Original Article

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Enhanced Serum Endogenous Ouabain in a Model of Sporadic Type of Alzheimer's Disease Induced by Intracerebroventricular Streptozotocin Injection

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ABSTRACT

BACKGROUND AND OBJECTIVE: Serum level of digitalis-like factors or sodium pump inhibitors increase under numerous pathogenic circumstances. Diminution of sodium pump activity was reported to be secondary to beta-amyloid oligomers, though the mechanism involved remains inconclusive. Therefore, this study was conducted to evaluate the alteration in serum levels of endogenous ouabain and cerebrospinal fluid (CSF) sodium in an animal model of Alzheimer's disease, induced by intracerebroventricular injection of streptozotocin (ICV-STZ).

METHODS: In this empirical study, 48 male rats were randomly divided into six groups of saline and STZ. Alzheimer's model was induced by ICV injection of STZ (3 mg/kg) on the first and third days. CSF samples were drawn from cisterna magna, and blood samples were collected from caudal vein. Thereafter, serum levels of ouabain and CSF sodium were evaluated 2, 7, and 14 days after STZ/saline injection in the test groups. Cresyl violet and Congo red stains were applied to study neuronal morphology and presence of amyloid plaques.

FINDINGS: The mean serum levels of ouabain in the STZ groups were 0.2±0.05 ng/mL vs. 0.54±0.04 ng/mL on the second day, 0.3±0.02 ng/mL vs. 0.59±0.06 ng/mL on the seventh day, and 0.2±0.07 ng/mL vs. 0.65±0.08 ng/mL on the fourteenth day. These results indicated a significant increase of endogenous ouabain in the experimental groups compared to the saline groups ($p<0.05$). On the second day, CSF sodium concentration in the STZ groups was significantly higher than the saline groups (142.6±2.9 vs. 150.6±3.2; $p<05.0$). However, despite the increasing trend, in the STZ groups there were no significant differences between the 7- and 14-day STZ groups.

CONCLUSION: In this study, elevated serum level of endogenous ouabain is a sign of early pathological changes in sodium pump activity and can be recommended as a potential therapeutic target.

KEY WORDS: *Alzheimer's, Ouabain, Streptozotocin.*

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Introduction

Low levels of sodium pump activity are noted in the brain of patients with Alzheimer's disease (1). The beta-amyloid concentration, at the nanomolar scale, can lead to decreased sodium pump activity in hippocampus slices. In this effect, probably caused by signaling pathway, membrane integrity is critical. Inhibition of the pump activity in the presence of beta-amyloid can initiate events that lead to cell death (2). In 1979, Fishman first identified ouabain-like compounds in the brain (3). Since then, various studies have evaluated different types of digitalis-like substances and their roles. In 2015, several review articles noted the characteristics and importance of monitoring these compounds in various pathological conditions, and proposed them as biomarkers (4). Simonini et al. illustrated that the plasma level of endogenous ouabain before and after surgery can determine disease severity and risk of mortality after the surgery (5).

Intracerebroventricular injection of streptozotocin (ICV-STZ) can progressively impair learning, memory, and cerebral glucose metabolism. This approach can be used as an animal model for inducing sporadic Alzheimer's disease (6). In case of pump activity diminution, there is a possibility of CSF sodium change, since molecular changes in the extracellular space and brain interstitium are reflected in CSF. Elevated CSF sodium concentration activates neuromodulatory pathways, which induces the release of ouabain through local production of aldosterone and affecting the epithelial sodium channel (7). Thus, in addition to measuring serum ouabain, CSF sodium concentration was evaluated in this study. Given the inhibition of sodium pump by digitalis-like substances and endogenous ouabain (8, 9), and considering the absence of studies on ligand changes of endogenous sodium pump in Alzheimer's disease model, the present study aimed to evaluate serum level of endogenous ouabain in Alzheimer's disease model induced by ICV injection of STZ.

Methods

In this applied-fundamental study, conducted during 2010-2011 in the Physiology Laboratory of School of Medicine, Tehran University of Medical Sciences, Tehran, Iran, 48 Sprague Dawley rats weighing between 250 and 300 gr were randomly divided into six groups. They were kept in the standard conditions (12 dark/light cycle, temperature 22±2°, humidity45-55%). They had free access to diet and water ad libitum. The principles of the 2008 Helsinki Declaration and ethical considerations in the use of laboratory animals were observed. First, the rats were anesthetized by ICV injection of sodium pentobarbital (60 kg/mg), and then they were placed on a stereotaxic frame, and the skull exposed. After identification of Bergma and lambda junctions, cannula guide was placed in spatial coordinates of the lateral ventricles. The stereotaxic coordinates for the lateral ventricle (10) were measured accurately as 0.8 mm posterior to bregma; 1.5 mm lateral to saggital suture; 3.6 mm beneath the surface of brain with the tooth bar set at ±3mm. After completion of the stereotaxic surgery and recovery process, 3 mg/kg b.wt. STZ was soluted in 2 µL saline was injected bilaterally to the brain ventricles on the first and third days.

The saline groups underwent stereotaxic surgery, and received ICV-saline injection. To evaluate the effects of subdiabetogenic dose of ICV-STZ on plasma glucose level, its level was measured using enzymatic glucose oxidase method and spectrophotometry. After 2, 7, and 14 days of ICV injection of STZ/saline in the STZ and saline groups, CSF sample was drawn from cisterna magna (11), and blood sample was obtained from caudal vein. After the intracardiac perfusion of saline and 4% paraformaldehyde (containing 1.0 M phosphate buffer, pH=2.7), the rats' brain was immediately removed and put in 4% paraformaldehyde solution for 48 hours. Coronal sections were prepared with a thickness of 10 µm, and morphological features of hippocampus neurons were evaluated after cresyl violet staining (12). Moreover, Congo red was used to identify amyloid in hippocampus (13). About 5 µM Congo red solution in potassium phosphate buffer with pH=4.7 was prepared.

After removal of paraffin, 8-µm sections were placed in Congo red and 5.0% ethanol solution for 20 minutes in the dark. After exposure to absolute alcohol and washing with water, they were immersed in hematoxylin solution for one minute. To measure ouabain, serum and standard solutions of ouabain were placed in microplate wells, which contained anti-ouabain rabbit antiserum, for two hours at 37°C. After washing, antibodies of anti-ouabain bound to ouabain-ovalbumin and Goat Anti-Rabbit IgG-Peroxidase were added, and were incubated for another hour. The presence of remaining peroxidase enzyme was determined through adding 200 µL tetra-methyl

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benzidine substrate solution to each well. Afterwards, in addition to protecting against the light, they were incubated for 15 minutes at room temperature. The reaction terminated with an acidic solution and the reabsorption was recorded at 450 nm (14). The standard curve was used to calculate the ouabain concentration. The sodium concentration was also determined by an autoanlyser (Medica EasyLyte analyzer, Bedfor, USA). The statistical significance was determined using the one-way analysis of variance (ANOVA) and a post hoc Tukey test. The null hypothesis was rejected at the 0.05 level of significance.

Results

There were no significant differences between the groups in terms of plasma glucose levels. In addition, despite weight variations, its decline was not significantly different in the STZ groups. Serum levels of ouabain in the STZ groups on days 2 (0.2±0.05 ng/mL vs. 0.54±0.04 ng/mL), 7 (0.3±0.02 ng/mL vs. 0.59±0.06 ng/mL), and 14 (0.2±0.07 ng/mL vs. 0.65±0.08 ng/mL) after STZ injection were significantly different from the saline groups (fig 1, $p<0.05$). However, the changing trend during these two weeks was reached a plateau.

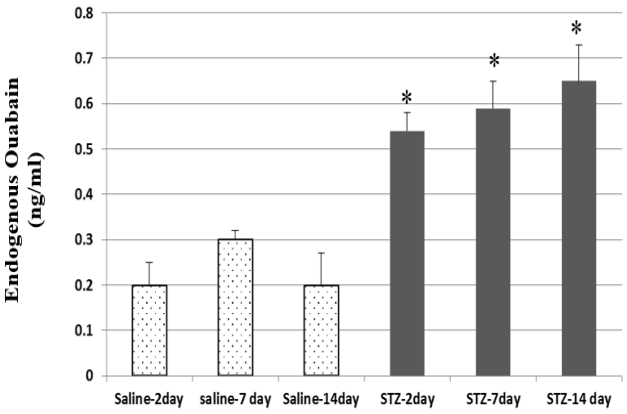


Figure 1. Alteration of serum level of endogenous ouabain in the studied groups
***p<0.05 compared to the saline groups**

The level of CSF sodium showed a significant increase after two days of STZ injection compared to the saline groups (142.6±2.9 MEq/L vs. 150.6±3.2 MEq/L, $p<0.05$; fig 2). Although there was an increasing trend in the above-mentioned index, there were no significant differences between the STZ and saline groups on days 7 (148.5±4.6 MEq/L) and 14 (147.9±3.9 MEq/L; fig 2). The results of histological analysis indicated enlarged ventricular space in the STZ groups compared to the saline groups (fig 3, B). Hippocampus cells were regularly observed in the saline groups (C). Degenerated and shrunken neurons were sporadically determined through increased extracellular spaces compared to healthy neurons (D). There was no apparent difference between the saline (E) and STZ (F) groups after staining the hippocampus region with Congo red.

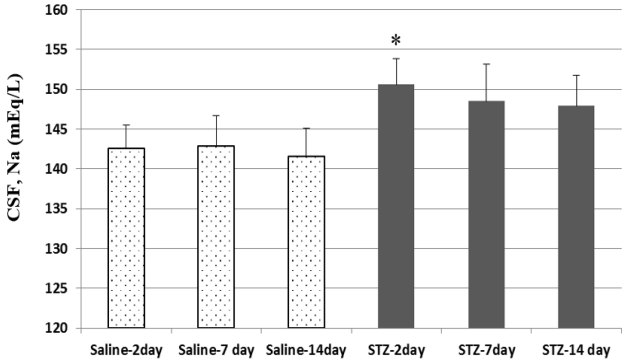


Figure 2. Alteration of the cerebrospinal fluid sodium concentration in the studied groups
***p<0.05 compared to the saline groups**

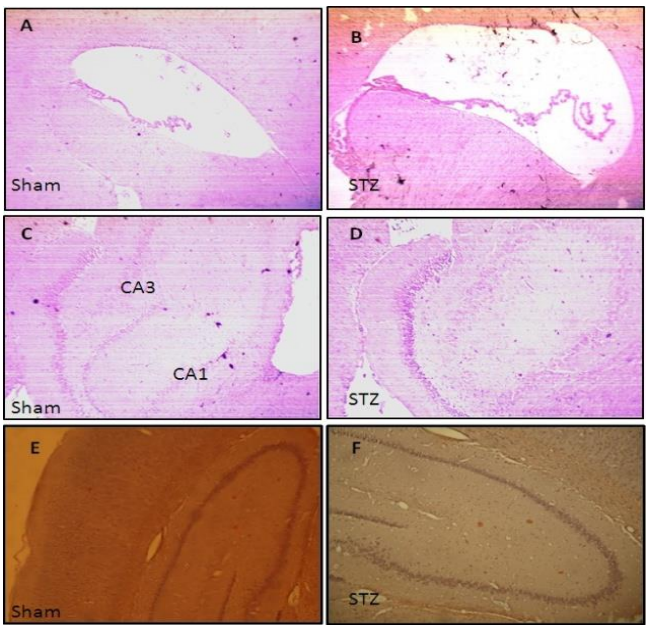


Figure 3. The effect of intracerebroventricular injection of streptozotocin on ventricular size.
(A-B) and the morphological features of hippocampus neurons (C-D) after Cresyl violet and Congo red staining (E-F) in the saline and streptozotocin groups. Degenerated and shrunken neurons were determined sporadically through increased extracellular spaces (D) compared to healthy neurons (C); magnification ×40

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Discussion

The results of the present study indicated that the rat model of sporadic Alzheimer's disease induced by ICV injection of STZ enhanced serum levels of, an endogenous sodium pump inhibitor, ouabain. ICV injection of STZ leads to diminution of energy metabolism, oxidative stress, and brain insulin resistance as well as progressive memory and learning deficits. This method can be considered as a proper animal model for inducing sporadic Alzheimer's disease (6). STZ is a nitrosamine methyl nitrous oriya, which is bound to d-glucose, and is metabolized after cellular absorption. Subsequently, N-nitrosoureide is released leading to DNA damage and cell death through production of reactive oxygen species (ROS) (15). Given the observed alterations in ventricular size and morphology of neurons in the STZ groups, degenerative changes in the mentioned model were confirmed.

In this study, ICV injection of subdiabetogenic dose of STZ had no significant effect on plasma glucose level in the STZ groups. In this study, increase of digitalis-like factor, ouabain, was in line with previous reports, which demonstrated its rise in various pathogenic conditions (16).

Digitalis-like factors are of steroid families, which are synthesized and released from the adrenal gland and brain. Their binding to sodium pump would inhibit ionic transfer, and in some cases, can activate various intercellular signaling pathways. Their effects were evaluated in physiological and pathological conditions such as hypertension, sodium homeostasis, regulation of vascular tone, and cancer (17, 18). On the other hand, there are several reports on reduced activity of sodium pump in cortical cells in culture medium and in hippocampus slices in the presence of beta-amyloid (2). A study by Bores et al. illustrated that nanomolar concentration of beta-amyloid declined sodium pump activity in hippocampus slices (2). This effect is not direct, and membrane integrity is vital for stimulating the effects of beta-amyloid on sodium pump. Thus, this effect may probably be exerted through signaling pathways. Moreover, reduction of sodium pump activity is not secondary to adenosine 5′-triphosphate (ATP) discharge, since this decline sustain in the presence of ATP. Inhibition of sodium pump activity in the presence of beta-amyloid initiates events and effects that lead to cell death (2). The reason for absence of changes in beta-amyloid in the present study may be due to short study duration. Beta-amyloid can decrease sodium pump activity through ROS production (19); however, beta-amyloid effects might be induced through the endogenous pump inhibitors.

Considering brain insulin resistance in this model, and given the significance of insulin as one of the important regulatory factors in sodium pump activity, it can be proposed that model induction stimulates internal organs to produce endogenous pump inhibitors through disrupting insulin signaling pathways and affecting the activity of endogenous pump inhibitors (20). Since molecular changes in extracellular area and brain interstitium are reflected in CSF, in case of pump activity diminution, CSF sodium might change due to lack of ion balance. Recently, there have been a number of reports indicating that elevated CSF sodium activates neuromodulatory pathways that through local aldosterone production and epithelial sodium channel function results in release of ouabain (7). Accordingly, increased CSF sodium concentration can be due to ionic gradient variation after pump dysfunction. On the other hand, it can act as a causative agent for releasing higher levels of endogenous pump inhibitors. The results of the present study revealed that the level of serum ouabain rises in sporadic Alzheimer's model induced by ICV injection of STZ.

Consequently, in pathological conditions such as Alzheimer's disease, which is accompanied with cellular metabolism disorder, endogenous sodium pump inhibitors might increase, even before development of beta-amyloid plaques; inhibition of this upturn is suggested as a novel therapeutic approach. One of the limitations of this study is small amount of collected CSF, which made the evaluation of digitalis-like factors problematic. It is recommended to examine other techniques such as high-performance liquid chromatography in future studies, which enable quantification of this substance even in small amounts.

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