ABSTRACT

Effect of Caffeine on Amyloid Precursor Protein and Sortilin-related Receptor in Zebrafish *Danio rerio* Model

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Biomedical studies employ various animal models for both better understanding about the pathogenesis of human diseases at the cellular and molecular levels and the development of new therapeutics. Human pathologies have mostly been modeled using higher mammalian systems including rats and mice. Although mammalian models have significant advantages, they are also expensive to maintain, difficult to manipulate embryonically, and limited for large-scale genetic studies. Furthermore, because of the ethical issue of using large number of experimental mammalian animals and the tendency to use the lower vertebrates as a model of human diseases, zebrafish has become attractive model in biomedical research.

The zebrafish, *Danio rerio*, have been used in molecular genetics and developmental biology. Currently, it attracts much attention in studies on the development of new drugs and modeling of various physiological and pathological processes, and more recently in the evaluation of genes function using modern technologies like transgenesis and knocking out of target genes. Due to the transparency of the zebrafish embryos, its development can be easily observed. Zebrafish larvae rapidly absorb low molecular weight compounds, diluted in the surrounding media, through the skin and gills and after 7 days of their development, oral rather than skin absorbance occurs. Taking full advantage of the fecundity, optical transparency and low cost of zebrafish embryos, various chemical assays can be performed in a relatively simple way.

Caffeine is one of the most comprehensively studied ingredients among the food supply. Despite our considerable knowledge about the caffeine and centuries of safe consumption in foods and beverages, questions and misperceptions about the potential health effects associated with caffeine persist, and recent studies suggest that caffeine consumption could be an effective therapeutic against Alzheimer's disease (AD).

The first part of the study was performed to investigate the effect of caffeine on cell damage-related genes expression at early developmental stages of zebrafish. The genes include heat shock protein 70 (HSP70) as indicator of oxidative stress; Cyclin protein coding gene (Cyclin G1) associated with mitochondrial metabolism; and two genes involved in apoptosis; B-cell lymphoma 2 (Bcl-2) and Bcl-2 associated X protein (Bax). After the treatment with caffeine, changes in the morphology, hatching rate and heartbeats were determined. For the study, 100 μM concentration of caffeine was chosen based on the

absence of locomotor effects from previous published studies. Neither significant mortality nor morphological changes were detected. Caffeine significantly increased heartbeat of tested embryos. Quantitative real-time polymerase chain reaction (qRT-PCR) revealed significant up-regulation of cell damage related genes by caffeine exposure; HSP70 at 72 hours post fertilization (hpf); Cyclin G1 at 24, 48 and 72hpf; and Bax at 48 and 72hpf. Significant down-regulation was found in Bcl-2 at 48 and 72hpf. The Bax/Bcl-2 ratio was increased significantly at 48 and 72hpf. Bax or Bcl-2 associated protein is reported to interact with and increase the opening of the mitochondrial voltage-dependent anion channel which leads to the loss in membrane potential and release of cytochrome C. The negative impact occurs when Bax (pro-apoptotic factor) expression increases and Bcl-2 (anti-apoptotic factor) expression decreases. Therefore, the results of this study demonstrate that caffeine treatment may induce apoptosis by modulating the Bax and Bcl-2 expressions via mitochondria-dependent pathway.

The second part of the study was conducted to test the effect of caffeine on Alzheimer's molecular factors and two cell communication systems involved in the disease, Adenosinergic and dopaminergic receptors (AR and DR), in developing zebrafish *Danio rerio*. All of these genes were expressed at early developmental stages. No morphological changes were observed at tested concentrations, 10 µM and 100 µM compared to the control group. Treatment with caffeine significantly down-regulated the expression of AD related genes at 24hpf, and had a pattern of fluctuation at other check points. At 7days post fertilization(dpf) treatments with caffeine down regulated amyloid pathway-associated genes; amyloid precursor protein (APP) and presenillin1 (psen1), and up regulated presenillin2 (psen2) gene. The down-regulation of APP and psen1 may be beneficial to AD patients since it decreases the accumulation of amyloid plaques in their brains. The up-regulation of psen2 gene may be also beneficial because it works with other enzymes to cut APP into smaller peptides. Adenosine receptor 2aa (A2aa) and adenosine receptor 2ab (A2ab) showed higher response for caffeine than adenosine receptor 2b (A2b). Overall expression of ARs was down-regulated by caffeine exposure. There is inverse relation between AR and DR. Caffeine significantly down-regulated the expression of dopamine receptor d2a (drd2a) and dopamine receptor d2c (drd2c), and almost blocked their expression at 24hpf. However it significantly stimulated the expression of DR at 96 and 168hpf. This phenomenon may be beneficial to AD patients. This study demonstrated that caffeine has effect on the tested genes and it may play protective role in AD by down-regulating the amyloid pathway genes, APP and psen1 expression and partially up-regulating psen2 expression. The study of the expression of the two cell communication systems and their interactions suggests that caffeine has protective effect against AD via its antagonistic function of AR and stimulation of dopamine expression.

The third part of the study utilized the transcription activator-like effector nucleases (TALENs) technology to design plasmids to disturb the first exon of zebrafish sorl1 gene. The sorl1 gene is genetically associated with AD by function as a switch in the APP processing pathway. In AD there is a decrease of sorl1, which directs APP toward the beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) arm, resulting in an increase of Aβ (a direct precursor of the characteristic Alzheimer's Plaques). The aim of this study was to detect the role of sorl1 in zebrafish development and to demonstrate the interaction with APP in zebrafish knockout model. Following the TALENs' messenger ribonucleic acids (TALEN mRNAs) injection into cytoplasm at 1 cell stage zebrafish embryos, several malformations were observed in the developing zebrafish. Abnormal growth, bent tail, curved body, cardiac edema and stunted structure were the frequent phenomena in microinjected zebrafish. Some embryos developed more than one disorder in same individual. Three mutations were found in TALEN's spacer sequence in genomic DNA extracted from malformed embryos that were injected with TALENs mRNAs. Those three mutations were replacement of cytosine nucleotide (C) to guanine nucleotide (G), and addition of two

thymine nucleotides (T) on the TALEN's spacacer sequence. The controls; wild type zebrafish and both single TALEN injected embryos did not show any morphological malformation. In order to check the apoptosis in the malformed embryos, acridine orange test of dechorionated malformed embryos at 24hpf were performed. The results showed that there were apoptotic cells under green fluorescence light in the TALEN microinjected embryos with bent tail shape at 24hpf around over the body. This study demonstrates that sorl1 gene has important role in zebrafish development and the interruption of its first exon with TALEN mRNAs causes mutations which leads to severe malformations. Taken together with previous research, it was suggested that the silencing of sorl1 gene by microinjection of TALENs causes a decrease or loss of APP function and therefore an increase in the percentage of APP that enters the late endosomal pathway. And this leads to produce the malformed body structure like stunted body and bent axis and may involve in other disorders.

My studies illustrate that caffeine has paradoxical effect on treated zebrafish under our experimental conditions at the molecular level. However, caffeine does not induce phenotypic defects in developing zebrafish. Caffeine induces apoptosis via mitochondrial dependent pathway. Caffeine has direct effect on amyloid pathway involved genes and may play protective role in AD by modulation of amyloid pathway involved genes; APP, sorl1, psen1, psen2 and apoeb. There is adverse relation between AR and DR in favor of the protective effect against AD. Reduction of sorl1 gene has vital role in zebrafish development perhaps by the reduction of APP which caused stunted structure and may play a role in other disorders.

Zebrafish could be a good alternative model that may be valuable for elucidating the molecular basis of human neurodegenerative diseases. Taking advantage of some unique features of the zebrafish, I anticipate its increased adoption as a vertebrate model for high-throughput drug screening. Further studies are required and should concentrate on the production of stable line of sorl1-knock out zebrafish that may be a promising model to study neurodegenerative disorders as well as to develop and screens of therapeutics and study extensively the interaction between APP and sorl1 genes in this model.

Keywords: Caffeine, Cell damage-related genes, Amyloid pathway, APP, sorl1, TALEN, Zebrafish *Danio rerio*.

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