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Effect of caffeine on Alzheimer's molecular factors in correlation with involved cell communication systems in developing zebrafish *Danio rerio*

Wirkung von Koffein auf die molekularen Faktoren der Alzheimer-Krankheit in Korrelation mit den beteiligten Zellkommunikationssystemen bei der Entwicklung von Zebrafisch *Danio rerio*

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Abstract

Background: Epidemiological studies suggested that caffeine/coffee could be an effective therapeutic agent against Alzheimer disease (AD). The mechanism has not been well established; however, molecular genetic analyses suggest that many genes influence it.
Methods: Using developing zebrafish (*Danio rerio*), we studied the regulatory effect of caffeine on AD molecular factors, APP, Psen1, Psen2, ApoE, and Sorl1, and on receptor expression of two cell communication systems involved in the disease, adenosine (AR) and dopamine receptors (DR).
Results: All genes are already expressed at early developmental stages. No morphological changes were found at tested concentrations and control. Caffeine significantly down-regulated the expression of all AD tested genes at 24 h post-fertilization (hpf) and APP, Sorl1, and Psen1 at 96 and 168 hpf. A_{2aa} and A_{2ab} receptors have higher affinity for caffeine than A_{2b}. Significant down-regulation occurred in A_{2b} at 168 hpf in both concentrations. Caffeine blocked the expression of drd_{2a} and drd_{2c} at 24 hpf but significantly stimulated the expression at 96 and 168 hpf.
Conclusions: Zebrafish is a promising organism in studying AD at the molecular level because all tested factors are already expressed at early developmental stages. Caffeine has a regulatory effect on all tested genes and may protect against the disease via amyloid pathway as well as AR and DR.

Keywords: adenosine and dopamine; Alzheimer disease; amyloid pathway; caffeine; zebrafish.

Zusammenfassung

Hintergrund: Epidemiologische Studien geben zur Annahme Anlass, dass Koffein/Kaffee ein wirksames therapeutisches Mittel gegen Alzheimer (AD) sein könnte. Der Mechanismus ist noch nicht klar genug nachgewiesen, jedoch deuten molekulargenetische Analysen darauf hin, dass viele Gene ihn beeinflussen.
Methoden: Mit der Entwicklung von Zebrafisch untersuchten wir die regulierende Wirkung von Koffein auf AD molekulare Faktoren; von APP, Psen1, Psen2, ApoE und Sorl1 auf Expressionen von Rezeptoren von zwei von der Krankheit befangenen Zellkommunikationssystemen und jeweils auf Adenosin und Dopamin-Rezeptoren.
Ergebnisse: Alle Gene sind bereits in frühen Entwicklungsstadien ausgedrückt. Es wurden keine morphologischen Veränderungen bei getesteten Konzentrationen und der Kontrolle festgestellt. Koffein hat den Ausdruck aller AD getesteten Gene signifikant nach 24 Stunden nach der Befruchtung (hours post-fertilization – hpf) herunterreguliert und APP, SORL1 und PSEN1 nach 96 hpf und 168 hpf herunterreguliert. A_{2aa}- und A_{2ab}-Rezeptoren besitzen eine höhere Affinität für Koffein als A_{2b}. Eine wesentliche Herabregulierung trat für A_{2b} bei 168 hpf in beiden Konzentrationen auf. Koffein blockierte die Expression von drd_{2a} und drd_{2c} bei 24 hpf, stimulierte jedoch die Expression bei 96 und 168 hpf deutlich.
Schlussfolgerungen: Zebrafisch ist vielversprechend als ein Organismus zum Studium von AD auf molekularer

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Ebene, da alle untersuchten Faktoren bereits in frühen Entwicklungsstadien ausgedrückt sind. Koffein wirkt regulierend auf alle getesteten Gene und kann gegen die Krankheit über Amyloid-Weg sowie auch durch Adenosin- und Dopaminrezeptoren seine Schutzwirkung entfalten.

Schlüsselwörter: Adenosin und Dopamin; Alzheimer-Krankheit; Amyloid Signal- und Leitweg; Koffein; Zebrafisch.

Introduction

Epidemiological studies have increasingly suggested that caffeine/coffee could be an effective therapeutic agent against Alzheimer disease (AD) [1]. AD is the most common form of neurodegenerative disease, with more than 20 million cases worldwide [2]. The mechanism in which caffeine may protect against AD has not been well established; nevertheless, molecular genetic analyses suggest that there are likely to be many genes that influence one's susceptibility to AD [3].

AD is generally characterized by the presence of neurofibrillary tangles and amyloid deposits that form plaques and cerebrovascular accumulations [4]. Extensive research on the mechanisms that underlie the disease has led to the identification of a number of genetic, environmental, and lifestyle factors that significantly contribute to increased risk of developing AD and play major roles in the pathogenesis of the disease [5]. However, those genes may increase the risk of developing the disease, and the most well-established link between AD and genetics is in familial early-onset AD. The discovery of genetic aberrances that either cause or increase the risk of AD heralded a rapid increase in the knowledge of the molecular and cellular alterations responsible for neuronal degeneration and cognitive dysfunction in AD [6]. One of the most intensively studied molecules is APP [7, 8]. It is known as the main controller of senile plaques that accumulate in the brain and cause neurologic disorders. Enormous scientific efforts have been put into APP-related studies mainly because of its vital pathophysiological functions in AD [9]. Two other genes linked to early-onset familial AD are those encoding Psen1 and Psen2. They are structurally similar integral membrane proteins with eight transmembrane domains and are localized mainly in the endoplasmic reticulum (ER). The presenilin proteins have been shown to play important roles in apoptosis, calcium homeostasis, cell cycle regulation, regulation of misfolded proteins in the ER, and cleavage of APP [10].

Also, one of the most widely studied risk factors for sporadic AD is apolipoprotein E (ApoE). An allele of this gene, ApoE4, has been associated with increased risk for late-onset AD. People who carry one or two copies of the ApoE $\varepsilon 4$ allele carry an increased risk of developing AD; however, the $\varepsilon 4$ allele is not necessary or sufficient to cause AD [11]. ApoE associates with lipoprotein particles and facilitates their interaction with lipoprotein receptors [12]. The gene coded by Sorl1 is a neuronal ApoE receptor. The lack of the ApoE receptor is suspected to be a contributory factor to AD [13].

For decades now, zebrafish (*Danio rerio*) have become a promising model in many research areas, including neuroscience, developmental biology, and toxicology [14–17]. Three distinct zebrafish adenosine receptors (ARs), A_{2aa}, A_{2ab}, and A_{2b}, were discovered [18]. Adenosines are biological endogenous purine nucleosides that modulate a variety of physiological processes. They play an important role in signal transduction, which increases drastically during brain ischemia caused by stroke. They are also potent anti-inflammatory agents that play an important role in tissue protection and repair [19]. In the central nervous system, adenosine is involved in regulating neurotransmitter release as well as postsynaptic neuronal responses [20].

The effects of the modulation of A_{2a} gene expression on normal aging and in pathological conditions as AD are still unclear, but the use of non-selective antagonists like caffeine to treat AD-related cognitive deficits is showing promising results [18, 21, 22]. In our present work, we tested the effect of caffeine on the mRNA expression of a package of confirmed AD-involved genes in two concentrations, 10 and 100 μM, and analyzed the correlation of two major transmitter systems in the cell communications systems, adenosine (A_{2aa}, A_{2ab}, and A_{2b}) and dopamine (drd_{2a} and drd_{2c}) receptors, with mRNA expression in order to understand the mechanisms of the disease in this model and improve the pharmaceuticals for this disease.

Materials and methods

Zebrafish maintenance

Zebrafish (*Dario rerio*) were obtained locally and maintained under our established laboratory housing system. Adult zebrafish were kept in glass aquaria under recirculation system with a photoperiod cycle of 14 and 10 h, light and dark, respectively, and temperature of 28 °C. They were fed three times a day with commercial dried pellet (Tetra Werke, Melle, Germany).

Breeding and egg collection

Two pairs of males and females were kept separately in spawning plastic boxes containing a mesh bottom to prevent the spawned eggs from being cannibalized. The mating boxes were incubated overnight for about 10 h in a 28 °C incubator. On the next day, the barriers were removed at the beginning of the light period to allow mating and spawning. We followed the Care and Treatment of the Animals guidelines by the Institutional Animal Care and Use Committee, Seoul National University (approval no. SNU-050418-2). The technical procedures were according to the zebrafish guidelines book [23].

Caffeine solutions and exposure

A stock solution with a concentration of 1 mM was prepared by dissolving caffeine powder (1,3,7-trimethylxanthine; Reagent plus W, powder, CAS no. 58-08-2; Sigma-Aldrich, Seoul, South Korea) in distilled water then diluted to obtain 10- and 100-μM concentrations.

Experiment design

The fertilized eggs were collected and washed twice with E3 medium (embryo medium) [23]. About 2 hpf (cleavage stage 32–64 cells), eggs were distributed randomly into three 12-well plates to start exposure, one plate for each checkpoint. The plates were filled with 3 mL of exposure solution 10 μM, exposure solution 100 μM, or distilled H_2O in three biological replicates for each treatment. At 24 hpf, the embryos from the first plate were prepared for molecular analysis. A 1.5-mL exposure solution of the other plates was replaced with a fresh one daily until the end of the experiment.

Monitoring assay

Hatching, survival rate, and phenotype abnormalities were monitored in all experimental sets. Monitoring assays were applied separately in another 12-well plate to check the morphological change from 24 hpf until 168 hpf, once every 24 h.

Molecular analysis

Embryos from each treatment were pooled and prepared for total RNA extraction using the protocol provided by Chen Laboratories, Department of Chemical and Systems Biology, Stanford University [24]. cDNAs were synthesized from extracted total RNA using TOPscript™ cDNA Synthesis kit (CAT EZ005S). Polymerase chain reaction (PCR) was performed according to kit provider guidelines (AccuPower® PCR PreMix, Cat: K-2016). The reactions were done by mixing 1 μL cDNA, 1 μL forward primer, and 1 μL reverse primer with distilled water until the mixture reaches 20 μL. The thermal cycling protocol was 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 40 s, then one cycle at 72 °C for 2 min. Band intensity was measured by ImageJ program (http://imagej.nih.gov/ij/index.html). Real-time (RT)-PCR (qPCR) was performed according to Takara Bio guidelines. A 25-μL RT-PCR reaction was done by adding 1 μL cDNA, 1 μL forward primer, 1 μL reverse primer, 12.5 μL SYBR Premix Ex Taq (Takara Bio, Shiga, Japan), and 9.5 μL of nuclease-free water (Ambion, Austin, TX, USA) to give the reaction a final volume of 25 μL. The reaction was performed using a Bio-Rad Real Time PCR System (CFX Connect™ Real-Time PCR Detection System and CFX Manager Version 2.1.1022.0523 to analyze data) according to the company's instructions. The thermal profile for RT-PCR was 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 40 s. We used β-actin as a housekeeping gene to normalize the results by eliminating variations in mRNA and cDNA quantity and quality. Three technical replicates of each RNA sample were performed. Relative mRNA expression for each gene was calculated as fold change compared with the control group using $2^{\Delta\Delta ct}$ formula method. All primers used in this experiment are described in Table 1.

Statistical analysis

Relative mRNA expression results were shown as the mean±SE of relative normalized expression of RT-PCR values. We performed two-way analysis of variance (ANOVA) to analyze the data, followed by Tukey multiple comparison tests. The results of the optical density of mRNA expression were also analyzed using two-way ANOVA followed by Bonferroni post-tests (*significant difference occurred for a given parameters when $p<0.05$; **high significance when $p<0.01$). The entire statistical analysis was carried out using Graphpad Prism (Version 4.03).

Table 1: Genes identification and specific primers used in PCR and Real Time – PCR analysis.

No.	Gene	Accession number	Forward primer	Reverse primer	Product size, bp
1	*β-Actin*	BC067566.1	AAGGCCAACAGGGAAAAGAT	AGGGCGTAACCCTCGTAGAT	176
2	*APP*	BC068375.1	CGACCAGTGTCTGGACTGAA	GCTTCTTCCTCAGCATCACC	205
3	*Psen1*	BC054639.1	CAGTCCCTCAGCAGGAGAAC	AAATCTCCCAAACCCAGCTT	223
4	*Psen2*	BC065382.1	ATTCTGTCCTCGCTGATGCT	ATGAAGATGAGGGCCATGAG	215
5	*ApoE*	BC154034.1	ATGCAGTGAAGGAAGGACCGTTTC	CGTAGGTTCTCGGCTGTCT	175
6	*Sorl1*	XM003200038.2	CCATACATGGGTCCTCCATC	GCTCTCGGTTTTTCGAACTG	195
7	*A2aa*	NM_001039815.1	ATCATCGTTGGTTTGTTCGCC	CCACTGAGTTTGCGTGTGAGA	139
8	*A2ab*	NM_001040036.1	CCGAGAGGAAGTCTCCTCCA	CCAGCCACATTCGGGTCAT	197
9	*A2b*	NM_001039813.2	GGATTCGCTCTACATCGCCA	AGTGATGGCAAAGGGGATGG	181
10	Drd_{2a}	AY183456.1	ACATCTTCGTCACCCTGGAC	CGCAATCACACAGAGAGCAT	242
11	Drd_{2c}	AY333792.1	TTATGCCCTGGGTGGTGTAT	CCCGTCTCTTGGAGCTGTAG	195

Results

Monitoring assay

A caffeine concentration of 100 µM, which has no effect on the locomotor activity, has been approved previously. Here, we did not observe morphological abnormalities during the experiment in both concentrations as well as in the control. Hatching started at about 48 hpf in all treatments and control. There was no difference in the overall hatching rate between treatments and control (data not shown). The overall survival rates were 84%, 78%, and 76% for control, 10 µM, and 100 µM, respectively, with no significant difference ($p>0.05$) (Figure 1).

Molecular analysis

Caffeine treatment was conducted to zebrafish embryos at three different ages, 24, 96, and 168 hpf, to assess the effects of all selected genes on mRNA expression. All tested genes are already expressed during early developmental stages.

Optical density of AR expression

We analyzed the expression of each AR by measuring the optical density of mRNA expression using ImageJ software. Caffeine altered the mRNA expression of all tested receptors (Figure 2). At 24 hpf, 10 µM of caffeine significantly up-regulated the expression of A_{2aa} and A_{2ab} genes ($p<0.05$) and highly significantly up-regulated the expression at 100 µM ($p<0.02$). The changes in expression at 96 and 168 hpf had no significant difference with the control, except at 100 µM ($p<0.05$). The effect of caffeine on AR A_{2b} was controversial because of the fluctuation of its expression at the different check points.

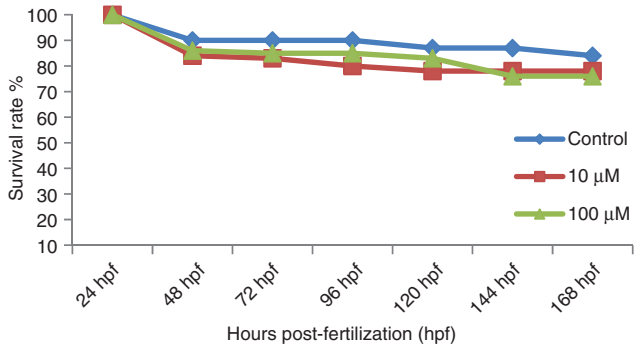

Figure 1: Survival rate of developing zebrafish after exposure to caffeine.
We checked the survival rate every 24 h from 24 to 168 hpf. Values represent the means±SE.

Optical density of dopamine receptors mRNA expression

The expression of dopamine receptors (DRs) was also analyzed by measuring the optical density of mRNA expression (Figure 3). Although the expressions of both receptors were low at 24 hpf in control, caffeine almost blocked the signal, especially in 100 µM, with significant difference compared with control ($p<0.05$). Interestingly, 10 and 100 µM of caffeine significantly up-regulated the expression of drd_{2a} at 96 and 168 hpf, respectively, and non-significantly at other checkpoints. Also, caffeine significantly up-regulated the expression of drd_{2c} at both 10- and 100-µM concentrations at 96 hpf and at 100-µM concentration at 168 hpf.

Relative quantitative of AD gene expression

All selected genes involved in the AD pathway, *APP*, *Psen1*, *Psen2*, *ApoE*, and *Sorl1*, are also already expressed at early developmental stages of zebrafish embryos. First, the control was adjusted to a value of 1, and the expression changes of all tested genes were calculated as fold change compared to the control. Caffeine altered the gene expression at most checkpoints (Figure 4). A down-regulation occurred at the expression at 24 hpf. A caffeine concentration of 10 µM significantly ($p<0.05$) down-regulated all tested genes except *APP*. A highly significant ($p<0.01$) down-regulation occurred in 100-µM caffeine treatment. The highest down-regulation was found in the *APP* gene, about 0.4-fold compared to control. Thus, at 24 hpf, caffeine down-regulated the expression all tested genes and the effect increases with the higher concentration, 100 µM. At 96 hpf, continuous exposure to caffeine showed a different effect on the expression of all the tested genes. With the 10-µM treatment, *Sorl1*, *ApoE*, and *Psen2* genes were down-regulated by 0.6-, 0.6-, and 0.5-fold change, respectively, with significant difference with control ($p<0.05$). At 100-µM treatment, the down-regulation was non-significant in *Sorl1* and *Psen1*, with ~0.9-fold changes with control. The 10-µM caffeine concentration significantly up-regulated the expression of *APP* and *Psen1* by 1.2- and 1.9-fold changes in control; however, the 100-µM treatment significantly up-regulated the expression of *APP* and *Psen2* by 2.6- and 1.2-fold changes, respectively, with control and non-significantly in ApoE by 1.1-fold change.

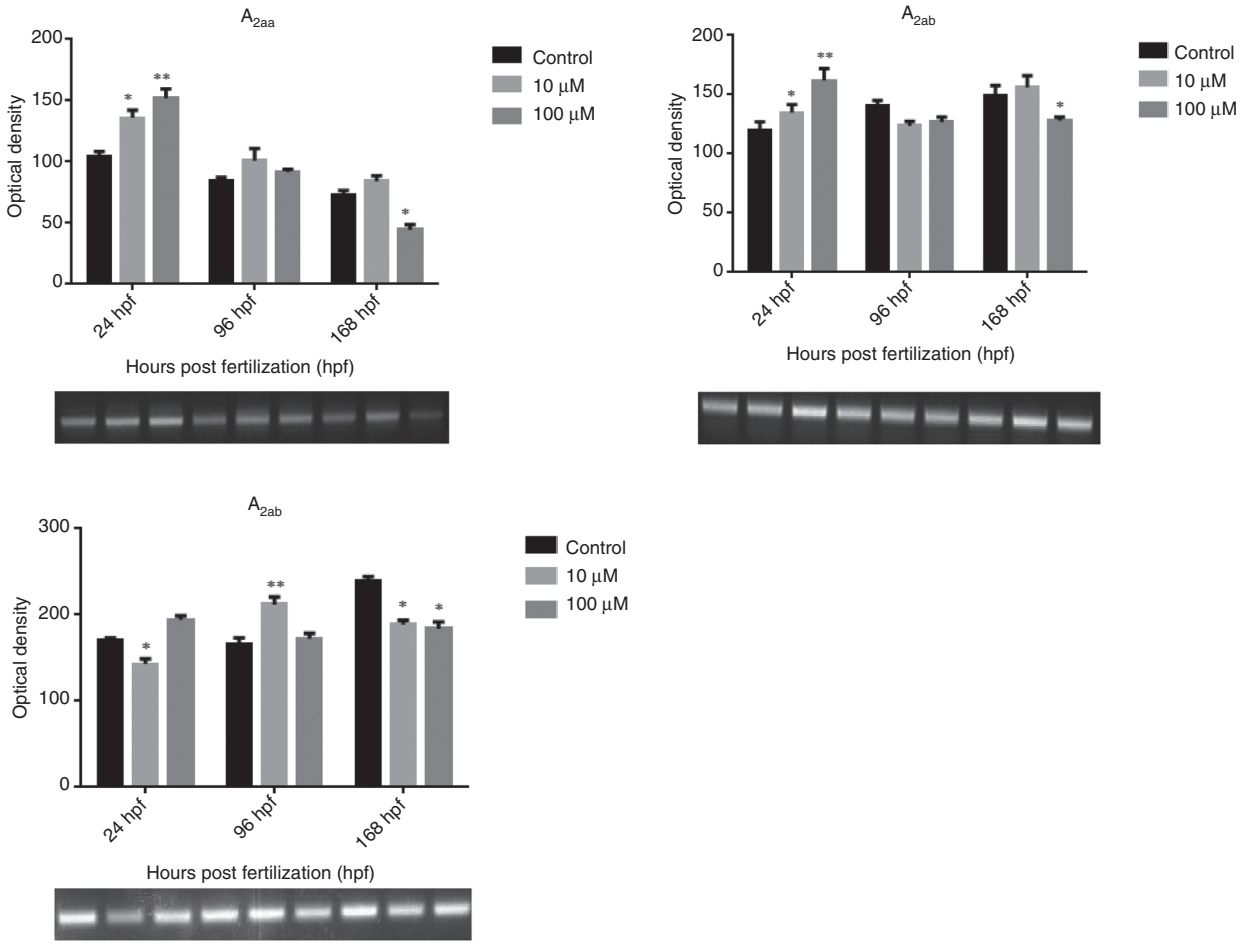

Figure 2: Effect of caffeine on the gene expression of ARs at 24, 96, and 168 hpf.
Embryos from each treatment were pooled and prepared for total RNA extraction. cDNAs were synthesized from extracted total RNA. PCR was performed according to kit provider guidelines. The reactions were done by mixing 1 µL cDNA, 1 µL forward primer, and 1 µL reverse primer with distilled water until the mixture reaches 20 µL. The thermal cycling protocol was (95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 40 s, then one cycle at 72 °C for 2 min. Band intensity was measured by ImageJ program. A_{2aa} is adenosine receptor 2aa, A_{2ab} is adenosine receptors 2ab, and A_{2b} is adenosine receptor 2b. *Significant difference with control ($p<0.05$); **high significant difference with control ($p<0.02$).

To identify expression pattern, mRNAs from developing zebrafish larvae at 168 hpf were analyzed after the completion of neurodevelopment. Caffeine significantly down-regulated *Sorl1* and *APP* at 10 and 100 µM, but *Psen1* was only down-regulated at a caffeine concentration of 100 µM. *ApoE* was down-regulated in the 10- and 100-µM treatments, with no significant difference with control. Also, the down-regulation of *Psen1* was non-significant in the 10- and 100-µM treatments.

Discussion

Caffeine was suggested as an effective therapeutic agent against AD. Confirmed genetic susceptibility factors for AD were studied in this present work. We analyzed the expression of *APP*, *Psen1*, *Psen2*, *ApoE*, and *Sorl1* genes in developing zebrafish embryos at 24, 96, and 168 hpf after exposure to two different concentrations of caffeine, 10 and 100 µM. We also studied the effect on the expression of two cell communication systems associated to AD, adenosinergic and dopaminergic systems, by analyzing the expression of their receptors. We found that all tested genes are already expressed at early phases of development, with a pattern of fluctuation during the development and as a result of caffeine treatment.

First, it was reasonable to use 100-µM concentration of caffeine because it has no effects on the locomotor activity and does not promote significant embryotoxicity or phenotypic features [25, 26].

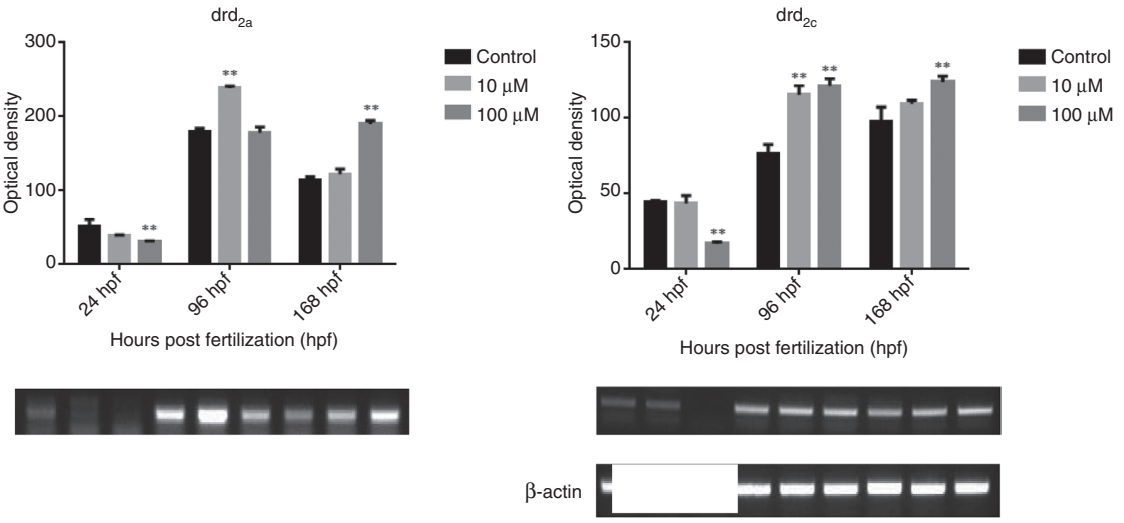

Figure 3: Effect of caffeine on the gene expression of DRs at 24, 96, and 168 hpf.
Embryos from each treatment were pooled and prepared for total RNA extraction. cDNAs were synthesized from extracted total RNA. PCR was performed according to kit provider guidelines. The reactions were done by mixing 1 μL cDNA, 1 μL forward primer, and 1 μL reverse primer with distilled water until the mixture reaches 20 μL. The thermal cycling protocol was 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 40 s, then one cycle at 72 °C for 2 min. Band intensity was measured by ImageJ program. drd_{2a} is dopamine receptor 2a and drd_{2c} is dopamine receptor 2c. *Significant difference with control ($p<0.05$); **high significant difference with control ($p<0.02$).

To study the direct effect of caffeine on AD gene expression, we choose a package of genes that have confirmed role in AD. The *APP* gene provides instructions for making a protein called amyloid precursor protein. This protein is found in many cells including the brain and the spinal cord. The normal functions of APP are not fully understood, but increasing evidence suggests that it has important roles in regulating neuronal survival, neurite outgrowth, synaptic plasticity, and cell adhesion [27]. A fundamental abnormality that plays a pivotal role in the dysfunction and death of neurons in AD is altered proteolytic processing of APP, resulting in increased production and accumulation of neurotoxic forms of Aβ in the brain. The evidence supporting the "amyloid hypothesis" of AD is extensive and has been reviewed [28]. The gene expression levels of *APP* and *Psen1* in the peripheral blood samples of patients with AD and their association with the disease are significantly high in AD patients than normal people [29]. Central to the disease is the altered proteolytic processing of *APP*, the genes involved in the amyloid pathway, which results in the production and aggregation of neurotoxic forms of Aβ. In our study, we found that caffeine has a direct effect on those genes because it down-regulated their expression during the early developmental stages of zebrafish embryos. The significant up-regulation in *APP* and *Psen1* expression occurred at 96 hpf, but after the completion of the neurodevelopment, caffeine significantly down-regulated their expression, compared with control.

We also found that caffeine regulates the expression of *Psen2* gene. This gene is known for its role in processing amyloid precursor protein [30]. Research suggests that *Psen2* works with other enzymes by cutting the amyloid precursor protein into smaller segments (peptides), β-amyloid peptide and soluble amyloid precursor protein (sAPP); the latter has growth-promoting properties and may play a role in the formation of neurons in the brain both before and after birth. Other functions of sAPP and β-amyloid peptide are under investigation [31, 32]. The up-regulation of this gene after caffeine treatment may increase the formation of neurons and/or help the generation of substantial numbers of new neurons in humans [33, 34].

The most widely studied risk factor for sporadic AD is ApoE [11]. *ApoE* gene mutation is predictive of AD [35]. A significant reduction in *Sorl1* expression, the receptor of ApoE gene, has been found in the brain tissue of AD patients [36]. The ApoE receptor has also been linked to the regulation of APP; faulty processing of which is implicated in AD [13]. A more recent study by a group of international researchers supports the proposition that *Sorl1* plays a part in the development of AD in seniors, the findings being significant across racial and ethnic strata [37, 38]. In addition to the link between Sorl1 and β-amyloid,

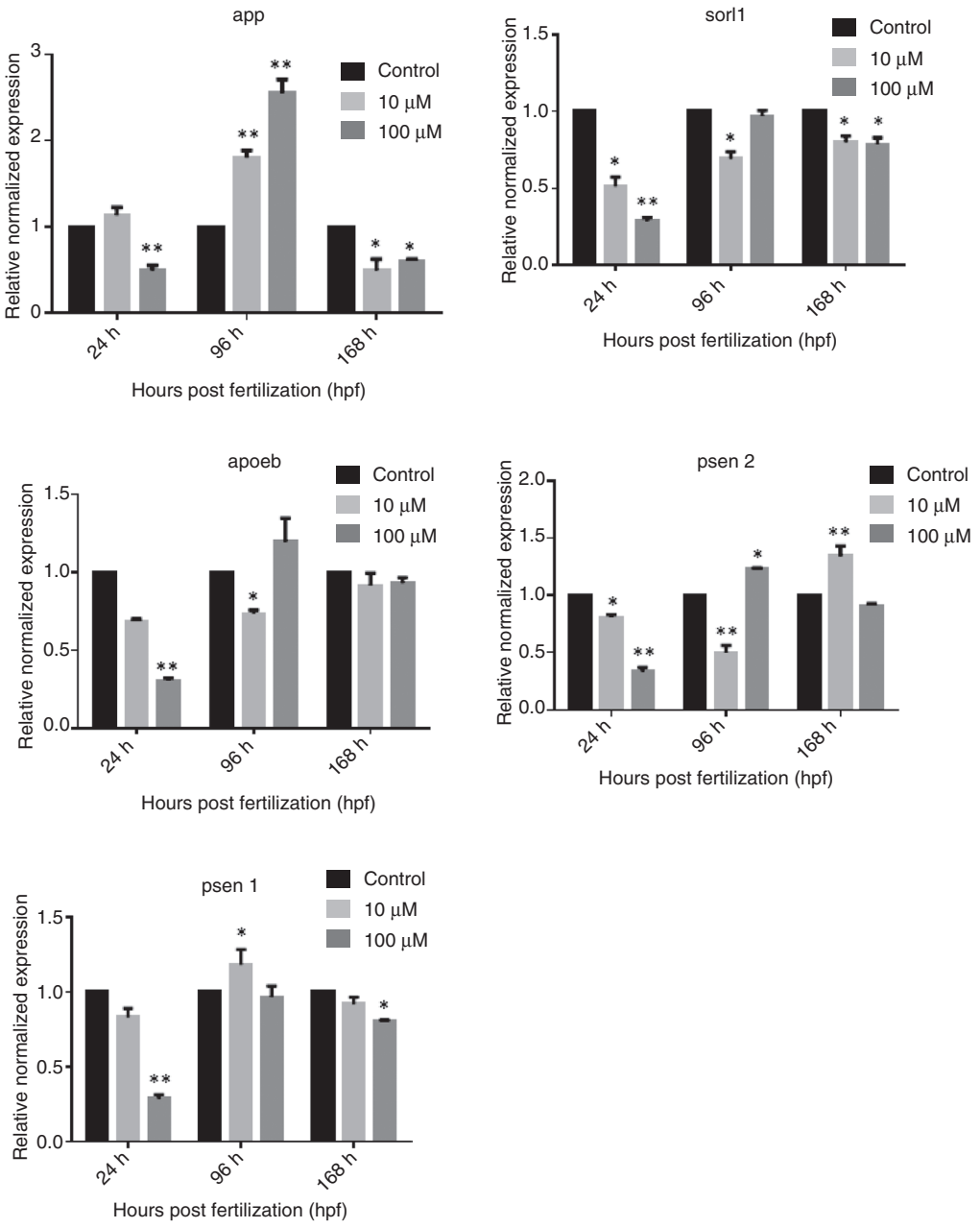

Figure 4: Effect of caffeine on the gene expression of AD-involved genes at 24, 96, and 168 hpf.
Bars represent the means±SE. RT-PCR (qPCR) was performed according to Takara Bio guidelines. A 25-μL RT-PCR reaction was done by adding 1 μL cDNA, 1 μL forward primer, 1 μL reverse primer, 12.5 μL SYBR Premix Ex Taq, and 9.5 μL of nuclease-free water to give the reaction the final volume of 25 μL. The thermal profile for RT-PCR was 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 40 s. Each mRNA level was expressed as a ratio to β-actin mRNA. Three technical replicates of each RNA sample were performed. Relative mRNA expression for each gene was calculated as a fold change compared with the control group using the $2^{\Delta\Delta ct}$ formula method. *Significant difference with control ($p<0.05$); **high significant difference with control ($p<0.01$). Two-way ANOVA was used followed by Tukey test. The represented values are the fold change of mRNA expression compared to control.

there is a connection between *Sorl1* and the *ApoE* gene known to influence the risk of developing AD [39]. The protein Sorl1 is a receptor that binds itself to a nearby molecule and then causes a reaction within the cell. The interactions between Sorl11 and ApoE have been surprisingly elusive [39]. However, it is difficult to believe that the functional links are merely coincidental. In this research, we observed that exposure to caffeine led to the down-regulation of *Sorl1*, but exposure to caffeine did not cause down-regulation of its target gene, *ApoE*. In contrast, we found

that the expression of *ApoE* was not significantly affected by caffeine treatment at 168 hpf at the two tested concentrations (0.94-fold change), compared with control; however, the expression of *Sorl1* gene, which is associated with the amyloid pathway, was down-regulated (0.8-fold change), compared with control. Accordingly, the continuous exposure to caffeine may protect and maintain the *ApoE* gene expression, which stems from the reduction of its receptor expression after completed neurodevelopment, and this may support the idea of that long-term intake of caffeine in moderate levels may protect against neurosystemic inflammation [40, 41].

Accordingly, caffeine has positive affect against AD through the reduction of the expression of the genes associated with the disease. Also, continuous exposure to caffeine may have a positive impact against the expression behavior of the *APP* and *Psen1* genes.

In order to study the correlation between caffeine and cell communication systems, we first studied the appeal of caffeine for AR. We analyzed the optical density of mRNA expression of three zebrafish adenosines, A_{2aa}, A_{2ab}, and A_{2b}. We found that caffeine up-regulated the expression of A_{2aa} and A_{2ab} at 24 hpf. Up-regulation of AR after the exposure to an antagonist is a known event described to occur in low intensities in a variety of species [42, 43]. In zebrafish embryos, the effects of caffeine on the studied genes appear to be selective to A_1 and A_{2aa}, which is probably because of the high affinity of these receptors to caffeine [44]. A_{2a} is the highest affinity receptor for caffeine in rodents and humans, while in zebrafish, we do not have this information. Additionally, zebrafish have two clones of A_{2a} with high similarity to the human A_{2a} [45]. According to our results, A_{2aa} and A_{2ab} may also have higher affinity for caffeine in developing zebrafish. This phenomenon must be confirmed by further experiments and studies.

The interaction between ARs and the dopaminergic system after caffeine exposure was studied in our present research. Wide research indicates that the neuromodulator adenosine interacts with dopamine A in the regulation of various behavioral functions, including locomotion [46–48]. Our tested concentrations have no effect on the locomotor activity of developing zebrafish, but we found that A_{2aa} and A_{2ab} up-regulation at 24 hpf causes a reduction in the expression of both DRs, especially at higher caffeine concentrations. Continuous exposure to caffeine up-regulated the expression of both DRs at 96 and 168 hpf, whereas the expression of ARs was significantly down-regulated at 100-μM caffeine concentration. This phenomenon shows the ability of adenosine A_{2a} antagonists to reverse the locomotor suppression that results from interference with dopamine transmission. Thus, caffeine can cause a stimulatory effect on the DR gene expression at later stages of zebrafish development and can be reasonable a therapeutic target for protection against AD. Hence, the stimulation of DRs is also an important event associated to AD; thus, dopamine is particularly involved in the regulation of cognitive processes associated with AD. The non-cognitive aspects of AD are usually linked to dopamine and serotonin, as these neurotransmitters most directly influence mood and emotional balance. According to previous research, dopamine may be low in people with AD [49]. Here we confirm that continuous exposure to caffeine stimulates the expression of DRs, and this may give an indirect protective effect against AD.

Conclusions

This is the first study on newly confirmed molecular factors in developing zebrafish. This model is promising because all tested genes are already expressed at early phases of development. Taking all the results together, we suggest that caffeine may protect against AD, directly via the regulation of amyloid pathway-involved genes and indirectly via stimulation of AR and DRs.

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