**Effect of recombinant insulin-like growth factor 2** **(IGF-II) injected into the hippocampus on memory impairment and IGF-II gene expression following hippocampal intracerebral hemorrhage in rats**

Running title: Effect of IGF-II on memory impairment following intracerebral hemorrhage

**Abstract:**

**Background:** Insulin-like growth factor 2 (IGF-II) is a growth factor and an anti-inﬂammatory cytokine that has also pivotal activity on memory. The aim of this study was to investigate the effect of recombinantIGF-II on memory impairment due to intracerebral hemorrhage (ICH). Hence, avoidance and investigation memory, locomotor activity, neurological deficit score and the level of IGF-II gene expression were evaluated.

**Materials and Methods:** To induce ICH, 100 μL of autologous blood was injected into the left hippocampus of male Sprague-Dawley rats. Recombinant IGF-II was injected into damaged hippocampus 30 min after induction of ICH. Then, over two weeks, the neurological deficit score (NDS) and locomotor activity and also passive avoidance and novel object recognition test (NOR) were evaluated. Finally, the level of IGF-II gene expression evaluated by RT-PCR technique.

**Results:** Our results indicated that recombinant IGF-II injection significantly increased step-through latency (STL) (P<0.001) and total time spent in dark box (P<0.01), but no significant difference was seen with novel object exploration time and neurological deficit score. Locomotor activity did not significantly change in any group. The level of IGF-II gene expression following IGF-II injection significantly reduced (P<0.05).

**Conclusion:** The results of this study show that recombinant IGF-II injection can improve avoidance memory but not investigation memory. IGF-II was not effective on locomotor activity. It seems that the reduction of the level of IGF-II gene expression is not related with memory improvement.

**Keywords:** Intracerebral hemorrhage; IGF-II; Learning and Memory; Hippocampus; Rats

**Introduction:**

Memory impairment is a debilitating factor in many diseases such as stroke, trauma, neuro-inflammatory and neurodegeneration diseases; it is also time-consuming and costly in today’s society [1]. In hemorrhagic stroke, blood pressure or trauma causes blood vessel rupture, intracerebral hemorrhage, and brain damage and when this damage is in the hippocampus, it causes disabling memory disorder in patients [2,3]. ICH can damage brain through driven complications of inflammation, upregulated neutrophil infiltration, glial activities, local cytokine imbalance and oxidative stress [3,4]. Among these, the inflammation plays a key role to develop the secondary brain damage and many studies have focused on anti-inflammatory factors involved in the treatment of stroke [5]. In the line of finding potent approaches to improve the memory impairment, the strategies affecting transcription factor cAMP-responsive element-binding protein (CREB) through medications are of great importance [6]. CREB is activated by various signaling pathways such as growth factors, activation of NMDA receptors, and other neurotransmitters; also CREB activates transcription targeted genes which play a role in memory enhancement [7–9]. The activity of these genes has increased learning and the expression of growth factors and anti-inflammatory factors, such as Insulin-like growth factors in the hippocampus [10]. Therefore, promoting an effective drug that can affect specifically the activity of genes involved in memory, reduce inflammation, act as a growth factor, speed up repair with minimal side effects is very important.

Insulin-like growth factor 2 (IGF-II) is a polypeptide that belongs to the insulin family. Studies have shown that IGF-II plays a key role in enhancing cognitive and memory activities [10]. The activation of IGF-II receptor causes responses such as regulating calcium homeostasis, increasing acetylcholine secretion and ultimately proliferating cells and helping them survive [11]. IGF-II can act as an effective medication for regulating calcium homeostasis and preventing neurotoxicity, and can play a role in increasing memory [12–14]. Schmeisser showed that IGF-II could increase the density of dendritic spine and also plays a role in the formation and regulation of synapses [15]. Lipopolysaccharides (LPS), a type of pro-inflammatory factor, can increase IGF-II in microglial cell cultures; in addition, the results of RT-PCR analysis and Western blot indicate that IGF-II significantly increases after inflammation, illustrating that IGF-II plays a key role as an anti-inflammatory factor [16,17]. Alberini et al. in studies on healthy mice showed that injecting IGF-II could activates the CEREB pathway and improve memory. Prior to these studies, it was thought that IGF-II was most important as a growth factor in evolution, but Alberini's research showed that IGF-II is not only important in the adult brain, but it also plays a key role in memory consolidation [1,7,9,10].

However, the effect of IGF-II on memory impairment in pathologic conditions and brain damage is still unknown. Our hypothesis in this study is that the injection of exogenous IGF-II into the hippocampus after brain injury following ICH is effective in memory consolidation and retention. In the present study, we injected IGF-II into the injured hippocampus 30 min after induced ICH, and performed behavioral tests after 2 weeks. Next, in molecular studies, we investigated the level of IGF-II gene expression.

**Methods and Materials:**

**Animals:**

The present study included 24 adult male Sprague-Dawley rats weighing 220–250 g that were prepared from the Center of Comparative and Experimental Medicine at Shiraz University of Medical Science in Iran. The rats were housed in accordance with standard conditions of ambient temperature (22 ± 2°C), 12:12 h light*–*dark cycle, and free access to water and food. The guidelines proposed by National Institutes of Health Guide for the Institutional Animal Care and Institutional Ethic Committee at Shiraz University of Medical Science (Ethical code: IR.SUMS.REC.1395.S767) were applied to perform animal experiments. Animals were handled 5 days before the behavioral experiments. On the test day, they were placed in the laboratory half an hour before starting the experiment to adopt with the environment.

**Taking orbital sinus blood samples:**

Following general anesthesia, blood samples were obtained from the rats using a capillary tube inserted into the eye medial canthus with 30° toward the nose. The tissue was punctured with thumb pressure to insert the plexus/sinus for holing blood flow into the capillary tube and taking samples. After removing the capillary tube, sterilized cotton was used to clean, and finger pressure was applied to prevent bleeding. After incubating the collected blood sample in room temperature (15 min), it was injected into the hippocampus [18].

**Surgical procedure:**

The animals were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and then they were placed in a stereotaxic frame for ICH surgery. A 2-cm incision was done in the shaven head skin to remove all soft tissue on the skull before drilling. According to Paxinos and Watson, a 26-G Hamilton needle was placed in the left hippocampus in the directions of 3.4 mm anterior (AP), 2.4 mm lateral (ML) and 3.6 mm ventral (DV) in relation to Bregma [19]. Autologous blood (100 µL) from orbital sinus was injected unilaterally into the left hippocampus gently within 5 min. The blood starts to clot after 10 min, which should be removed to impede the backﬂow. Acrylic dentalcement was used to seal the drilled hole and then the skin was sutured. Meloxicam (0.1 mg/kg) was injected to maintain the post-operative analgesia. The sterilized saline (vehicle) was replaced with blood in the sham group and IGF-II with concentration of 250 ng/1 µL (R&D, USA), according to our pilot study, was injected into the hematoma, 30 min after injecting the blood in ICH–IGF-II group. After the end of surgical procedure, the operated rats were housed aseptically again in the same cage and controlled until they recovered from anesthesia [20–22].

**Behavioral test**

**Neurologic deficit score:**

All 6 parameters of the Neurologic deficit score includingbody symmetry, climbing, gait, circling behavior, compulsory circling, and front limb symmetry were measured after 1, 3, 7, and 14 days of post-ICH for each rat. Each item was scored from 3 to 18 that 3 refers to maximum deficit and 18 to normal animal. [23].

**Assessment of wire-hanging test:**

A wire (2 × 60 mm) was installed between two platforms with a height of 50 cm and then the animals were placed midway for a maximum of 5 min. In this way the grip strength and balance were observed on days 1, 3, 7, and 14 post-ICH. To control any injury caused by possible falls, a pillow was placed under the animal. The suspension time of the rat on the wire was recorded [24,25].

**Novel object recognition (NOR) task:**

An open ﬁeld apparatus (72 × 72 × 35 cm) with white wall was used to test the behavioral function. Each animal was left in the box for 5 min for the first time to familiarize with the environment without any stimuli after 14 days after induced ICH. The following day, the rats were trained for 10 min through two identical objects (A1 and A2). After an hour rest, they were allowed to be familiar with one of either object A1 or A2 in the box, or one novel object (B) with different shape and color, for 5 min. The total time of exploring the novel and old objects was recorded for each animal, including time in direct contact and time within the object area. If the nose of the animal was directed at the object at a distance of less than 2 cm, the rat was regarded as in the object area. The discrimination ratio was calculated as the time spent with the novel object divided by the total time spent exploring either object [26,27].

**Passive avoidance test:**

The rats underwent passive avoidance training on day 14 after surgery, using a shuttle box with two connected chambers of equal size separated by a guillotine door. All animals were initially left in the apparatus to be familiar with the environment without any stimuli. The control and experimental groups were guided individually into the lighted chamber facing away from the entrance to the dark side for 10 s. The installed door was opened to note the latency to enter the dark chamber. If any animal didn’t enter the darkness within 60 s, it was replaced with a new rat. After 5 min, the habituation step was done repeatedly for the same interval. The third adaption trial was conducted after 2 hours where the animals’ feet were exposed to an electrical stimulation (0.5 mA, 50 Hz, 2 s once) delivered through the stainless-steel floor in the dark chamber for 20 s. Next, the animals were returned to their own cage. This test was repeated after 5 min. In this way, the successful acquisition of passive avoidance response was considered in case the animals avoided entering the darkness up to 300 s. After a day, the rats were re-entered into the illuminated chamber to test the retention trial, without any foot shock. After entering into the darkness, the latency to re-enter the dark chamber and total time spend in dark box were recorded. The time of going into the dark compartment was regarded as the step-through latency (STL) in the two learning and retention trials; as mentioned, the maximum cut-off time for the STL was 300 s in the retention test [28,29].

**IGF-II Gene Expression**

**Collection of tissue to perform real-time (RT) PCR**

Once the animals were decapitated, the brains were separated immediately. Their hippocampus were isolated from the left hemisphere immediately and were frozen using liquid nitrogen inside microtubes and kept at a temperature of –80°C.

**Extraction of RNA**

A homogenizer handle was used to homogenize the samples that were then incubated in ice for 5 min. Afterward, the homogenates were centrifuged for 15 min at 12,000 × g at 4°C. To isolate RNA, 1 mL of TRIzolTM reagent (Invitrogen™, made in São Paulo, Brazil) in five parts of 200 µL was appended for five times, for a total of 50 to 100 mg of tissue. The mixture was shaken for 15 min each time. To completely dissociate the nucleoprotein complex, the homogenized samples were incubated for 5 min at the RT. Then, 250 µL of chloroform was added for 1 mL of TRIzol reagent.

The tubes were completely shaken for 15 s then were incubated at the RT for up to 2 to 3 min. The samples were centrifuged for 15 min at 12,000 × g at 4°C, and 0.4 mL of the obtained aqueous phase was poured into another tube. Precipitation was achieved by 0.4 mL of cold isopropanol per 1 mL of TRIzol reagent. The samples were incubated over 10 min at RT, then were centrifuged for 10 min at 12,000 × g at 4°C. Afterwards, the samples were incubated overnight at –20°C.

The supernatant was discarded and the pellet was rinsed with 1 mL of DEPC-treated ethanol 75% once in the presence of 1 mL of TRIzol reagent. The pellet containing RNA was air-dried for 5–10 min and subsequently with a hot block for 1–2 min. Next, the pellet was resuspended in the DEPC-treated water. A spectrophotometer (λ260, UV light) was used to measure the quantity of RNA; thus 1 μL of RNA was diluted in 99 μL of DEPC-treated water. The stock concentration was obtained on the basis of 1 OD = 40 μg/mL of RNA and the dilution of 1/100. RNA was kept at–80°C.

**The synthesis of cDNA:**

All-in-One™ First-Strand cDNA Synthesis Kit (GeneCopoeia, Inc., USA, Cat. No. AORT-0050) was applied to synthesize the cDNA; thus, total RNA (1 μg) was considered as template. The incubation of final volume of 13 μL RNA was performed in the presence of 1 μL of oligo (dT), 1 μL of random primer and ddH2O (RNase/DNase-free) at 65°C for 10 min, followed by instant temperature dropping on ice. Next, the final volume of 25 μL was set using RNase/DNase-free ddH2O, 13 µL of RNA-Primer Mix, 5 µL of RT Reaction Buffer, 1 µL of dNTP, 1 µL of RNase Inhibitor, and 1 µL of M-MLV RTase. The resulting reaction mix was incubated at 37°C for 60 min. Finally, the reaction process was ended by exposing it to 85°C for 5 min and kept at –20°C until testing.

**Real-time PCR**

The StepOnePlus™ thermocycler (Applied Biosystems™, Foster City, CA, USA) was used to process the reactions in 96-well plate containing PCR master mix (Applied Biosystems™, São Paulo, Brazil) of SYBR™ green (12.5 μL), forward and reverse primers (each 0.5 μL), of cDNA (100 ng), and the rest nuclease-free water in a total volume of 25 μL.

PCR primers were designed with Gene Runner online software and controlled by BLAST search to ensure no cross reactivity. In all reactions, the housekeeping β-actin gene was run separately with the same experimental conditions to obtain the RNA integrity and quantity in the onset of RT reaction. The rat IGF-II (forward sequence of 5′-TGTCATTGCTTCAGTGCTCTCT-3′ and reverse sequence of 5′-TTCTGTTCCTCTCCTTGGGTTC-3′ with the product size of 163 bp) and rat GAPDH (forward sequence of 5′-AAGTTCAACGGCACAGTCAAGG-3′ and reverse sequence of 5′- CATACTCAGCACCAGCATCACC-3′ with the product size of 121 bp) were the primers used in the PCR process.

The protocol of PCR procedure was denaturation at 95°C for 15 min, 50-cycle amplification with denaturation (95°C, 15 s), annealing (60°C, 60 s), and extension (72°C, 60 s). The specificity of PCR products was determined by a melting curve analysis. The algorithm enhancements provided by the equipment estimated the experimental threshold cycle (Ct). All samples were tested twice and the calculated mean values were analyzed. The Ct values were achieved with the aid of instruments in each reaction using default parameters. The reactions were separately continued in the tubes for the same samples to test the levels of IGF-II and β-actin. The equation of 2-ΔΔCt was recruited to calculate the relative quantiﬁcation of the IGF-II mRNA expression levels.

**Statistical analysis**

Data for each group were analyzed by SPSS software (SPSS Inc) and described using graphs, means and standard error of mean; Kolmogorov-Smirnov test was used to determine normal distribution of data; one-way ANOVA with the Tukey-Kramer post hoc test was used to fulfill the inter-group comparison. To analyze the NDS statistically, nonparametric Mann-Whitney test was applied. In all tests, statistically significance level was *P* values <0.05.

**Results:**

**Behavioral assessment test**

**IGF-II effects on neurological analysis and locomotor function**

We measured the neurological deficits using an 18-point scoring system in which lower scores mean greater deficit. The motor deficit was significantly higher in the sham rats than in the ICH group (15.17 ± 0.26, 16.83 ± 0.40) in days 1 and 3, and significantly lower in the IGF-II treatment group (15.64 ± 0.31) than in the sham group just in day 1 (Fig. 1A). The wire-hanging test indicating the gripping and forelimb strength measured the locomotor deficits. Based on the findings, no significant differences in gripping and forelimb strength were found among all groups (Fig. 1B).

**IGF-II effects on passive avoidance learning**

Based on the passive avoidance test results, one or two foot shocks made the rats in all groups to learn the avoidance task. No statistical difference was seen in the training trials (data not shown). IGF-II treatment significantly prolonged the STL time and improved memory retention (211.43 ± 29.31) compared with the control (85.75 ± 14.01). Further, time in dark compartment (TDC) was significantly higher in ICH group (104.63 ± 24.47) than in the sham group (4.29 ± 4.29). In addition, TDC was significantly less in the treatment group (38.14 ± 13.07) than the control group (Figure 2).

**IGF-II effects on novel object recognition test in assessing learning and memory**

The NOR test was used to evaluate recognition memory. According to the data obtained, discrimination ratio of the test; the duration of the object investigation by the rats in the training session was not found to be significant (data not shown).

The ICH group (0.26 ± 0.062) had less exploration time in the novel object than the sham group (0.69 ± 0.078). Further, time was spent by IGF-II group to seek the novel objects (0.41 ± 0.089) didn’t show any significant difference compared with ICH groups (Fig. 3).

**The decreased expression of IGF-II gene due to recombinant IGF-II injection**

The level of IGF-II mRNA in the hippocampus was measured using real-time PCR technique (Figure 4). All study groups exhibited the expression of IGF-II, but IGF-II mRNA level was significantly reduced (1.6 ± 0.91) after injecting the 20 ng of IGF-II into the left hippocampus. IGF-II mRNA was obvious in ICH group (103.22 ± 30.25) but, there was no significant difference with the sham group (174.12 ± 44.48), suggesting reduced expression of IGF-II due to ICH-induced brain damage and suppressed expression of IGF-II in the presence of higher exogenous IGF-II.

**Discussion**

In this study, we observed that recombinant IGF-II could significantly increase avoidance memory, but did not have a significant effect on investigation memory caused by ICH. However, our data is in line with studies by Alberini et al. that suggest IGF-II administration—both intrahippocampal and systemically—significantly improves memory types [1,9,10]. Pascual-Lucas et al. in a study on a transgenic mouse model showed that IGF-II could improve memory and reduce synaptic deficiency [30]. To our knowledge there isn’t any other study on the effect of IGF-II on memory impaired due to acute cerebral injury, such as stroke and trauma. Considering that IGF-II is a growth factor and a potent anti-inflammatory factor without any reported adverse effect, it can be considered as an effective treatment. IGFs are secreted as endogenous anti-inflammatory factors of M2 microglia after injury [16,31]. Human culture studies have shown that the main source of IGF-II is microglia. In addition, IGF-II production has been shown to increase following the upregulation of inflammatory factors such as IL-4 and IL-13. Increasing LPS in the cell culture also increases the production of IGF-II [16,32,33]. In this study, neurological deficit score (NDS), which did not show any significant changes between the groups. It is likely that the inflammatory factors have changed, but this difference has not been sufficient to have an effect on the NDS.

The IGF-II connection to its receptor activates the downstream PI3K/Akt pathway, this pathway affects the growth and survival of neurons. In addition, IGF-II induces an upregulated C/EBPβ target gene, which plays a key role in memory stability. Studies have shown that training increases the release of IGF-II, and this increase persists for up to 4 days after training [1,10,34]. IGF-II plays a key role in hippocampal-dependent memories, another reasons for the effectiveness of IGF-II in the memory enhancement is the expression of activity-regulated cytoskeletal protein (Arc) following the activity of the IGF-II receptor. Arc – a protein with an important role in learning and memory-related molecular process- was induced by synaptic activity[10,35]. Our results showed that avoidance memory was significantly improved after single-dose administration of IGF-II. Investigation memory in the NOR test did not show a significant change, but the trend was increasing. However, both of these tests evaluate hippocampal-dependent memories, there are differences in the retention, consolidation pathways between these tests which can be suggested as a possible reason for our results.

In addition, the activation of the CREB pathway following the performance of the IGF-II receptor has been shown to be involved in synaptic plasticity and memory formation. The CREB-C/EBP cascade enhances the expression of the IGF-II gene and subsequently increases IGF-II levels in the environment [9,36]. Our results showed that the expression of IGF-II gene in the IGF-II group was significantly decreased in comparison with the sham group. Because IGF-II works as a growth factor, in addition to cell survival, it can also cause an increase in cancer cells. The brain has defensive mechanisms that prevent excessive function of this factor and its receptor. One of these mechanisms is that the IGF-II receptor has a short life span and is rapidly degraded [36,37]. This causes the IGF-II to remain in the tissue, but its receptor shows less activity. The reduction in IGF-II gene expression in the treatment group can be due to the possible increase of IGF-II levels in the tissue and the probable subsequent suppression in the expression of the IGF-II gene. However, in the sham group, in contrast to the other two groups, normal function of the receptors and significant expression of the IGF-II gene is observed.

**Conclusions**

In summary, the results of this study indicate that injection of recombinant IGF-II into hippocampus 30 min after the ICH can improve avoidance memory in passive avoidance test but it doesn’t have any positive effect on investigation memory. In addition, IGF-II was not effective on motor activity assessed by wire hanging test, which indicates that the information obtained from the memory tests was not due to changes in motor activity. IGF-II injection reduces the IGF-II gene expression; regarding the improvement of memory and the effective role of the IGF-II, it seems that the expression of the IGF-II gene does not correlate with memory changes, which requires further studies.

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**Figure legends:**

Figure 1 (A). IGF-II effect on NDS. Data are shown as mean ± SEM (n=8). \* *P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001 compared with the sham group. (B). IGF-II effect on locomotor deficits evaluated by wire-hanging test. There were no significant difference between groups.

Figure 2 (A). Effect of IGF-II on step-through latency (STL) in shuttle box test. Data are shown as mean ± SEM (n=8).\* *P* < 0.05 and \*\*\**P* < 0.001 as compared to Sham, +++ *P* < 0.001 in comparison with ICH group. (B). Effect of IGF-II on total time spent in dark chamber (TDC) in shuttle box test. \*\**P* < 0.01 as compared with sham group; +*P* < 0.05 as compared with ICH group.

Figure 3. Effect of IGF-II in novel object recognition test. Data are shown as mean ± SEM (n=8). In contrast to sham, ICH and IGF-II groups spent less time exploring novel object than old ones. No significant difference was observed between IGF-II and ICH groups. \**P* < 0.05 and \*\**P* < 0.01 compared with the sham group.

Figure 4. Effect of recombinant IGF-II on the level of IGF-II gene expression in a damaged hippocampus following intracerebral hemorrhage; the mRNA level of IGF-II measured using real-time PCR was normalized by GAPDH. The statistics are shown as mean ± SEM (n=4). \**P* < 0.05 compared with sham group.