



## Research Paper: Effect of Epigallocatechin Gallate and Catechin on Overexpression of *GSK-3β* and *IR* Genes Induced by Streptozotocin in Rat Brain



Marzieh Zamani<sup>1</sup>, Kambiz Rohampour<sup>1\*</sup>, Samira Rashtiani<sup>2</sup>, Masoume Dolati<sup>3</sup>, Faranak Fallahian<sup>3</sup>, Naser Kalhor<sup>4</sup>

1. Department of Physiology, School of Medicine, Neuroscience Research Center, Guilan University of Medical Sciences, Rasht, Iran
2. Student Research Committee, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran
3. Cellular and Molecular Research Center, Qom University of Medical Sciences, Qom, Iran
4. Highly Specialized Jihad Daneshgahi Infertility Center, Qom University of Medical Sciences, Qom, Iran



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**Running Title** EGCG Reduced the Expression of *IR* and *GSK-3β*

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### ABSTRACT

**Background:** Type 2 Diabetes Mellitus (T2DM) is one of the significant risk factors for Alzheimer Disease (AD). Defects in insulin signaling pathway induce AD hallmarks mainly through activation of Glycogen Synthase Kinase-3β (*GSK-3β*) pathway.

**Objectives:** In this study, we investigated the expression of *GSK-3β* and Insulin Receptor (*IR*) genes in the hippocampi of an animal model of sporadic AD and assessed the preventive effect of Catechin (CAT) and Epigallocatechin Gallate (EGCG) on their expression.

**Materials & Methods:** Adult male Wistar rats were treated by intracerebroventricular Streptozotocin (STZ) injection (3 mg/kg) at day 1 and 3 after cannulation. CAT was administered at a dose of 40 mg/kg for 10 days per gavage, and EGCG was administered at a dose of 3 mg/kg for 14 days into drinking water. Then the animals were decapitated, and their hippocampi were removed. Real-time Polymerase Chain Reaction (PCR) was used to evaluate the alteration in gene expression.

**Results:** There was overexpression in *GSK-3β* gene in STZ-treated rats ( $P \leq 0.05$ ), which was brought back to normalcy by EGCG ( $P \leq 0.01$ ). The *IR* gene also increased after STZ treatment, but CAT reduced *IR* expression ( $P \leq 0.05$ ). However, the suppressive effect of EGCG on *IR* expression was stronger ( $P \leq 0.01$ ).

**Conclusion:** The neuroprotective activity of EGCG might be due to its influence on *IR* and *GSK-3β* expression.

**Keywords:** Catechin; Alzheimer disease; Receptor Insulin; Glycogen synthase kinases; Memory

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#### \* Corresponding Author:

**Kambiz Rohampour**

**Address:** Department of Physiology, School of Medicine, Neuroscience Research Center, Guilan University of Medical Sciences, Rasht, Iran

**Tel:** +98 (912) 4358587, **Fax:** +98 (13) 33690036

**E-mail:** [rohampour@gums.ac.ir](mailto:rohampour@gums.ac.ir)

## Highlights

- The expression of *IR* and *GSK-3β* genes increased in the hippocampi of animals with STZ-induced dementia.
- Oral administration of epigallocatechin gallate reversed the overexpression of *IR* and *GSK-3β* genes.

## Introduction

**A**round the world 35.6 million people have dementia [1]. The most common form of dementia is Alzheimer Disease (AD) characterized by the accumulation of Amyloid-beta ( $A\beta$ ) plaques, and its cardinal symptoms include language breakdown, memory loss, and cognitive impairment [2]. The pathological features of AD are intracellular Neurofibrillary Tangles (NFT) composed of hyperphosphorylated tau protein and extracellular accumulation of neurotic plaques comprising misfolded amyloid peptides [3].

Many reports indicate that oxidative stress is high in the brain of patients with AD or mild cognitive impairment [4]. Oxidative stress may be the first sign in the brain of patients with AD, which appears before  $A\beta$  deposition [5-7]. Transgenic mice bearing a mutant Amyloid Precursor Protein (APP) also show the primary presence of oxidative stress before  $A\beta$  deposition [8]. Evidence suggests that cells try to protect themselves from oxidative stress by secretion and deposition of  $A\beta$  plaques in vulnerable neurons [9]. Abnormal metabolism of APP and  $A\beta$  in mitochondria will damage the mitochondrial function by increasing Reactive Oxygen Species (ROS) and decreasing Adenosine Triphosphate (ATP) production [10]. Specifically, secretion of  $A\beta$  (to sequester ROS) eventually destroys cellular integrity; the peptide becomes subject to oxidative damage that causes its oligomerization and aggregation, which in turn produces neuroinflammation [11, 12]. Other consequences of cellular oxidative damage include cell cycle aberration and tau hyperphosphorylation, leading to the formation of NFTs [13, 14].

Catechin (CAT) is a polyphenolic flavonoid which has neuroprotective effects against oxidative stress [15]. Recently this class of antioxidants has drawn attention because of their benefits, especially in the prevention and treatment of cancer, as well as cardiovascular, inflammatory, and neurodegenerative diseases [16, 17]. Some reports claim that CAT protects cultured mesencephalic neurons against 6-hydroxydopamine (6-OHDA) treatment and also prevents primary hippocampal neurons

from  $A\beta$  toxicity [18, 19]. Evidence suggests that damages caused by  $A\beta$  can be undermined by antioxidants or polyphenols [20]. Epigallocatechin-3-Gallate (EGCG), the most abundant CAT in tea, also has neuroprotective effects. Some reports argue that CATs are more effective than vitamin E and C in the destruction of free radicals [21]. These antioxidants could be the primary candidates for the prevention and treatment of AD.

Insulin Receptors (IRs) are widely distributed in the brain with variable densities in different brain areas, the highest concentration being found in the olfactory bulb, cerebral cortex, hippocampus, cerebellum, and hypothalamus [22]. Some evidence suggests that insulin signaling plays a role in synaptic plasticity and long-term memory consolidation [23]. Activated *IR* phosphorylates Insulin Receptor Substrate (IRS), which in turn activates Phosphoinositide 3-Kinase (PI3-K). Activated PI3-K/Akt inhibits both  $\alpha$  and  $\beta$  forms of Glycogen Synthase Kinase-3 (GSK-3) [24]. Insulin inhibits abnormal tau hyperphosphorylation by stimulating Akt-induced inactivation of *GSK-3β* [25]. Therefore, disturbance in insulin signaling cascade increases tau hyperphosphorylation and thus NFT formation [26].

In this study, we evaluated the effects of CAT as a potent antioxidant on insulin signaling cascade. Notably, we examined whether CAT and EGCG could affect the hippocampal expression of insulin receptor (*IR*) and *GSK-3β* genes as potential signaling pathways involved in AD pathogenesis.

## Materials and Methods

### Animals

Adult male Wistar rats (weight: 250-300 g) were obtained from the Pasteur Institute of Iran. Rats were housed four animals per cage at a temperature-controlled room ( $22\pm 2^\circ\text{C}$ ), with a 12-12 hour light-dark cycle. Rats had access to food and water ad libitum. Efforts were made to minimize animal suffering and the number of animals used. All studies were performed following the ethical guidelines of the "Ethics Committee of Faculty of Medicine, Qom University of Medical Sciences", which

were based on the “NIH Guide for the Care and Use of Laboratory Animals.”

### Experimental procedure

Rats were divided into 5 groups (n=3 for each group): Intact, CAT, Streptozotocin (STZ), STZ+CAT, and STZ+EGCG. The STZ group received 3 mg/kg Intracerebroventricular (ICV) STZ. The CAT group received 40 mg/kg CAT by daily gavage for 10 days. STZ+CAT group received CAT after the first ICV STZ injection by gavages for 10 days, and in the STZ+EGCG group, after the first ICV STZ injection, the EGCG was administered 3 mg/kg for 14 days into drinking water. This dose of CAT prevents oxidative stress and can reverse oxidative markers in the liver and kidney [27].

### Surgical procedure

Rats were anesthetized with a mixture of ketamine (80 mg/kg, Intraperitoneally IP) and xylazine (20 mg/kg, IP) and were bilaterally implanted with cannulae (23-gauge) in the lateral ventricles with the following coordinates; AP: -0.8 mm from Bregma, ML: ±1.5 mm from midline and Dorsoventral (DV): -2.6 mm from the skull surface according to the atlas of Paxinos and Watson [28]. Two screws were inserted into the skull, and the cannulae fixed to them with dental cement. Three days after the operation, the rats received ICV STZ by an injection needle.

### Microinjection procedure

Intracerebroventricular injections were made via guide cannulae with injection needles (30-gauge) that were connected by polyethylene tubing to a 10 µL Hamilton microsyringe. The injections (5 µL per site) were delivered over 2 min, and the injection needles (extending 1 mm from the end of the guide cannulae) were left in place an additional 3 min before they were slowly withdrawn. On days 1 and 3, after the surgery, the animals received 5 µL per site, vehicle Artificial Cerebrospinal Fluid (aCSF), or 3 mg/kg STZ dissolved in aCSF.

### Gene expression assay using a quantitative polymerase chain reaction

All animals were decapitated, and their brains were incubated in chilled aCSF. The total Ribonucleic Acid (RNA) was extracted from hippocampus samples using the TRizol reagent (Invitrogen Technology, Carlsbad, CA, USA) according to the manufacturer’s protocol and stored at 80°C until further use. The RNA concentration was quantified by Ultra Violet (UV) spectrophotometer

at 260 nm, and its purity and integrity were determined using the A260/A280 ratio. Total RNA was treated with DNase and then reverse transcribed into the first-strand cDNA in a 20 µL reaction volume using RevertAid M-MuLV Reverse Transcriptase, according to the manufacturer’s protocol (Vivantis Inc, Malaysia).

Quantitative Real-time Polymerase Chain Reaction (RT-PCR) assays of *GSK-3β* and *IR* cDNA were carried out using the SYBR Green kit (Qiagen, Inc.) in an Applied Biosystems (ABI) 7500 Sequence Detection System in accordance with the manufacturer’s recommendations. A dissociation curve was generated at the end of each PCR reaction to verify the amplification of a single product. Identical PCR conditions were performed using 1 µg of cDNA, and the relative expression levels of genes were normalized to the endogenous housekeeping gene Glyceraldehyd 3-phosphate Dehydrogenase GAPDH. The relative mRNA expression level was determined using the 2- $\Delta\Delta C_t$  analysis method. Table 1 presents the primers used for real-time RT-PCR.

### Statistical analysis

The obtained data were analyzed in SPSS V. 20. The normal distribution of the data was examined by the Shapiro-Wilk test, and the non-parametric equivalent of One-Way Analysis of Variances (ANOVA). The Kruskal-Wallis test was used to assess the significance of the results. The study values were expressed as Mean±SEM and P<0.05 was considered as the level of significance in all comparisons.

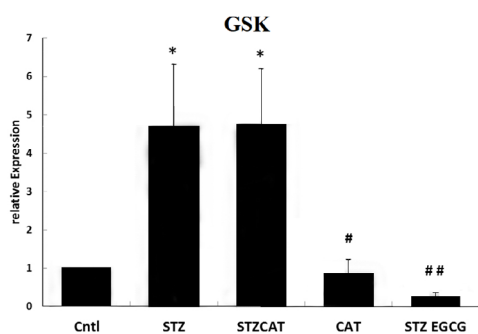
### Histology

The animals were deeply anesthetized, decapitated, and their brains were removed and fixed in formalin. The place of cannulae and injection needle were verified by histological examination. For this purpose 100 µm thick sections were taken, and cannulae and needle traces were examined for each side with light microscopy. Only the obtained data from the animals whose cannulae and injections were in the lateral ventricles were used for analysis.

### Results

#### Effect of STZ on *IR* and *GSK-3β* expression in the hippocampus

Using qRT-PCR, we determined the expression level of the *IR* and *GSK-3β* genes in the hippocampus, 10 days after ICV injection of STZ. As shown in Figures



**Figure 1.** Relative expression of *GSK-3β* gene in the hippocampus

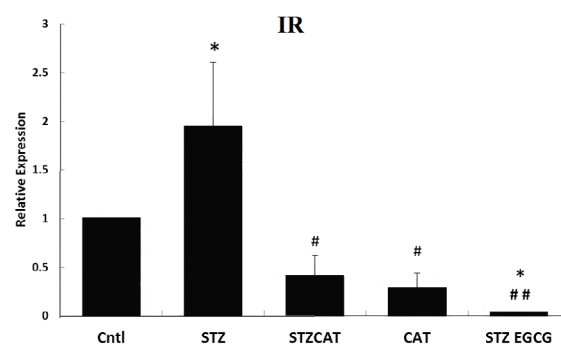
STZ treatment increased the expression of *GSK3β*; while EGCG treatment after STZ reversed the expression of *GSK3β* (Kruskal-Wallis test; \* $P \leq 0.05$  in comparison to control; # $P \leq 0.05$  in comparison to STZ group; ##  $P < 0.01$  in comparison to STZ group)

1 and 2, two STZ injections in the ventricles led to a significant ( $P < 0.05$ ) upregulation in both the *GSK-3β* and *IR* mRNA levels compared to the untreated rats. The enhancing effect of ICV STZ treatment on *GSK-3β* gene expression was much more than its influence on *IR* expression.

#### Catechin treatment decreased *IR*, but not *GSK-3β* expression in the hippocampus

CAT gavage for 10 successive days did not affect *GSK-3β* mRNA content in the intact rats or the STZ-treated ones (Figure 1), but it could prevent the *IR* overexpression induced by ICV STZ treatment. CAT gavage significantly ( $P < 0.05$ ) reduced the *IR* gene expression in comparison to the ICV STZ-injected rats (Figure 2).

#### EGCG treatment decreased *IR* and *GSK-3β* expression in the hippocampus



**Figure 2.** Relative expression of Insulin Receptor (*IR*) gene in the hippocampus

STZ treatment increased the expression of *IR*; CAT and EGCG treatment prevented the increase in *IR* (Kruskal-Wallis test; \* $P \leq 0.05$  in comparison to control; # $P \leq 0.05$  in comparison to STZ group; ##  $P < 0.01$  in comparison to STZ group)

There was overexpression in *IR* and *GSK3β* values in STZ group ( $P < 0.05$ ), which was reversed to normal level by EGCG administration ( $P < 0.01$ ). The suppressive effect of EGCG on both genes was stronger in comparison to CAT effect (Figures 1 & 2).

### Discussion

The present study was designed to evaluate the effect of CAT and EGCG on *IR* and *GSK-3β* expression in the model of dementia, which could further elucidate the impact of oxidative stress in AD pathophysiology. Our results in other studies showed memory impairment after two sets of STZ injections (3 mg/kg). The administration of ICV STZ is characterized by progressive deterioration of memory, cerebral glucose, and energy metabolism that leads to cognitive dysfunction [29, 30]. STZ enters the cells via the Glucose Transporter-2 GLUT-2, which is most abundant in the limbic system [31]. Insulin reduces tau phosphorylation by inhibition

**Table 1.** Primer sequences used in real-time PCR

Gene	Primer Sequences (5' to 3')	Primer Length (bp)
GSK-3β	Forward: 5'-GAGCTGATCTTTGGAGCCAC-3'	20
	Reverse: 5'-CCAAGTATCCACACCACTG-3'	
IR	Forward: 5'-CAGCTGGAAAAGGCTACAGG-3'	20
	Reverse: 5'-TCCCTTACATCGATGCCTTC-3'	
GAPDH	Forward: 5'-TGGCCTCCAAGGAGTAAGAA-3'	20
	Reverse: 5'-TGTGAGGGAGATGCTCAGTG-3'	

of *GSK-3β* via the PI3-K pathway [32]. Therefore, disturbance in insulin signaling cascade leads to an increase in tau hyperphosphorylation potentiating and the formation of NFT [26].

Prolonged consumption of green tea extract, rich in CAT, could also prevent age-related biochemical and morphological changes in the rat hippocampal formation [33]. Prolonged CAT administration prevented either age-related reductions of Postsynaptic Density proteins (PSD-95) and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II, suggesting that synaptic structural changes may also be involved in its mechanism of action [34].

Many reports suggest that Aβ formation is a protective response to oxidative stress [35]. An initial free radical-induced injury would exacerbate a vicious cycle in which the amyloidogenic processing of APP would be further enhanced, generating more Aβ that, in turn, would cause more oxidative stress [36]. CAT supplementation could significantly decrease lipid peroxidation, H<sub>2</sub>O<sub>2</sub> generation, and protein carbonyl contents in tamoxifen-induced oxidation, which could explain, at least in part, the memory-improving effect of CAT [27, 37].

Emerging evidence has suggested that insulin signaling cascade alters gene expression that is required for long-term memory consolidation [23]. Insulin dysfunction, which occurs in AD leads to *GSK-3β* activation, which in turn phosphorylates tau and reduces its affinity for microtubules and thereby potentiating NFT formation [38]. Training-induced upregulation of *IR* mRNA is reported in the Cornu Ammonis (CA1) region of hippocampus [39]. Agrawal R, et al. reported a significant decrease in *IR* mRNA and protein level in CA3 region of hippocampus after ICV STZ treatment [39]. Our data showed a significant increase in *IR* mRNA in STZ-treated rats, which was reversed by CAT gavage and EGCG administration. This event could be caused by our mRNA sampling, extracted from the whole hippocampus. Osmanovic J, et al. showed a significant increase in *IR* mRNA content in the hippocampus of ICV treated STZ rats after two weeks, which was not accompanied by a change in protein expression [40]. Agrawal R, et al. showed that *IR* levels in the CA1 and Dentate Gyrus (DG) regions remained unchanged after ICV STZ injection [39].

Our results demonstrated a significant increase in *GSK-3β* mRNA after STZ treatment, which is consistent with expected sporadic AD pathogenesis, namely, tau hyperphosphorylation, NFT and amyloid formation [41]. However, CAT did not reverse its overexpression, but EGCG could reverse it. Other reports indicate that

total *GSK-3β* mRNA did not change shortly after ICV STZ injection, but an acute activation of this enzyme occurs as early as 2 weeks after ICV STZ treatment [40]. Decreased phosphor- to total- (p/t) *GSK-3β* is reported by others up to 1 month after STZ injection. Although the expression of tau in the hippocampus is reported to increase even 2 weeks after ICV STZ treatment, the p/t tau ratio remains unchanged until 1 month, which may be because of the activation of Protein Phosphatase 2A (PP2A), the major tau phosphatase [40, 42, 43].

## Conclusion

The results of this study emphasize that CAT treatment could reduce *IR* expression induced by ICV STZ, while it did not reverse *GSK3β*. Therefore CAT treatment could affect insulin signaling by preventing alterations in *IR*, but not *GSK-3β* mRNA expression induced by ICV STZ treatment. On the other hand, EGCG could reverse *IR* and *GSK3β* expression, which increased by STZ. Thus it can be hypothesized that the neuroprotective activity of EGCG might be due to its influence on *IR* and *GSK3β* expression.

## Ethical Considerations

### Compliance with ethical guidelines

The study protocol was approved by the Ethics Committee of Qom University of Medical Sciences (No. 91281). All study procedures were in compliance with the ethical guidelines of the Declaration of Helsinki 2013.

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### Authors contributions

Design of the study: Kambiz Rohampour; Data gathering: Marzieh Zamani, Masoumeh Dolati, Naser Kalhor; Initial draft: Marzieh Zamani, Samira Rashtiani; Writing, revision, and editing the article, and final approval of the study: All authors.

### Conflict of interest

The authors declared no conflict of interest.



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