discussion

Our findings indicated that 10 min global cerebral ischemia exerted serious deficits in attention, learning and memory functions as indicated by the significant treatment effects of statistical analysis of spontaneous alternation, novel object recognition and spatial learning task. Attention and memory function proved to be more sensitive to the ischemic insult at the later ages compared to the 4 mo old young age. Spontaneous alternation and novel object recognition decreased markedly at the age of 9 mo, and remained highly impaired at 18 mo in ischemic gerbils. This finding indicates that attention component of learning could be seriously destroyed as early as 9 mo of age, and it did not go through further impairment at later ages in response to the same insult.

Learning performance during spatial memory task showed gradual decrement with ageing except reference memory, which did not show remarkable alteration. These results suggest that memory imprinting is sensitive to ischemic insult, and this sensitivity is

growing gradually with age. Long-term memory proved to be quite resistant to ischemia, as it was indicated by the relatively stable reference memory occurred during spatial learning task, which is in line with previous studies on older gerbils [39]. As it was shown also by our earlier publication, the learning performance of control animals did not show remarkable changes in course of the ages examined in this study.

Following brain injury old animals recover more slowly and less completely compared to the young in most cases [2,33,55,56]. In some of simpler motor asymmetric tests, like corner test or foot-fail test, ageing did not influence the functional outcome after cerebral ischemia [33]. In our tests age-related behavioural changes were not consequent either, as we discussed above. The complexity of the tests together with the anatomical background of different behaviours can influence the functional outcome in every rodent model. Most of our behaviour tests are associated with the function of the hippocampus. The processing mechanisms, however, are complex, as the thalamus, the perirhinal cortex, the medial prefrontal cortex together with the basal forebrain and the basal ganglia are involved in it with various (cholinergic, dopaminergic, glutamatergic and serotoninergic, etc.) synapses [13,30]. The age of the animals also influences the vulnerability of different brain structures [5].

Hippocampal cell loss was not more severe at older age in our work. In previous studies accelerated infarct development and neuronal degeneration, larger infarction volume were usually associated with stroke in older animals [2,10,32,33,35]. In case of smaller infarct size or following later time point in rat or shorter ischemic period (5 min) in gerbils no difference was detected in the number of surviving neurons in middle-aged or old animals compared to their young counterparts [21,25,39]. According to these we can say that in different models of stroke increased age is not always associated with increased cell death.

This is the first study to report that a single dose of E2 (using the same method as Chen et al. [4]) was efficient to prevent ischemic deterioration of learning ability in gerbils at every age in each behavioural test examined. In the affected hippocampus, cells were also preserved in every age group we used. E2 pre-treatment compensated the hyperactivity of our ischemic animals, which was only a mild hyperactivity at 4 mo and gradually disappeared at later ages. Animals pre-treated with E2 showed significant improvement in spontaneous alternation, in novel object recognition, as well as in spatial learning as compared to ischemic gerbils. The effectiveness of E2 pre-treatment, however, showed characteristic changes among different age groups. In spontaneous alternation, less markedly in novel object recognition and in total time for solving spatial learning task, E2 pre-treatment exerted the strongest beneficial effect at the age of 9 mo. The learning performance of ischemic animals pre-treated with E2 was reached, even surpassed those of sham-operated controls. This result suggests that the brain of 9 mo gerbils is especially sensitive to estrogen and probably to estrogen deprivation by OVX. Although there are no data on reproductive ageing in female gerbils and we do not know the estrogen levels of the animals used in the present or any previous studies [6,15], high sensitivity of 9 mo animals is probably due to the decreased or fluctuated endogenous estrogen supply of the brain in intact gerbils, and to those mechanisms, which are dedicated to compensate the climacteric steroid level fluctuations.

In most of the previous studies chronic estradiol pre-treatment was examined and was proved to be beneficial following cerebral ischemia in younger and older animals [1,16,19,28,29,41,46,49,50,53,54] except a gerbil global brain ischemia model using middle-aged animals [12]. The difference can be explained by the differences in stroke model or the differences in intervals from OVX to estradiol treatment in these studies [9,40,45]. To date only a few earlier studies examined the effectiveness of a single-dose estradiol on stroke [4,8,20,37,49] using young animals, and mainly immediate or short-term effect was examined – except in a study of Gulinello et al., who also examined functional outcome following a single-dose intracerebroventricular estrogen administration [20].

In summary, our results suggest that middle-aged and elderly female gerbils had worst functional outcome following cerebral ischemia in Y-maze and in novel object recognition tests that were accompanied with an impaired working memory in the hole-board spatial learning task in these groups. On the other hand no age effect was found in reference memory performance following stroke in our hole-board test, as long-term memory proved to be more resistant to brain injury. A single dose of estradiol, however, was neuroprotective at every age, which means that the ageing brain is also amenable to neuroprotection by estrogen.

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