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ORIGINAL PAPER

# Effects of treadmill exercise on brain insulin signaling and β-amyloid in intracerebroventricular streptozotocin induced-memory impairment in rats

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**[Purpose]** The purpose of the study is to explore effect of 6 weeks treadmill exercise on brain insulin signaling and β-amyloid(Aβ). **[Methods]** The rat model of Alzheimer's disease(AD) used in the present study was induced by the intracerebroventricular(ICV) streptozotocin(STZ). To produce the model of animal with AD, STZ(1.5mg/kg) was injected to a cerebral ventricle of both cerebrums of Sprague-Dawley rat(20 weeks). The experimental animals were divided into ICV-Sham(n=7), ICV-STZ CON(n=7), ICV-STZ EXE(n=7). Treadmill exercise was done for 30 min a day, 5 days a week for 6 weeks. Passive avoidance task was carried out before and after treadmill exercise. **[Results]** The results of this study show that treadmill exercise activated Protein kinase B(AKT)/ Glycogen synthase kinase 3α (GSK3α), possibly via activation of insulin receptor(IR) and insulin receptor substrate(IRS) and reduced Aβ in the brain of ICV-STZ rats. More interestingly, treadmill exercise improved cognitive function of ICV-STZ rats. Finally, physical exercise or physical activity gave positive influences on brain insulin signaling pathway. **[Conclusion]** Therefore, treadmill exercise can be applied to improve AD as preventive and therapeutic method. **[Keyword]** intracerebroventricular-streptozotocin injection, treadmill exercise, β-amyloid, brain insulin signaling

## INTRODUCTION

Alzheimer's disease (AD) is the most general form of dementia shows the memory loss and cognitive impairment. It reported by aggregation of senile plaque which is made up causing the brain's toxicity, the amyloid-beta: Aβ and apoptosis which is cause by aggregation of neurofibrillary tangles: NFTs what is made up hyperphosphorylated tau protein's development are the main reason of pathologic [1].

AD fall into two categories. One is familial AD (FAD) which is occurred by specific gene's mutation, Also second is sporadic AD (SAD). The FAD is caused by less than 5% of AD patients, most of another patients are affiliated to SAD. There aren't no exact reason of SAD, but It is reported to expedite memory loss because of metabolic disorder by senescence and obesity, aggregation of hyperphosphorylated tau protein & Aβ, oxidative stress and brain energy metabolism's lack which is cause by damage of brain insulin signal transduction [2-7].

Some studies show the relation of AD and the damage of insulin signal transduction. So the glycose metabolism AD and the insulin signal transduction has many concern by scientists, recently [8,9]. In the past, insulin is registered on periperal hormone which can't have an effect central nervous system, also can't pass the Blood-Brain Barrier (BBB). However, it is reported that the insulin created by pancreas moves toward the brain through cerebrospinal fluid and it has some big role like that neuroplasticity, survival of brain's nerve cells, controling memory and cognitive ability [10-15]. This insulin revitalizes two hypothalamus's signal transduction categorized PI3K&MAPK through signal transduction caused by combing insulin and it's receptor. Especially, some studies say hypothalamus modulator : GSK3 that is connected to PI3K&AKT on signal transduction's route causes apoptosis which is caused by aggregation of neurofibrillary tangles: NFTs and senile plaque which is the main lesion of AD [16-19].

Recently, the animal models use to experiment for study of AD. The ICV-STZ rat that is poured streptozotocin (STZ) into intracerebroventricular (ICV) is used as SAD's studies a

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lot. Because It has the same pathology's symptom of AD like Aβ, revelation of hyperphosphorylated tau protein, damage of insulin signal transduction, lack of glucose, oxidative stress and memory loss [20-22]. So it is reported as destroying memory that ICV treatment induces damaging of PI3K/AKT/GSK3 signal transmission, decreasing of glucose-metabolizing, increasing of oxidant stress and Aβ deposition which were caused by decreasing insulin receptors susceptibility of cerebrum [17,23-25]. Thus these kinds of preceding research underpin the hypothesis which is one of the major factor causing AD that insulin deficiency in the central nervous system and decrease expression of insulin receptors.

Although many scientists had developed various medicine for repressing AD, but their effectiveness was reported to temporary expedient. So changing lifestyle such as physical activity is magnified as alternative method which can minimize side effects and improve AD. The physical performance not only supplies oxygens and nutritive substance to brain cell through blood, but also enhance removal rate about body wastes and carbon dioxide [26]. Also it be known as improving brain function to fetch out nerve growth factors and cognition-enhancing effect about aging [28-30].

Especially the exercise can induce a positive change about creation of nerve cell, nerve growth factor and cerebrovascular with decrease of neurofibrillary senile plaque which is a major lesion of AD. So it is reported to improve cognitive ability [31-36]. As previously stated, one of the major risk factor which induces SAD, but the study about the effect of exercise as a alternative method that can prevent or treat to AD on insulin signaling has been insufficient.

Thus this study try to examine the effect of 6-week treadmill exercise with animals which are SAD models injected STZ in their ICV where the Aβ of brain, protein express about insulin signaling & cognitive functions.

## METHODS

### *Experimental animal*

This study submits H university's ethics committee of animal experiment, so receives approval. Animal used experiment is a Sprague-Dawley male white rat which is 20 weeks olds. The white rat is kept the light and shade at an interval of 12 hours, maintain around 22~24℃ temperature, also keep 40~60% humidity following‘ The guiding principles for the care and Use of Animal' which is based by declaration of Helsinki announced on 1964. Also, the rat is housed 2 animals each dispersed in cages with free supply of tap water and solidity feed for rat. The experimental animal is classed as ICV-Sham (n = 7), ICV-STZ CON (n = 7) and ICV-STZ EXE (n = 7).

### *Intracerebroventricular injection of streptozotocin*

This study use the Jee *et al*. [37] 's way of surgical operation to make a rat model of AD. Fixed to the head of the rat stereotaxic frame and intraventricular (ICV) was injected to STZ. It is perforated with small drill with a diameter of 0.5 mm, from the surface of skull right point 1.5 mm, rear side 0.8mm with the bregma as a center. that time the micro injection manipulator (10 μl, 26 G, Hamilton. USA) is needled deep into the 3.6 mm after that, dilute the STZ by 1.5 mg per weight and inject 20 μl in saline. Whereas, the control group (ICV-sham) was injected only the salin.

### *Treadmill exercise*

Treadmill exercise use for the experimental animal's treadmill (Rodent, Treadmill, Dae-myung Scienific Co, Ltd, Korea) 2 weeks after ICV-STZ treatment and also their adaptive training of one week in advance is progressed by this sequence followed cumulative intensity like that gradient of 0% to the fixed, start exercising 5 minutes 2 m/min, and then 5 m/min the next five minutes, and lastly make 8 m/min 20 minutes. After the adaptive training, The main exercise was performed 5 days a week, 6 weeks. Gradient of 0% to the fixed, the study is progressed the order after the start of exercise, first 5 minutes 8m/min and next 5 minutes to 11 m/min, finally 20 minutes to 14m/min. Exercise protocol which is used this experiment is carried out on the basis of medium strength exercise program that is offered by Kim *et al*. [38] and Jee *et al*. [37].

### *Passive avoidance task*

To investigate about damages of memory, this study conducts passive avoidance task after two weeks for ICV-STZ treatment. The equipment of passive avoidance task consists of 2 rooms. The front-room is a white box with full of light (18 × 18 × 25 cm). And the behind room, which is connected to front room, is a dark box blocked light (18 × 18 × 25 cm). Steel which can give them electric shock was installed on the floor of dark room. The wall between front room and behind room has a circle hole (4 cm in diameter). This hole has a door which can opens and shut like a guillotine. After leaving each experimental animal in anther cage during one minute, the put them in front room for 10 seconds to adapt

the room. Then opening the guillotine door, let them free to move. When the experimental animals enter the darkroom completely, shut the door quickly and give them electronic shock (0.5 mA) for 2 seconds. 5 seconds later, take them out and put them in their cage. The experiment is conducted by the same method 72 hours later. And retention latency time is recorded until 300 seconds as maximum. To verify the improvement of memory through the treadmill exercise, the experiment is conducted by the same method after six weeks-treadmill exercise.

### *Tissue preparation*

After inject anesthetic (Mixed Rompun / Zoletil, 10 mg/kg in a 2:1 ratio) in the peritoneal, 4 rat, after are removed the hippocampus and extracted to their brain tissues, are kept at the -80℃, (Bio-Freezer, Forma Science, USA) until next analysis to measurement of protein expression. Also, After give an anesthetic, to analyze dyeing of Immunohistochemistry, the others 3 rat, which are opened thoracic cage, are injected to (50 mM phosphoate buffer saline PBS), through left ventricle during 10 minutes and are perfused 4% paraformaldehyde (PFA) fixatives dissolved at 100 mM phosphoric buffer. After perfusion fixation, The extracted brain is immersed in 4% PFA fixative and done postfixation during 12 hours at 4℃, and finally, after paraffin-embedding, cut as much 40 μm through method of preparing the specimens.

### *Western blot*

At cryogenic freezer -80℃, the stored brain tissues homogenize their tissues through using the lysis buffer and homogenizer. Electrophoretic protein conducts sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). 30 μg of protein extracted from brain tissue has an electrophoresis in 10% SDS-polyacrylamide gel. After that make them metastasis with PVDF membrane (Millipore) at 45 volt during two hours. When the metastasis is completed, make membrane to block to 5% skim milk solution (in PBS-T: 10 mM Tris-base pH 8.0, 150 mM NaCl, 0.05% Tween-20) on rocker platform for 1 hour. Then each primary antibody, anti-Aβ-42 (Covance, SIG-39320), anti-Insulin Receptor β (Cell signaling, #3025), anti-pInsulin Receptor (Abcam, ab60946), anti-IRS1 (Cell signaling, #3407), anti-pIRS1 (Cell signaling, #2381), anti-PI3K (Cell signaling, #4257), anti-pPI3K (Cell signaling, #4228), anti-AKT (Cell signaling, #9272), anti-pAKT (Cell signaling, #4051), anti-GSK-3α (Cell signaling, #9338), anti-pGSK-3α (Cell signaling, #9316) is diluted to 1:10000 in skim milk solution and shaken for 12 hours. After shaking, it is cleaned by TBS-T solution five times by 10 minutes. And secondary antibody (goat anti-rabbit ZYMED, 65-6120; rabbit anti-goat ZYMED, 81-1620; goat anti-mouse Santa Cruz, sc-2005) is diluted with blocking solution to 1:5000 and shaken during one hour. After that cleaned by TBS-T solution five times by 10 minutes. The last stage, put membrane into WBLR solution Western Blotting Luminol Reagent (Santa Cruz, sc-2048), and scanning Resultant solution which ager for 1 minute through using molecular Imager ChemiDoc XRS System (Bio-Rad, USA). After scanning, calculate protein using Quantity One 1D Analysis Software (Bio-Rad, USA). And we digitize concentration of quantitative protein and which ICV-Sham group as a standard, put it on a percentage basis of distinction of other groups.

### *Immunohistochemistry*

Immunohistochemistry use 0.01 M PBS to wash 3 times for 5 minutes each, using the free-floating method. To remove peroxidase, It is shacked for 30 minutes in 50% ethyl alcohol and wash 3 times for 5 minutes each with 0.01 M PBS. And each pieces are put in the beaker which is contained 0.01M sodium citrate, and incubate on boiling water (100℃) for 8 minutes. After that, during 40 minutes, it is carried out by blocking on 10% Normal Donkey Serum (Millipore, 2309032). After blocking, Aβ-42 (Covance, SIG-39320) is overnighted 12 hours at 4℃. After wash 3 times for 5 minutes each with 0.01 M PBS, It is reacted as goat anti-mouse (Santa Cruz, sc-2005) for one hour at room temperature. Again wash 3 times for 5 minutes each at 50mM PBS, and then, to RT Incubate using by Vectastain-Elite ABC kit (Vector Laboratories, PK-6200) for 30 minutes, Next, wash 3 times for 5 minutes each at 0.01 M PBS, To use DAB Peroxidase Substrate Kit (Vector Laboratories, SK-4100) in order to dilute to DAB (3,3'-diaminobenzidinetetrahydrochloride) to 0.05M tris-buffer (pH 7.6) about 0.02% and 0.02% hydrogen peroxide was added and color to 5 minutes. The tissue that is completed color, is washed with 0.01M PBS, three times, after that, attach gelatin-coated slide and completely dry at room temperature. Dehydrated at ethyl alcohol increased by 80%, 90%, 100% in the concentration. At the end of the dehydration process, make it transparent by using xylene and seal it by permount.

### *Data processing method*

All data were analyed with SPSS version 18.0 (SPSS, Inc.,

Chicago, IL, USA). All values are expressed as mean ± SE. Statistical significance was determined using a one-way ANOVA when comparing the groups. A Fisher'S LSD post hoc test was followed for all pairwise multiple comparisons if a statistically significant group main effect was found. The differences were considered statistically significant at $\alpha = .05$.

## RESULTS

### *The Effect of treadmill exercise on behavioral tests of ICV-STZ rat*

For 6 weeks, the result that effect of treadmill exercise on behavioral tests of ICV-STZ rat's memory is as <Fig. 1> is presented. In case of passive avoidance time, it has significant differences by the groups [$F_{(2,20)} = 108.013$, $p = .001$]. The result of post-verification, statistically, there are the significant differences between group of ICV-Sham (284.78 ± 5.88) and group of ICV-STZ CON (136.08 ± 7.04). Also, there are another significant differences between group of ICV-STZ Con and group of ICV-STZ EXE (163.20 ± 9.49).

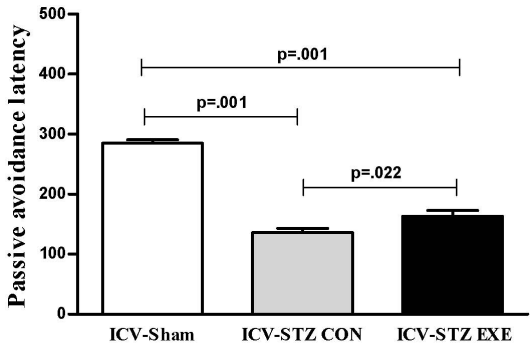


**FIG. 1.** EFFECT OF TREADMILL EXERCISE ON THE LATENCY TIME OF THE PASSIVE AVOIDANCE TASK. VALUES ARE MEAN ± SEM 7 ANIMALS/GROUPS

### *The Effect of treadmill exercise on β-amyloid accumulation of ICV-STZ rat*

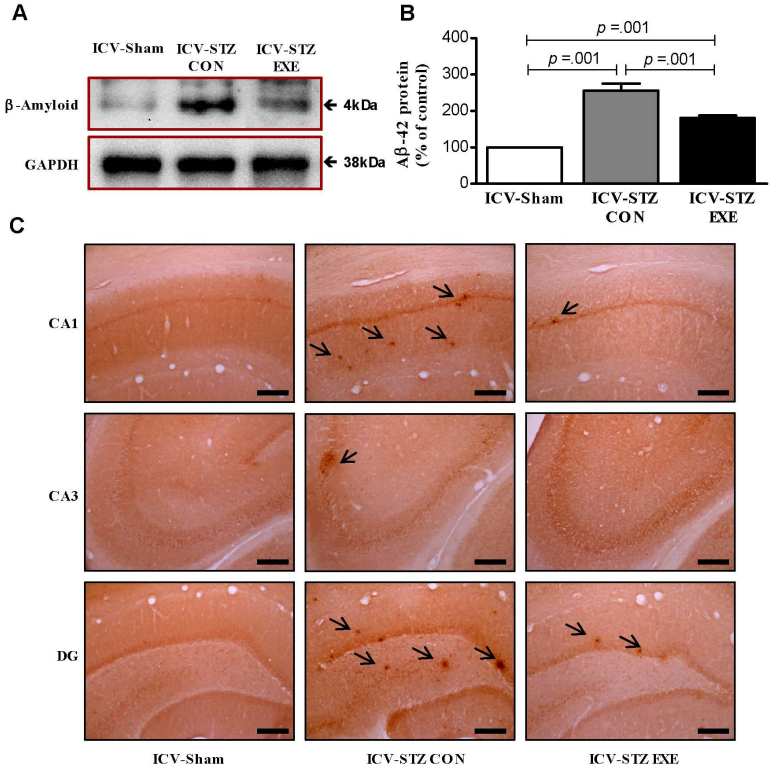


**FIG. 2.** EXPRESSION AND IMMUNOSTAINING ANALYSIS OF B-AMYLOID LEVEL IN THE HIPPOCAMPUS OF ICV-SHAM, ICV-STZ CON AND ICV-STZ EXE. WESTERN BLOT (N = 4) AND IMMUNO STAINING ANALYSIS (N = 3) OF B-AMYLOID DEPOSITION. A,B) PVDF FILTERS TRANSFERRING 30MG OF PROTEIN FROM THE CORTEX AND HIPPOCAMPUS OF EACH GROUP, INCUBATED WITH ANTI-HUMAN B -AMYLOID ANTIBODY. THE BANDS WERE QUANTIFIED BY DENSITOMETRY TO OBTAIN RELATIVE LEVELS OF B-AMYLOID. GAPDH WAS PROBED AS AN INTERNAL CONTROL. C) IMMUNOSTAINING OF AB-42. 40MM-THICK SECTIONS OF BRAINS FROM EACH GROUP WERE INCUBATED WITH ANTI-HUMAN B-AMYLOID PRIMARY ANTIBODY AND HRP-CONJUGATED GOAT ANTI-RABBIT IGG. A SCALE BAR REPRESENTS 100㎛. VALUES ARE PRESENTED AS MEANS ± SEM.

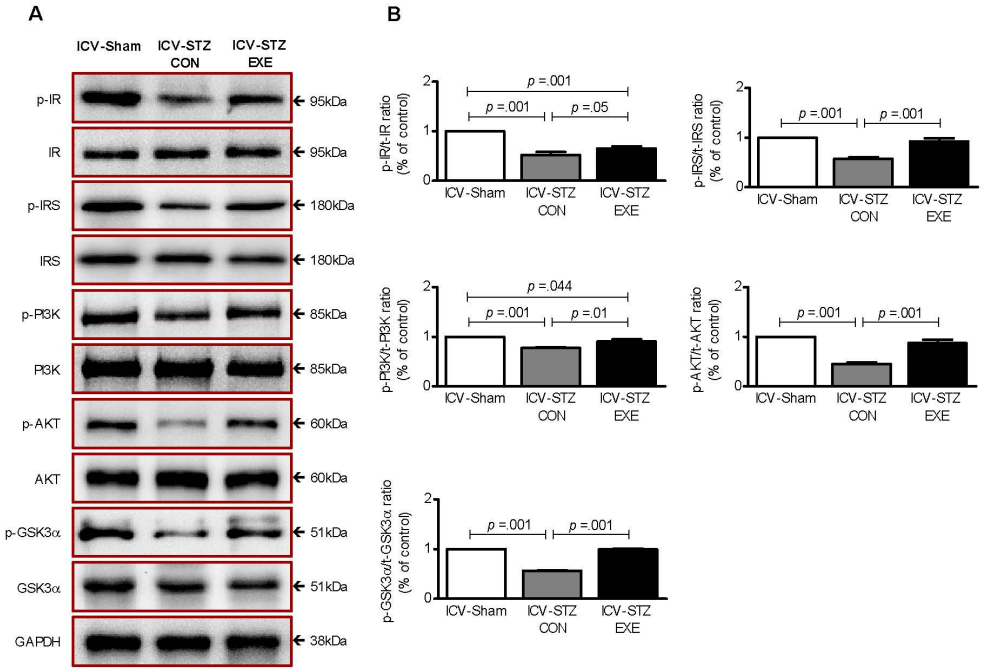


**Fig. 3.** Effect of treadmill exercise on activation of brain insulin signaling in the hippocampus of ICV-Sham, ICV-STZ CON and ICV-STZ EXE. A) The levels of brain insulin signaling in the hippocampus was analyzed by western blots. GAPDH was probed as an internal control. B) Acticvation of brain insulin signaling was normalized with respect to ICV-Sham. Values are mean ± SEM 4 animals/groups.

For 6 weeks, the result of treadmill exercise on β-amyloid accumulation of ICV-STZ rat is as <Fig. 2> is presented. The revelation of β-amyloid has the significant difference between there groups [$F_{(2,11)} = 44.553$, $p = .001$]. The result of post-verification, statistically, there are the significant differences ($p = .001$) between group of ICV-Sham (100%) and group of ICV-STZ CON (255.68 ± 19.16%). And also, there are the significant differences ($p = .001$) between group of ICV-STZ Con and ICV-STZ EXE (180.98 ± 6.40%).

### *The Effect of treadmill exercise on the brain Insulin signaling of ICV-STZ rat*

For 6 weeks, the result of treadmill exercise on the brain insulin signaling of ICV-STZ rat is as <Fig. 3> is presented. IR-phosphorylation have significant differences between their groups [$F_{(2,11)} = 38.189$, $p = .001$]. There are the significant differences between by the group of IR-phosphorylation. The result of post-verification, there are statistically significant differences ($p = .001$) between the group of ICV-Sham (1.00%) and group of ICV-STZ CON (0.53 ± 0.06%) and also significant differences ($p = .05$) between group of ICV-STZ CON and group of ICV-STZ EXE(0.65 ± 0.08%). IRS-phosphorylation have significant differences between their groups [$F_{(2,11)} = 32.433$, $p = .001$]. The result of post-verification, there have statistical and significant differences ($p = .001$) between the group of ICV-Sham (1.00%) and group of ICV-STZ CON (0.58 ± 0.07%) and the group of ICV-STZ CON have a significant difference to group of ICV-STZ EXE (0.93 ± 0.06%). In addition, PI3K-phosphorylation have the significant differences by their groups [$F_{(2,11)} = 15.893$, $p = .001$]. Continue to, the result of post-verification, statistically, there are the significant differences ($p = .001$) between group of ICV-Sham (1.00%) and group of ICV-STZ CON (0.77 ± 0.05) and also statistically significant differences ($p = .01$) between group of ICV-STZ CON and group of ICV-STZ EXE (0.91 ± 0.09%). AKT-phosphorylation have the significant differences by their groups [$F_{(2,11)} = 39.522$, $p = .001$]. After post-verification, the result that there are the significant differences ($p = .001$) between group of ICV-Sham (1.00%) and group of ICV-STZ CON (0.45 ± 0.03%) and also, statistically, between the group of ICV-STZ CON and group of ICV-STZ EXE (0.88 ± 0.07%) have the significant differences ($p = 0.01$). GSK3α phosphorylation have the significant differences by their groups [$F_{(2,11)} = 534.665$, $p = .001$]. Finally, the result of post-verification, statistically, there are the significant differences ($p = .001$) between the group of ICV-Sham (1.00%) and group of ICV-STZ CON (0.57 ± 0.01%) and also have the differences ($p = .001$) between group of ICV-STZ CON and group of ICV-STZ EXE (0.99 ± 0.02%).

## DISCUSSION

The deposition in Aβ cells which is the main cause of the AD cause unbalance of energy metabolism & infection. and It makes death of cells and to loss efficiency of synaptic transmission between nerve cells. So it can be the reason to loss of cognitive abilities or learning abilities of behavior [39]. Therefore, in this study, the passive avoidance task is done to know whether the treadmill exercise have the positive effects to develop the cognitive abilities of rats which are modeled ICV-STZ AD. As the result, the cognitive abilities of ICV-STZ EXE is more progress than ICV-STZ CON.

With these reasons, it has the same effect which can improves rat's (ICV-STZ AD models) cognitive abilities between the result of treadmill exercise and Muller *et al*. [40]. Also, it has same result to Cho *et al*. [32] and Um *et al*. [36], Kang *et al*. [41] reported that the treadmill exercise can improve cognitive abilities of transformation-animals which is the model of AD.

In this way, the improve of recognition & behavior learning abilities of the rat's induced by ICV-STZ is explained by smooth transfer oxygen and nutrients through bloods of brain cells, Improve of physiological function caused by increasing of body waste and carbon dioxide removal rate, through this, oxidative stress & decreaing of inflammation, efficiency increasing of insulin & glucose metabolism, suppressing apoptosis caused by nerve growth factor's increasing and plasticity of brain, interation of nerve cell's generation which is also caused by decreasing of Aβ which is the main leison factors of AD.

The main lesion, Aβ deposition which induces AD, is known as increasing cytotoxicity to accelerates oxidation stress and inducing apoptosis through destroy of insulin signaling [42]. Also, this study showed that Aβ deposition is considerably high in group of ICV-STZ CON and express of protein about insulin signaling (IR, IRS, PI3K, AKT, GSK3 α) markedly decrease.

This result show us it coincides with existing research result which Aβ deposition in brain of rat same ICV-STZ CON AD model, accelerates oxidation stress and decreases express of protein about insulin signaling [23,25,44,45]. Although we did not analyses Aβ deposition by ICV-STZ treatment, we can explain as increasing of activity of β-secretase and γ -secretase which are concerned directly in Aβ producing [45,46], this Aβ deposition induces phosphorylation decrease of insulin receptors and decreases phosphorylation of tyrosine of insulin receptor substrare (IRS), decreases protein PI3K/ AKT/GSK3α which are concerned in sub-signaling systems. In short, decrease of protein about insulin signaling by Aβ deposition means that intracellular metabolism like glucose drink and glycogenesis isn't been controlled and induces neuronal cell death by extension [47,48].

In contrast, following the deposition of Aβ made by produced abnormal caused by ICV-STZ treatment, the result of treadmill exercise about the rats which have decrease of protein's express about insulin signal delivery, appears remarkably decrease of Aβ's expressing and contrary, the expression of proteins about signal delivery (IR, IRS, PI3K, AKT, GSK3α) appears increasing remarkably. In this way, decrease of Aβ's deposition is explained by decrease of β -secretase's and ϒ-secretase's vitality on amyloid precursor protein (APP)'s metabolism course by treadmill exercise and expression's increase of protein (IR, IRS, PI3K, AKT/GSK3α) about insulin signal delivery is resulted by promotion of insulin receptor's phosphorylation by treadmill exercise and tyrosine's phosphorylation-promotion of IRS protein. So these vitality of IR, IRS, PI3K, AKT suppress the GSK3α's activity and they are regarded as increasing of living of nerve cells & protective effects.

All that result, through ICV-STZ treatment, the activity of IR and IRS decreases in rat's brain cell and by suppressing the activity of PI3K/AKT/GSK3α which is the downstream signaling pathway, we can confirm the increase of β -amyloid's expression and damage of memory. However, through treadmill exercise for 6 weeks, the activity of IR & IRS is increased, we can confirm the decrease of β-amyloid's expression and improve of memory through activity of PI3K/AKT/GSK3α. Finally, physical exercise or physical activity gave positive influences on brain insulin signaling pathway. Therefore, treadmill exercise can be applied to improve AD as preventive and therapeutic method.

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