A Dissertation for the Degree of Doctor of Philosophy

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

제브라피쉬를모델에서 아밀로이드전구단백질과 소르틸린수용체에 대한 카페인의 효과

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August 2015

Department of Veterinary Medicine
Graduate School of Seoul National University

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ABSTRACT

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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 (as pro-apoptotic factor) expression increases and Bcl-2 (as 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 Alzheimer's disease (AD), 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 7 days post fertilization (dpf), treatments with caffeine down regulated amyloid pathway-associated genes; amyloid precursor protein (APP) and presenillin1 (psen1) and up regulated presenillin2 (psen2) genes. The down-regulation of APP and psen1 may be beneficial to AD patients since it decreases the accumulation of amyloid plaques in their brain. 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 but 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, Heartbeat, Cell damage-related genes, Amyloid pathway, APP, sorl1, TALEN, Zebrafish *Danio rerio*.

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LIST OF ABBREVIATONS

Aβ	Amyloid beta
A nucleotide	Adenine nucleotide
A2aa	Adenosine receptor 2aa
A2ab	Adenosine receptor 2ab
A2b	Adenosine receptor 2b
AD	Alzheimer’s disease
Apoeb	Apolipoprotein receptor b
APP	Amyloid precursor protein
BACE1	Beta-site amyloid precursor protein cleaving enzyme 1
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
C nucleotide	Thymine nucleotide
DNA	Deoxyribonucleic acid
Dpf	Days Post Fertilization
Drd2a	dopamine receptor 2a
Drd2c	dopamine receptor 2c
E3	Embryo water 3

FAD	Familial Alzheimer’s disease
FET	Fish embryo toxicity Test
G nucleotide	Guanine nucleotide
Hpf	Hours post fertilization
Hsp70	Heat shock protein 70
LC50	Lethal concentration
LOEC	lowest observed effect concentration
mRNA	Messenger ribonucleic acid
NHEJ	Non-homologous end joining
NOEC	No observed effect level
Psen1	Presenilin-1
Psen2	Presenilin-2
Sorl1	Sortilin-related receptor
TAU	T Proteins
T nucleotide	Cytosine nucleotide
TALENs	Transcription activator-like effector nucleases
ZET	Zebrafish embryo toxicity
ZFNs	Zinc finger endonucleases

LITERATURE REVIEW

1. Zebrafish

1.1 General characteristics

Zebrafish, *Danio rerio*, is tropical freshwater fish and a very popular model organism for scientific research in the fields of development, vertebrate processes, genetics, and more. Zebrafish is an omnivorous vertebrate and consumes zooplankton, insects, insect larvae and phytoplankton. Zebrafish has several features that make it attractive model for scientific research. As an oviparous species, zebrafish have external fertilization and development, which allow for easy detection of morphological alterations and manipulation of the embryos. Zebrafish has rapid growth (with all major organs formed within 2-4 days), high egg yield, and a short generation time (Xu, Z., 2010). Female zebrafish spawn every 2-3 days and produce several hundred eggs in each clutch. Males must be present at spawning and for successful ovulation. Zebrafish eggs are relatively transparent characteristic that makes it a very desirable model organism for development biology study. Eggs hatch within 48-72 hours post-fertilization. With five days of fertilization, larvae display food seeking and avoidance behaviors. The generation time of zebrafish is approximately 3-4 months, which is convenient for selection experiments (Spence et al., 2007 and

Keller et al., 2008).

All these features render the zebrafish embryos/larvae as a convenient platform for pharmacological assessment and screening for potential adverse effects of drugs, particularly with regard to their potential adverse effects during the developmental process (Busquet, et al., 2008; David and Pancharatna, 2009; Gurvich, et al., 2005; Hillegass, et al., 2008; Menegola, et al., 2006)

1.2 Molecular characteristics

The sequenced zebrafish genome is being assembled and annotated, and many molecular techniques have been developed to study gene function in zebrafish, including the production of transgenic and mutant fish and the use of transient antisense gene-knockdown methods (Nasevicius and Ekker, 2000; Udvadia and Linney, 2003).

Fish seem to be very different from humans, with different body structures and living environments. This makes them a seemingly strange choice as a comparator for humans. Like us, though, they are vertebrates and as such share a common ancestor and a remarkable biological similarity. Sharing the majority of genes with humans makes them an important model for understanding how genes work in health and disease (http://www.redorbit.com, online article, 2013).

Approximately, 70% of protein-coding human genes are related to zebrafish genes and 70% of all human disease genes have functional homologs in zebrafish (Langheinrich, 2003). These findings highlight the importance of the zebrafish model in human disease research. Several small molecules discovered using the zebrafish system, have recently entered into clinical trials. The availability of the genome sequence, coupled with the rapid expansion of disease models and chemical screening ability, ensures that the zebrafish system has a major place in biomedicine.

2. Toxicity test

Over the past twenty years, the zebrafish (*Danio rerio*) has emerged as a pre-eminent vertebrate model for the screening of chemicals and therapeutic drugs (Penberthy et al., 2002; Sumanasa and Lin, 2004). The OECD method has been developed for environmental and hazard classification applications. Pesticides and whole effluent would be prime examples of the types of compounds tested. However, some European countries are now moving away from fish LC_{50} testing and adopting alternative methods such as the fish embryo test (FET) (Nagel, 2002; Braunbeck et al, 2005). Fast-track zebrafish embryo toxicity (ZET) testing is a much more robust and sensitive model for evaluating vertebrate development

pathways and toxicity than small-animal models. Currently, the FDA requires biocompatibility testing for medical devices according to ISO 10993. Extracts of medical devices (or components) are tested according to standard procedures. These procedures utilize small animal models or cell cultures. The testing encompasses systemic toxicity, irritation, sensitization, and implantation requirements. These tests do not give one a complete toxicological profile. Most tests are subjectively scored. In contrast, ZET is ideal for biocompatibility testing of medical devices and polymers as a preliminary screening method (http://www.microtestlabs.com/zebrafish-embryos-testing/ online article, 2014).

2.1 Developmental toxicity

Fish embryos toxicity test (FET) intends to define lethal effects of chemicals and pharmaceuticals on embryonic stages of fish and constitute an alternative test method to the acute toxicity tests with juvenile and adult fish (OECD, 1992), thus providing a reduction in fish usage. Developmental toxicity and even Genotoxicity are readily viewable through the transparent embryo during development. The FET testing samples are diluted to various concentrations to determine the LC50, NOEC, and LOEC. The standard incubation time is 96 hours with sample renewal every 24 hours. The test is

initiated immediately after fertilization. Assessment of toxicity is determined by apical end points that include coagulation, heart beat presence/absence, formation of a somite, and detachment of the tail from the yolk sac. Thorough controls and requirements for high fertilization and viability rates of the embryos ensure that observed toxic effects are in fact linked to the test compound, and also ensure quality and consistency of results (http://www.microtestlabs.com/zebrafish-embryos-testing/ online article, 2014).

2.2 Cell damage related genes

One of the ways to study the damages happens after exposure to chemical or any other substances is by studying the cellular damage at the molecular level by identifying the regulation of genes involved in cell damage.

Heat shock protein 70 (HSP70) is a molecular biomarker of cellular stress that induces denaturation of other proteins (Sanders, 1990; Lewis et al., 1999) because it is involved in protecting and defending cells from environmental insults (Sanders, 1990); its induction is much more responsive than traditional indices of contaminant effects (Feder and Hofmann, 1999). Also the relationship between oxidative stress and proper mitochondrial function (Krunschnabel et al., 2005; Risso-de-Faverney et al., 2004) is important event.

Cyclin G1 is one of the genes that involved in cell cycle arrest to allow time for DNA repair when DNA is damaged (Ford, 2005; Kohn, 1999). Mitochondrial membrane potential change is directly associated with apoptosis (Weber et al., 2003). Bax and Bcl-2 are the major effectors of mitochondria-mediated apoptosis. Both Bcl-2 and Bax are transcriptional targets for the tumor suppressor protein, p53, which induces cell cycle arrest or apoptosis in response to DNA damage (Basu and Haldar, 1998). These genes regulate changes in the mitochondrial membrane potential and permeability, which play important roles in apoptotic processes (Shimizu et al., 2000).

Bcl-2 plays an important role in apoptosis, similar to cysteine proteases that regulate mitochondrial membrane potential changes and the release of cytochrome C by modulation of the outer mitochondrial membrane permeability (Martins et al., 1997; Nicholson; Thornberry, 1997; Bernardi et al., 2001and Gottlieb 2001). It is located in the inner membrane of the mitochondria and plays an important role in the regulation of ROS production and anti-oxidation. It localizes to sites where free radicals are generated and functions as an apparent antioxidant against oxidative stress to prevent apoptosis (Hockenbery et al., 1993). The negative impact occurs when Bax expression increases and Bcl-2 expression decreases (Basu and Haldar, 1998; Elizabeth et al., 2000).

The other gene, Bax, affects mitochondria-mediated apoptosis. Bax is a pro-apoptotic Bcl-2 member gene, which triggers a mitochondrial pro-apoptotic pathway. The up-regulation expression of Bcl-2 shows the activation of apoptotic cell death. However, many researchers have presented not expression data for both Bax and Bcl-2 genes but the ratio of Bax/Bcl-2 is an indicator for cell apoptosis (Jung et al., 2011; Basu and Haldar, 1998).

The up-regulation of Bax/ Bcl-2 ratio shows cell apoptosis (Pavlovic et al., 2006; Salakou et al., 2007; Jin et al., 2011). Therefore, the change in the Bax/Bcl-2 ratio is an important issue to demonstrate the occurrence of apoptosis (Pavlovic et al., 2006). Specifically, a high Bax/Bcl-2 ratio is associated with a lower threshold of apoptosis, while a low ratio represents a higher apoptotic threshold (Chan et al., 2007; Chan, 2007).

3. Model of human diseases

As mentioned previously that zebrafish is one of the most important vertebrate models organisms in genetics, developmental biology, neurophysiology and biomedicine (Vascotto, Beckham & Kelly, 1997; Grunwald & Eisen, 2002; Rubinstein, 2003; Amsterdam & Hopkins, 2006). It has a number of attributes that make it particularly tractable to experimental manipulation.

3.1 Molecular genetics

For many years zebrafish has been used in developmental biology and prominence as a model organism stems from the work of Streisinger (1981), who pioneered its use to apply molecular genetics to the study of vertebrate embryology, and Kimmel (1989, 1993) ; (Kimmel et al., 1990), who published detailed descriptions of cell differentiation and nervous system organization (Grunwald & Eisen, 2002). The zebrafish was the subject of the first large-scale random mutagenesis screens to be conducted in a vertebrate (Granato & Nüsslein-Volhard, 1996). These screens conducted by Driever et al., 1996 and Haffter et al., 1996, generated over 4,000 mutations and led to the identification of over 400 genes controlling vertebrate development. Since then there have been numerous technological advances (Patton & Zon, 2001;Golling et al. 2002; Udvadia & Linney, 2003; Chen & Ekker, 2004; Guo 2004; Eggert & Mitchison, 2006; Sood et al., 2006), culminating in the zebrafish genome project, based at the Sanger Institute in Cambridge, which began in 2001 and will shortly be completed (www.sanger.ac.uk).

The zebrafish is increasingly important in biomedical research (Dooley & Zon, 2000; Shin & Fishman, 2002), particularly as a model of human disease (Berghmans et al., 2005; Guyon et al., 2006). Its strength as a model organism is

that as a vertebrate it is more comparable to humans than invertebrate model species such as Drosophila (Postlethwait et al., 1998; Barbazuk et al., 2000), while being more tractable to genetic and embryological manipulation than mammalian model species such as mice, in which such procedures are both more complicated and costly.

Over 400 labs worldwide now routinely use the zebrafish in fundamental and applied research (www.zfin.org) and there is an increasing interest in its use as a model for understanding the genetic basis of behaviour (Guo, 2004; Miklósi & Andrew, 2006). Despite this interest, it has attracted little attention from the behavioral ecology community, possibly because little is known about its natural ecology and few studies have been conducted on wild populations. Most laboratories lines of zebrafish are the product of many generations in captivity, which is likely to have resulted in selection for reproductive capacity, while relaxing selection for other traits, such as predator avoidance (Robison & Rowland, 2005; Wright et al., 2006).

3.2 Knock-out genes

Reverse genetic tools are available, although it was more challenging and time-consuming to identify mutations in specific genes of interest and virtually

impossible to induce mutations in a targeted manner (Sharon, 2008). Currently, there are a variety of genetic or genomic manipulation technologies available like Zinc finger endonucleases (ZFNs).

ZFNs are chimeric fusions between DNA-binding zinc finger proteins and the nonspecific cleavage domain of the FokI endonuclease. They can induce double-strand breaks in a specific genomic target sequence, which are imprecisely repaired by non-homologous end joining (NHEJ) (Lieber, 2010). ZFN mRNA is injected into one-cell–stage embryos to generate zebrafish carrying the desired genetic lesions. It has been shown that germ cell mosaicism in the identified ZFN allele–bearing founder fish is up to 50% (Urnov et al, 2010). The bottleneck of this approach was the generation of ZFNs with high and specific activity in vivo (Zhu C et al, 2011).

In a similar strategy, researchers have recently fused transcription activator-like (TAL) effectors to a FokI cleavage domain generating custom-made transcription activator-like effector nucleases (TALENs) to disrupt target genes in zebrafish (Li, 2011). TALENs are precise and efficient genomic engineering tools, which have been successfully used in many fields of scientific research (Joung and Sender, 2013). TALENs are fusion proteins and work in pairs, consisting of a modular DNA-binding domain and a FokI endonuclease monomer (Moscou and

Bogdanova, 2009; Boch et al, 2009). When two TALENs bind to their DNA targets, the FokI monomers will dimerize and introduce a DNA double-strand break within the specific binding site (Boch and Bonas, 2010). The DNA break can either be repaired by NHEJ or homologous recombination (HR), which results in deletion/insertion mutations and specific site mutations or specific sequence additions, respectively. However, the conventional TALENs screening systems are mainly based on b-galactosidase or a single fluorescence reporter (Huang et al, 2011; Zhang et al, 2011) which functions in an indirect manner and limits the assessment of the transfection efficiency.

4. Alzheimer’s disease

4.1 Definition

Alzheimer's disease is a progressive disease that destroys memory and other important mental functions. It's the most common cause of dementia; a group of brain disorders that result in the loss of intellectual and social skills. These changes are severe enough to interfere with day-to-day life. In Alzheimer's disease, the brain cells themselves degenerate and die, causing a steady decline in memory and mental function. Current Alzheimer's disease medications and

management strategies may temporarily improve symptoms. This can sometimes help people with Alzheimer's disease maximize function and maintain independence.

4.2 Causes

Scientists believe that for most people, AD results from a combination of genetic, lifestyle and environmental factors that affect the brain over time. Less than 5 percent of the time, Alzheimer's is caused by specific genetic changes that virtually guarantee a person will develop the disease. Although the causes of Alzheimer's are not yet fully understood, its effect on the brain is clear. Alzheimer's disease damages and kills brain cells. A brain affected by Alzheimer's disease has many fewer cells and many fewer connections among surviving cells than does a healthy brain (Goldman et al., 2013 and Lonogo et al., 2014).

As more and more brain cells die, Alzheimer's leads to significant brain shrinkage. When doctors examine Alzheimer's brain tissue under the microscope, they see two types of abnormalities that are considered hallmarks of the disease: Plaques. These clumps of a protein called amyloid-beta (Aβ) may damage and destroy brain cells in several ways, including interfering with cell-to-cell communication. Although the ultimate cause of brain-cell death in Alzheimer's

isn't known, the collection of Aβ on the outside of brain cells is a prime suspect tangles. Brain cells depend on an internal support and transport system to carry nutrients and other essential materials throughout their long extensions. This system requires the normal structure and functioning of a T protein called tau (Grabowski et al., 2014)

In Alzheimer's, threads of tau protein twist into abnormal tangles inside brain cells leading to a failure of the transport system. This failure is also strongly implicated in the decline and death of brain cells (Papadakis et al., 2014).

4.3 Molecular risk factors

Alzheimer`s disease is a multifactorial disease and genetic as well as environmental factors are included in AD pathology. In the last decades, several genes involved in AD have been identified. The majority of Alzheimer's cases are late-onset, usually developing after age 65, and this form of the disease shows no obvious inheritance pattern (Eva et al., 2011).

The molecule seems to contribute to Alzheimer's through two distinct pathways, one of which is amyloid-dependent. A gene called Apolipoprotein E (apoe) appears to be a risk factor for the late-onset form of Alzheimer's. There are three forms of this gene: apoe2, apoe3 and apoe4. In both animals and humans,

apoe4 strongly promotes Aβ deposition in the brain, compared with apoe3, long considered the 'neutral' form when it comes to Alzheimer's risk. apoe2, which is considered the protective form, decreases the build-up (Kim *et al*, 2009).

Roughly one in four Americans has apoe4 and one in twenty has apoe2. While inheritance of apoe4 increases the risk of developing the disease, ApoE2 substantially protects against it. Some current research is focused on the association between these two forms of apoe and Alzheimer's disease. Several other genes also appear to influence the development of Alzheimer's disease, and more detailed information is available in the Heredity section (http://www.brightfocus.org/alzheimers/about/risk/?referrer=https://www.google.co.kr/ online article, 2014).

Familial AD (FAD) or early-onset Alzheimer's is an inherited, rare form of the disease, affecting less than 10 percent of patients. FAD develops before age 65, in people as young as 35. It is caused by one of three gene mutations on chromosomes 1, 14 and 21 (http://www.nia.nih.gov/alzheimers/publication/alzheimers-disease-genetics-fact-sheet, online article, 2015).

Scientists believe that several other genes may influence the development of AD. Two of these genes, UBQLN1 and Sortilline related receptor1 (SORL1),

are located on chromosomes 9 and 11.

Researchers have also identified three genes on chromosome 10, one of which produces an insulin degrading enzyme that may contribute to the disease. A gene, called TOMM40, appears to significantly increase one's susceptibility to developing Alzheimer's when other risk factors are present, such as having the ApoE-4 gene. Several recently discovered genes that influence Alzheimer's disease risk are CLU (also called APOJ) on chromosome 8, which produces a protein called clusterin, PICALM on chromosome 11 and CR1 on chromosome 1.

In October 2013, an international group of researchers reported on the identification of 11 new genes that offer important new insights into the disease pathways involved in AD add to a growing list of gene variants associated with onset and progression of late-onset Alzheimer's. However, the genetic risk factors alone are not enough to cause the late-onset form of AD; researchers are actively exploring education, diet and environment to learn what role they might play in the development of this disease.

Recent studies demonstrated that SORL1 is able to interact with both amyloid precursor protein (APP) and VPS 35 to direct whether APP enters the retromer recycling pathway or instead enters the late endosomal pathway. Scientists have demonstrated that over expression of SORL1 leads to an

increase in the percentage of APP entering the recycling pathway and therefore a decrease in Aβ production. Likewise, the silencing of sorl1 leads to an increase in the percentage of APP that enters the late endosomal pathway (Rogaeva, 2007). Taken together, this data indicates that SORL1 plays an important role in the determination of the path APP takes. This is important because the late endosomal pathway leads towards the accumulation of Aβ peptide, which has previously been shown to play a substantial role in the pathology of AD (Mattson, 2004).

5. Caffeine

5.1 Source and consumption

Caffeine is one of the most comprehensively studied ingredients in the food supply. Yet, despite our considerable knowledge of caffeine and centuries of safe consumption in foods and beverages, questions and misperceptions about the potential health effects associated with caffeine persist.

Caffeine is a naturally occurring substance found in the leaves, seeds and/or fruits of at least 63 plant species worldwide and is part of a group of compounds known as methylxanthines. The most commonly known sources of

caffeine are coffee, cocoa beans, kola nuts and tea leaves. (Barone and Roberts, 1996; Frary et al., 2005).

The amount of caffeine in food products varies depending upon the serving size, the type of product, and preparation method. On average, a five-ounce cup (150 ml) of percolated or drip coffee has 120 mg caffeine. Chocolate contains a small amount of caffeine announce of milk chocolate or cup of cocoa has about 7 mg. The content of caffeine in over-the-counter medications is as follows: stimulants (such as No-Doze®) 100-200 mg, menstrual aids (such as Midol®) 20-100 mg, analgesics (such as Anacin® or Excedrin®) 30-65 mg, and cold preparations 30-65 mg of caffeine. The average consumption of caffeine in the US and Canada is about 2.4 mg/kg/day for adults and 1.1 mg/kg/day for children 5-18 years old (Wells, 1984).Daily intake of caffeine may be quite high in some individuals, exceeding 15 mg/kg in heavy coffee drinkers. It is noteworthy that a 27-kg child drinking three colas and eating three small chocolate bars in a day will ingest 7.2 mg/kg (Gilbert et al, 1976).

5.2 Chemical properties and biosynthesis

Pure anhydrous caffeine is a white odorless powder with a melting point of 235–238 ℃. (Caffeine pubchem, 2014 and caffeine chemspider, 2014). Caffeine

is moderately soluble in water at room temperature (2 g/100 mL), but very soluble in boiling water (66 g/100 mL). (Susan, 1996). It is also moderately soluble in ethanol (1.5 g/100 mL). (Susan, 1996) It is weakly basic (pKa = ~0.6) requiring strong acid to protonate it. (Harry G, et al, 2007). Caffeine does not contain any stereogenic centers (Klosterman L, 2006) and hence is classified as an achiral molecule. (Vallombroso T, 2001). The xanthine core of caffeine contains two fused rings, a pyrimidinedione and imidazole.

The pyrimidinedione in turn contains two amide functional groups that exist predominately in a zwitterionic resonance form where the nitrogen atoms are double bonded to their adjacent amide carbons atoms. Hence all six of the atoms within the pyrimidinedione ring system are hybridized and planar. Therefore the fused 5, 6 ring core of caffeine contains a total of ten pi electrons and hence according toHückel's rule is aromatic (Keskineva N, 2014).

Caffeine is synthesized in plants from the purine nucleotides AMP, GMP, and IMP. These in turn are transformed intoxanthosine and then theobromine, the latter being the penultimate precursor of caffeine.(Ashihara et al, 1996) Being readily available as a by-product of decaffeination, caffeine is not usually synthesized chemically. (Simon T, 2009). If desired, it may be synthesized from dimethylurea and malonic acid (Swidinsky J and Baizer MM, 1957 and

Zajac MA et al., 2003)

5.3 Mechanism of Action

The molecular recognition of caffeine in both solution and solid state is important to understand their fundamental roles in biological systems (Parliament TH et al, 2000 and Vinchurkar MS et al, 1997). Recognition of such biomolecules with abiotic synthetic receptors has great significance to understand the enzyme reactions (Stoddart JF, 1984; Lehn JM, 1985) (enzyme–substrate interactions), immunological reactions in vivo, (Franco MIR et al., 1999 and Marchel E, et al., 2013) selective host–guest complexion and catalytic reactions in bio-mimetic chemistry.

Pharmaceutical drugs work the same way Enzymes work (Tymoczko, JL, 2006). They bind to a specific binding site that either inhibits or activates a specific biological action. The reason behind the relationship between caffeine and alertness and wakefulness is the big structural similarity between the caffeine molecule, adenosine and cyclic adenosine phosphate. The structures similarity allows caffeine molecules to bind to the same binding site of receptors or enzymes that reacts with adenosine derivatives. Adenosine has a very important role in the regulation of brain activity. The human brain builds up adenosine molecules

during the day. When the level of built up adenosine increases in the human brain, adenosine starts binding to its binding sites (receptors) in the human brain which activates mechanisms that lead to drowsiness and sleep. Since caffeine has the same structural molecule it binds to the same receptors that adenosine binds to, preventing adenosine to bind to that specific receptor in the human brain and delaying the sleeping and drowsiness process.

Adenosine is a locally released purine hormone that acts on different receptors (AI and A2) that can increase or decrease cellular concentrations of cyclic AMP. In general, adenosine inhibits adenyl cyclase via high-affinity (AI) receptors and stimulates adenyl cyclase via low-affinity (A2) receptors. Adenosine receptors are found throughout the body including the brain, in the cardiovascular, respiratory, renal, and gastrointestinal systems, and in adipose tissue. Caffeine non-selectively blocks both adenosine receptors and competitively inhibits the actions of adenosine at concentrations found in people consuming caffeine from dietary sources. Adenosine acts presynaptically to inhibit neuronal release of acetylcholine, norepinephrine, dopamine, gamma amino butyric acid, and serotonin; it also reduces spontaneous firing of neurons in many regions of the brain, produces sedation, and has anticonvulsant activity. Caffeine releases norepinephrine, dopamine, and serotonin in the brain and increases circulating

catechol amines consistent with reversal of the inhibitory effects of adenosine on these systems.

5.4 Central Nervous System

Caffeine is primarily a stimulant, increasing arousal and vigilance, reducing fatigue, and decreasing motor reaction time for some tasks (Lieberman, HR et al., 1987).

At least some of the stimulation experienced by habitual coffee drinkers is a result of reversal of the lethargic state induced by caffeine withdrawal.

Caffeine reduces EEG power, particularly in the alpha and theta frequencies. In higher doses, caffeine may produce insomnia, anxiety, tremors, and seizures. Caffeine may interfere with sleep-increasing sleep latency and decreasing total sleep time but these effects are seen primarily in light caffeine users. Caffeine also decreases cerebral blood flow, presumably by antagonizing adenosine-mediated cerebral vasodilation (Mathew RJ and Wilson WH, 1985).

5.5 Risk of Caffeine

Caffeine or coffee consumption has been extensively investigated as a risk factor for human disease. Caffeine consumption was associated in various studies

with pancreatic and urinary tract cancer and fibrocystic breast disease, but the studies were flawed in design and more recent studies have not confirmed these associations (Council of scientific affairs, 1984). At present there is· no basis to conclude that caffeine contributes to cancer. Despite its effect to increase gastric acid secretion, caffeine has not been found to be a risk factor for peptic ulcer disease. Coffee drinking can produce symptoms of esophageal acid reflux, which may be a result of relaxation of the lower esophageal sphincter, increased gastric acid secretion, and/or a nonspecific irritant effect of coffee. The major health concerns concerning caffeine and human disease at present are coronary heart disease, reproductive disorders, and psychiatric disturbances. Other concerns include interactions of caffeine with sympathomimetic and analgesic drugs to cause cardiovascular and renal injury, respectively.

5.6 Reproductive disturbances

Despite the teratogenicity demonstrated in some animal studies, there is no evidence that coffee or caffeine is teratogenic in humans (Council of scientific affairs, 1984).

A study of 389 women found that caffeine consumption during pregnancy decreases, in a dose-related manner, the birth weight of the newborn although an

earlier case-control study did not find such an association (Martin, TR and Bracken MB, 1987; Linn S et al., 1982). Another study found that consumption of caffeinated beverages decreased fertility in women followed for a year after discontinuing birth control (most using barrier methods) (Wilcox A, et al., 1988). There is no clear explanation for the association between caffeine and low birth weight or decreased fertility, and further research is needed to confirm that these reproductive problems are due to caffeine and to define mechanisms of reproductive toxicity. Significant concentrations of caffeine are present in the umbilical cord blood of newborns (Van't HW, 1982), and a neonatal withdrawal syndrome, consisting of irritability, jitteriness, and vomiting, has been reported in infants born to mothers who consumed large amounts of caffeine during pregnancy (McGowan D, et al., 1988).

5.7 Caffeine versus Alzheimer's

Epidemiologic studies have increasingly suggested that caffeine/coffee could be an effective therapeutic against AD (Arendash and Cao, 2010). Although studies have historically shown the "negative" effects of coffee, recent studies indicate how it can actually improve your health from boosting brain power, to delaying AD and improving memory as you age results from another recent study

show that caffeine has a positive effect on the tau protein. Caffeine is an adenosine receptor antagonist, which means it blocks some receptors in the brain that contribute to the build of and entanglement of tau. (Alzheimer's.net, 2014). Caffeine appears to provide its disease-modifying effects through multiple mechanisms, including a direct reduction of Abeta production through suppression of both beta- and gamma-secretase levels. In acute studies involving AD mice, one oral caffeine treatment quickly reduced both brain and plasma Abeta levels - similarly rapid alterations in plasma Abeta levels were seen in humans following acute caffeine administration. "Caffeinated" coffee provided to AD mice also quickly decreased plasma Abeta levels, but not "decaffeinated" coffee, suggesting that caffeine is critical to decreasing blood Abeta levels (Arendash and Cao, 2010).

6. Future perspectives of zebrafish in biomedical research

Zebrafish are an important model for unlocking the mechanisms of disease, and have already played a central role in helping to unravel the biological processes behind muscular dystrophies. New studies should aid in uncovering the processes that underlie both common and rare diseases, reveal the gene or genes that cause the diseases, and may lead to eventual new treatments. The future challenge is to develop a comprehensive, functional understanding of all human

genes as quickly as possible. The systematic analysis of zebrafish gene function will advance understanding of human disease.

The zebrafish is rapidly emerging as an attractive model for studying AD research. Zebrafish is ideal model for drug testing prior to clinical testing in rodents. However, there are still aspects of this model that require better understanding. For the zebrafish system to be used to model aspects of AD pathobiology we need to better understand zebrafish brain structure and function and also gain a deeper understanding of adult zebrafish brain physiology. Work so far has revealed that the zebrafish brain does have a reasonable level of conservation of basic structure when compared to mammals as well as similar neuroanatomical and neurochemical pathways to those that play roles in human disease (reviewed in Santana et al., 2012). Various aspects of presenilin gene biology were revealed using the zebrafish that would otherwise be difficult to observe/analyze in other models. However, to analyze effectively future transgenic and mutant zebrafish models of AD we need to strengthen our understanding of the functions in zebrafish of some of the orthologs of the key genes implicated in human AD pathogenesis such as MAPT and APOE.

Whether the zebrafish can be employed to model a late-onset disease like AD is debatable since zebrafish have a profound capacity for regeneration and this

must impact on the development of neurodegenerative phenotypes. Neurogenesis in the adult zebrafish brain is much more abundant than is observed in mammals (Kizil et al., 2012) consequently making analysis of neuronal loss difficult. Despite these limitations the recent availability and feasibility of using genome editing technologies presents an exciting opportunity to develop zebrafish genetic models of neurodegenerative diseases such as AD. ZFNs, TALENs and CRISP/Rs have been validated for use in the zebrafish (reviewed by Hwang et al., 2013; Schmid and Haass, 2013) and it is inevitable that FAD mutations will be introduced into zebrafish FAD gene orthologs.

Due to their versatile and unique features, I believe zebrafish will play an increasingly prominent role in the identification and study of disease genes and therapeutic discovery.

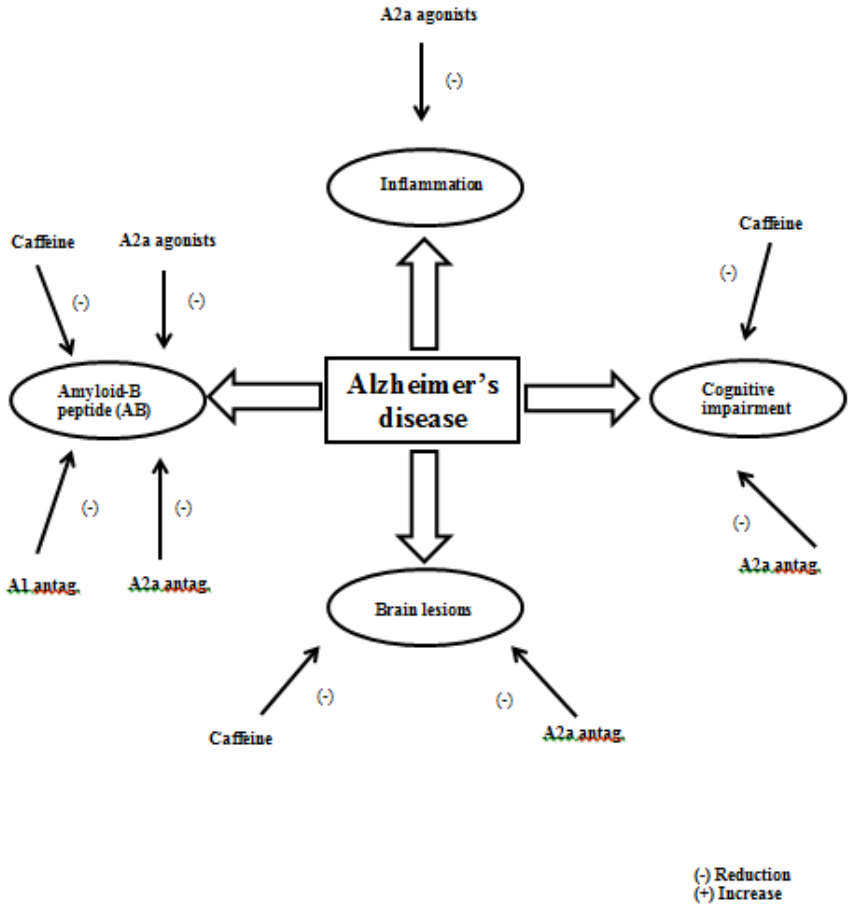

Figure 1. Summary of the recently reported results of using adenosine receptor agonists and antagonists to treat the main detrimental effects caused by AD (Marla and Manuel, 2014).

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GENERAL INTRODUCTION

Due to the physical and ethical issues associated with performing experiments on humans or other mammalians, biomedical research utilizes primarily animal models to study biologic processes conserved between humans and lower vertebrates. The most common model organisms are rats and mice. Although these models have significant advantages, they are also expensive to maintain, difficult to manipulate embryonically, and limited for large-scale genetic studies. The zebrafish, *Danio rerio*, has emerged as a popular vertebrate model in different areas of research such as developmental biology, genetics, pharmacology, and toxicology (Alestrom et al., 2006; Beis and Stainier, 2006; Ingham, 2009).

This study aims to identify the molecular impact of caffeine on zebrafish at early stages of development. Determine the role and mechanism of action of caffeine in Alzheimer’s Molecular factors. Introduce zebrafish model of AD for further research of neurodegenerative disorders for the identifications and and screens of therapeutics.

CHAPTER I

Caffeine affects heartbeat and cell damage-related genes expression with no morphological malformations at early developmental stages of zebrafish *Danio rerio*

1.1. Abstract

Caffeine is white crystalline xanthine alkaloid that is naturally found in some plants and can be produced synthetically. It has various biological effects, especially during pregnancy and lactation. This study was performed to investigate the effect of caffeine on cell damage-related genes expression at early developmental stages of zebrafish. The genes include HSP70; Cyclin G1; and Bcl-2 and Bax. After the treatment with caffeine, changes in the morphology, hatching rate and heartbeats were determined. For the study, 100μM concentration of caffeine was chosen based on the absence of locomotor effects. Neither significant mortality nor morphological changes were detected. Caffeine significantly increased heartbeat of tested embryos. 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.. 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.

1.2. Introduction

Caffeine is white crystalline xanthine alkaloid that acts in human as a stimulant drug for central nervous system and metabolism. It is most commonly used medicine indicated for headache and other pain relievers and considers the most widely consumed psychoactive drug in the world (Carlsen et al., 2005). Caffeine is naturally found in varying quantities in the seeds, leaves, and fruit of some plants and also can be produced synthetically (Chiranjib, 2011). It is used also as additive in many products.

There are many beverages containing caffeine, such as coffee, tea, soft drinks, and energy drinks (Lovett, 2005). It was found that a cup of coffee content near about 60 to 80 mg caffeine, however it varies according to cup size, coffee preparation method and filtration (Cook et al., 1996). A recent review of national dietary consumption between 1994 and 1998 estimated that 87% of the population consumes caffeine, with an average of 193 mg/day. Among adults, 18 years and older, the daily intake ranges between 166 and 336 mg/day (Frary et al., 2005). The caffeine level is estimated as 21.22μM in the blood circulation of people imbibing 2427 mg caffeine for 36 weeks (Cook et al., 1996). Caffeine has shown a wide array of pharmacological and biological effects. The manner in which caffeine triggers pleiotropic effects is still largely unknown (Calvo et al., 2009). It

was found that caffeine induces cell death in human osteoblasts which attributed primarily to apoptosis (Pin-Zhen, 2008). Sometimes caffeine prescribed to pregnant woman, and it is reported that it can cause problems to the fetus as well as the mother (Carlsen et al, 2005).

Recently, the zebrafish embryos and larvae is one of the most extensively used tools for investigating the negative impact of chemicals and pharmaceuticals (Kristensen, 1995 and Luckenbach et al., 2001). Zebrafish is a small, freshwater, aquarium species. It has a short generation time, breeds almost all of the year-round. They are easy to grow, very sensitive and can be maintained in different environments. Furthermore, the developmental stages of embryos are external and transparent, the effect of treatments can be observed clearly and distinctly (Chiranjib, 2011). Thus, zebrafish is considered an excellent model for studying the effect of drugs and pharmaceuticals (Ulrike 2003; Holmberg et al., 2006; Bello et al., 2004 and Brion et al., 2004), especially at the early development stages.

The aim of our research is to study the effect of different exposure durations of caffeine on zebrafish embryos at first developmental stages; 24, 48 and 72hpf. In this study, we adopted a real-time polymerase chain reaction (PCR) to determine the effects of caffeine on the expression levels of some cell damage-related genes in the embryo-larval zebrafish exposed for different durations at

about 2hpf. We selected a linked package of genes which included oxidative stress gene (HSP70), mitochondrial metabolism (Cyclin G1) and apoptosis (Bcl2 and Bax), to study the effect of caffeine on the expression behavior of these genes. Also, we observed the effect on survival rate, hatching rate and heartbeat of the developing zebrafish embryos.

1.3. Materials and methods

Zebrafish maintenance and egg collection

Zebrafish *Dario rerio* were obtained locally (Green Fish, Korea) and were raised at the laboratory housing system (Daejong instrument industry, Korea). Adult zebrafish *Dario rerio* were maintained in glass aquariums with continuous re-circulating system, 14hr. light: 10hr. Dark photo-cycle. The temperature was 28.5℃ during all stages of the experiment. Adult zebrafish were fed three times a day with a combination of dried blood worms (TetraWerke, Melle, Germany) and new hatched brine shrimp *Artemia* (San Francisco Bay Brand, Inc., Newark, CA, USA). Two pair's ripe fish males and females were separated with a barrier in a spawning box containing a mesh bottom to prevent the spawned eggs from being cannibalized and then the boxes were incubated overnight in 28.5℃ incubator. In

the next day, the barrier was removed at the beginning of light period and the zebrafish started spawning. Fertilized eggs were obtained from matured zebrafish according to the manual by (Westerfield, 1995). We followed the Care and Treatment of the Animals guidelines established by Institutional Animal Care and Use Committee, Seoul National University (Approval no. SNU-050418-2).

Chemical preparation and exposure

Caffeine 1, 3, 7-trimethylxanthine, (Reagent Plus®, powder) was purchased from Sigma-Aldrich (CAS: 58-08-2). The exposure solution was prepared in a concentration of 100 µM by dissolving Caffeine powder in distilled water. The tested concentration was selected according to (Kitiucia et al, 2011). We washed the fertilized eggs twice and started exposure at the cleavage stage 32-64 cells (about 2hpf). For the monitoring assays; hatching rate, survival and heartbeat, a 6-wells cell culture plate was used. We put 30 eggs in each well filled with 3 ml of 100 µM caffeine solution or distilled water, in three replicates, to give a total of 90 eggs per each treatment. About 30 % of the solution was replaced daily with fresh one, for 96hpf. For molecular analysis, the remained fertilized eggs were separated and handled carefully under temperature control to avoid heat-shock. We divided them into two groups and exposed immediately to

caffeine solution or distilled water. Separate control for each treatment.

Embryo-larvae monitoring assay

The morphological changes were observed using an Olympus IX70 microscope during the experiment. Embryo and larvae were counted for survival rate and hatching rate at 36, 48, 60, 72, 84 and 96hpf. The heartbeat was counted at 48, 72 and 96hpf. The petri-dishes were placed under a stereomicroscope (SZ-PT, Olympus, Japan) to count the heartbeat rates per 1 minute of larvae using a stop watch and counter (Zhu et al., 2007) under temperature control. About 30 embryos from each treatment were used in this study.

Total RNA extraction and cDNA synthesis

At 24, 48 and 72hpf, about 60-70 embryos from Caffeine treatment and control were pooled and prepared for total RNA extraction using the following protocol provided by (Chen Lab, Department of chemical and systems biology, Stanford University). Briefly, wash the embryos using E3 Buffer then PBS. Flash freeze in liquid nitrogen then homogenize in 500µl Trizol reagent (Invitrogen, USA). Add 150µL chloroform and shake vigorously for 30sec then centrifuge at 10000 rpm at 4℃ for 15 min. Transfer the RNA-containing upper aqueous phase

to a new tube and precipitate the RNA by adding 0.8 volumes of isopropanol. Mix well and incubate for 10 minutes at room temperature then centrifuge at 10,000 rpm for 15 minutes at 4 ℃. Remove the supernatant and wash the RNA pellet with 750 μL of 75% RNase-free ethanol by centrifuging at 8,000 rpm for 15 minutes at 4 ℃. Air dried the pellet for 10 minutes and re-suspend RNA in 50 μL RNase-free H2O. cDNAs were synthesized using M-MLV cDNA synthesis kit (Enzynomics, Korea) following the supplier's instructions.

Relative quantification (RQ) of transcripts by real-time RT-PCR (qPCR)

Real-Time RT-PCR was done according to the Takara Bio Inc. guidelines. Total 22 μL PCR reaction was made by adding 2 μL cDNA, 1 μL forward primer, 1 μL reverse primer, 8 μL SYBR Premix Ex Taq, 0.4 μL ROX Reference (Takara Bio Inc. Shiga, Japan) and 9.6 μL of Nuclease-free water (Ambion Inc., Austin, TX). The reaction was done by using 7300 Real Time PCR System (Applied Biosystems, Forest City, CA) according to the company instructions. The thermal profile for real-time RT-PCR was 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 10 sec, 60 ℃ for 20 sec, and 72 ℃ for 40 sec. We used GAPDH As a housekeeping gene to normalize the results by eliminating variations in mRNA and cDNA quantity and quality, and each mRNA level was expressed as a ratio to

GAPDH mRNA. Three replicates (pools of embryos) and 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. All primers are described in Table 1.

Statistical Analysis

The effects of caffeine treatment on the hatching rate, survival rate, heartbeat rates and relative quantitative expression of tested genes were determined using one-way analysis of variance (ANOVA) followed by tukey test. Significant difference occurs for a given parameter when $p<0.05$. The entire statistical analysis was carried out using Graphpad Prism (version. 5).

1.4. Results

Morphological changes and monitoring assays

We didn't observe morphological changes during the experiment. There was no significant difference ($P> 0.05$) in the survival rate between control and caffeine treatment from 24 to 72hpf. The final survival rate (%), at 72hpf, was 94% in control group and 92.6% in caffeine treatment. Hatching rate (%) started at 48hpf

and the over-all hatching rate was calculated at 96hpf (Fig. 1). At 60 and 72hpf hatching rate was 50% and 80% in control group but it was 30% and 60% in caffeine treatment, respectively with significant difference ($P<0.05$) with control. Over-all hatching rate was 94% in control group and however it was 93% in caffeine treatment with no significant difference ($P>0.05$). Therefore, there is no significant difference between the control and caffeine treatment in the over-all hatching rate (96hpf), but there was a significant decrease of hatching rate in the caffeine treatment at 60 and 72hpf.

Effect of caffeine treatment on heartbeat

The effect of caffeine exposure on the heartbeat was studied at 48, 72 and 96hpf. We allocated control for each treatment. In the control groups, the heartbeat rate min^{-1} was 110, 110 and 112 at 48, 72 and 96hpf, respectively. Significant higher heartbeat ($P<0.05$) was observed in caffeine treatment at 72 and 96hpf which were 122 and 136 heartbeat rate min^{-1}, respectively (Fig. 2).

Gene expression

Effect of caffeine different exposure durations on cell damage-related genes expression was studied using the real time PCR. Quantitative mRNA level of the

genes related to oxidative stress (HSP70), mitochondrial metabolism (Cyclin G1), and apoptosis (Bcl-2, Bax) were determined at early development stages of zebrafish embryos; 24, 48 and 72 hpf after exposure to 100 µM caffeine. mRNA level of HSP70, one of the oxidative stress related genes, showed slightly down-regulation at 24hpf with no significant difference with the control. The up-regulation of mRNA level, 1.43 and 11.75 folds was found at 48 and 72hpf treatments, respectively. There was no significant difference ($P>0.05$) between control and 48hpf treatment but there was significant difference ($P<0.01$) at 72hpf, (Fig.3A).

The mRNA expression level of mitochondrial metabolism-related gene, Cyclin G1, increased significantly directly after exposure to caffeine at 24, 48 and 72hpf treatments (1.34, 1.2 and 1.35 folds-increase, respectively) (Fig.3B). The mRNA expression levels of apoptosis-related genes, Bax and Bcl-2 expression levels were affected also in caffeine treatments. Bax expression increased directly after caffeine exposure but with significant difference with control only at 48 and 72hpf treatments (1.7 and 2.5 folds-increase, respectively) (Fig.3C). The mRNA expression level of Bcl-2 gene slightly increased at 24hpf treatment (1.05 fold) with no significant difference with control ($P>0.05$). The expression level decreased significantly ($P>0.01$) at 48 and 72hpf treatments (0.65 and 0.45 folds-

decrease, respectively) (Fig. 3D).

Also, we analyzed the Bax/Bcl2 ratio. We found that all values of Bax/Bcl2 ratio increased at 24, 48 and 72hpf treatments with significant difference with control at 48 and 72hpf treatments (3.05 and 4.7 folds-increase, respectively), but there was no significant difference at 24hpf treatment (1.54 fold) with control (Fig. 4).

1.5. Discussion

We investigated the effect of different exposure durations of caffeine on zebrafish embryos at early development stages 24, 48 and 72hpf. Zebrafish embryos and larvae are extensively used for investigating the negative impact (Kristensen, 1995; Luckenbach et al., 2001). As they are easy to grow, very sensitive, can be maintained in different environments and all the developmental stages are transparent and ex-utero, they are very suitable organisms for toxicological studies. We studied the effect on the expression behavior of some cell damage-related genes. We also observed the effect of caffeine on heartbeat, survival and hatching rate. We started exposure at the cleavage stage, 32-64cells (about 2hpf). Samples were pooled for the molecular analysis at 24, 48 and 72hpf. As known, zebrafish can’t eat until about 7dpf because of the late development of its mouth and the diminishment of yolk ball. Therefore, the most suitable way to expose it to

chemical is by diluting the chemical in maintenance water (Katiucia et al., 2011). We used 100 μM (~19.4 μg/ml) concentration of caffeine in our experiment. The rationale to use this concentration based on the absence of locomotor effects, especially in regard to the fact that this concentration is unable to promote significant embryo toxicity, effects on heartbeat, blood circulation, number of somites, hatching, tactile sensibility and other phenotypic features (Chen et al., 2008 and Selderslaghs et al., 2009).

In this study and under our experimental conditions, there was no visible morphological alteration in caffeine treatment. Also, there was no significant difference ($P>0.05$) between control and treatment groups in survival rate and over-all hatching rate. Although it is reasonable that caffeine cannot enter in the complete concentration to the embryo during the chorionated phase (Kitiucia MC. et al, 2011), we observed a delay of hatching rate occurred at 60 and 72hpf in the caffeine treatment with significant difference with control ($P<0.05$). This finding is agree with (Chiranjib et al, 2011) who found that the increase of caffeine concentration, decreases hatching rate of zebrafish embryos. Therefore, the small amount of caffeine able to affect the developing embryos even complete concentration didn't reach to the embryos inside the eggs.

Although the previous studies, in which the concentration of 100μM caffeine is

unable to affect the heartbeat of zebrafish larvae (Chen et al., 2008; Selderslaghs et al., 2009), we found that continuous exposure to this concentration increased the heartbeat at all durations 48, 72 and 96hpf with significant difference with control at 72 and 96hpf.

Previous studies of which caffeine has various biological effects on all the tested organisms, showed that caffeine induces various cell responses, including cell death (He et al., 2003). In our experiment, we studied the expression behavior of some cell-damage related genes in order to know the mechanism of caffeine toxicity after exposure at different durations during the early development stages. We selected a linked package of genes related to cell damage including the oxidative stress, HSP70; mitochondrial metabolism, Cyclin G1; and apoptosis, Bax and Bcl2. Our results showed significant changes in all tested genes. Several studies reported an induced expression of these genes after exposure to different genotoxic compounds (Gonzalez et al., 2006 and Jung et al., 2011). HSP70, a molecular biomarker of cellular stress that induces denaturation of other proteins (Sanders, 1990; lewis et al., 1999) because they are involved in protection and defending cells from environmental offenses (Sanders, 1990) and their induction is much more responsive than traditional indices of contaminant effects (Feder and Hofmann 1999). There is no documented evidence to show that caffeine

directly provokes oxidative stress (Pin-zhen et al., 2008). In our results, we offer evidence that the caffeine directly provokes oxidative stress. First, we found a down-regulation of HSP70 at 24hpf after treatment (0.84 folds). The reason of this phenomenon is that the chorionated phase prevented the complete concentration of caffeine to reach to the embryos. So, that little amount which entered to the embryos affected as anti-oxidant. As known, caffeine has anti-oxidant properties if taken in moderate dose (Nikolic et al., 2003 and Jaya et al. 2010). Also it has been reported that it acts as a protective substance on cellular damage (Kamat et al., 2000; Krisko et al., 2005). So, the first embryo-response to caffeine may enhance a natural tolerant after exposure to caffeine for short time as if taken in a little amount (Kristensen, 1995; Luckenbach et al., 2001 and Chiranjib et al, 2011). At 48hpf treatment, the up-regulation of HSP70 was occurred (1.35 folds-increase) but with no significant difference ($P>0.05$) with control. The high significant up-regulation expression of HSP70 (11.75 folds-increase) was found after continuous exposure to caffeine up to 72hpf, as an indicator of the high stress which occurred to the cells of the developing embryo. Our result is agreed with and confirm the results obtained by (Pin-zhen et al., 2008), who demonstrated that caffeine stimulates intracellular oxidative stress in human osteoblasts.

One of the main targets of toxicity is mitochondria and there is a close

relationship between oxidative stress and proper mitochondrial function (Krunschnabel et al., 2005; Pourahmad and O'Brien, 2000; Risso-de Faverney et al., 2004). The expression behavior of a gene related to mitochondrial metabolism, Cyclin G1, was studied also in order to demonstrate the role of mitochondria in the pathway of caffeine toxicity and apoptosis. Cyclin G1 is a gene which involved in cell cycle arrest to allow time for DNA repair when DNA is damaged (Ford, 2005; Kohn, 1999). Mitochondrial membrane potential change is directly associated with apoptosis (Li et al., 1997 and Weber et al., 2003). Our results showed direct up-regulation of Cyclin G1 expression with significant difference with control immediately after exposure and at all durations 24, 48 and 72hpf (1.37, 1.21 and 1.35 folds-increase), respectively. The slight decrease of Cyclin G1 expression at 48hpf treatment was not significant ($P>0.05$) and may because of a possibility that embryos can induce a tolerance to this amount (Kitiucia et al., 2011 and Chiranjib et al, 2011), or zebrafish embryos may developed a natural tolerance capacity of toxicity from the natural systems (Kristensen, 1995 and Luckenbach et al., 2001). There is evidence that the cytotoxicity of caffeine may due to its ability to trigger apoptosis (Fernandez et al., 2003). Our finding regarding the expression of Cyclin G1 may support the idea of that caffeine triggers apoptosis via a mitochondria-dependent pathway (Pin-zhen et al., 2008).

In order to verify this idea, Two genes involved in apoptotic mechanisms were selected, Bax and Bcl-2 (Fernandez et al., 2003; Sandrini et al., 2009). Bax and Bcl2 are the major effectors of mitochondria-mediated apoptosis. Both Bcl2 and Bax are transcriptional targets for the tumor suppressor protein, p53, which induces cell cycle arrest or apoptosis in response to DNA damage (Basu and Halder, 1998). These genes regulate changes in the mitochondrial membrane potential (MMP) and permeability, which play important roles in apoptotic processes (Shimizu et al., 2000). Bcl-2 gene play important role in apoptosis, similar to cysteine proteases which regulate mitochondrial membrane potential changes and the release of cytochrome C by modulation the outer mitochondrial membrane permeability (Martins et al., 1997; Nicholson and Thornberry, 1997). It is located in the inner membrane of the mitochondria and plays an important role in the regulation of ROS production and anti-oxidation. It localizes to sites where free radicals are generated and functions as an apparent antioxidant against oxidative stress to prevent apoptosis (Hockenbery et al., 1993). The negative impact occurs when Bax expression increases and Bcl-2 expression decreases (Basu and Haldar, 1998; Elisabeth et al., 1996; Miyashita et al., 1994). Our results showed significant down-regulation of Bcl2 with the increase of exposure time at 48 and 72hpf. The other gene affects mitochondria-mediated apoptosis, Bax.

Bax is a pro-apoptotic Bcl-2 member gene, which triggers a mitochondrial pro-apoptotic pathway by promoting the mitochondrial release of cytochrome C (Bernardi et al., 2001; Gottlieb 2001). The up-regulation expression of Bax means activation of apoptotic cell death. However, many researchers have presented not expression data of both Bax and Bcl-2 genes but the ratio of Bax/Bcl2 is an indicator for cell apoptosis (Jung et al., 2011; Basu and Haldar, 1998). The high ratio of Bax/Bcl2 means cell apoptosis (Pepper et al., 1997; Pavlovic et al., 2006; Salakou et al., 2007; Jin et al., 2011). Therefore, the change in the Bax/Bcl2 ratio is important issue to demonstrate the apoptosis occurrence (Pavlovic et al., 2006). Specifically, a high Bax/Bcl-2 ratio is associated with a lower threshold of apoptosis, while a low ratio represents a higher apoptotic threshold (Chan et al., 2007; Chan 2007). Our results demonstrated that the Bax/Bcl-2 ratio increase with the increasing of exposure time in the caffeine treatment and significantly higher than the control. Thus, the results in (Fig 4) indicate a potential increase of apoptosis, and the last was happened because of oxidative stress, which is induced by increasing the exposure time of caffeine (Deng et al., 2009; Echtay et al., 2002; Nègre-Salvayre et al., 1997).

In conclusion, the above results together with the previous studies, we conclude that the increase of the exposure time of caffeine, increase the heartbeat, and

affects the expression of cell-damage related genes of the developing zebrafish embryos and may make damage to the cell. By analyzing the expression behavior of all the selected genes, we can illustrate that the oxidative stress which caused by caffeine exposure, affects mitochondrial metabolism, which may induce cell apoptosis. Therefore, our results demonstrate that caffeine may induce apoptosis by modulating the Bax/Bcl2 ratio, the major effector of mitochondria-mediated apoptosis via mitochondria-dependent pathway. Use of caffeine during pregnancy and lactation may affect the developing fetus and cause cell-damage by affecting the expression of cell-damage related genes. Finally, zebrafish embryos as a model for examining and analyzing the negative impact of chemicals and pharmaceuticals, are promising and have many advantages more than the other laboratory animals in this regard.

Table1. Pimers used for quantitative real time RT-PCR.

Gene	Mechanism	Forward primer (5'-3')	Reverse primer(5'-3')	Accession number
HSP70	Oxidative stress	CATCGACGCCAACGGG	CCAGGGAGTTTTTAGCAGAAATCTT	AB062116
Cyclin G1	Mitochondrial metabolism	TCTCTCCTTGACTCGATTCTTTG	AATATTCAACCAGGCACTTAGCA	BC052125
Bcl2	Apoptosis	TTGTGGAGAAATACCTCAAGCAT	GAGTCTCTCTGCTGACCGTACAT	BC133848
Bax	Apoptosis	GAGCTGCACTTCTCAACAACTTT	CTGGTTGAAATAGCCTTGATGAC	BC055592

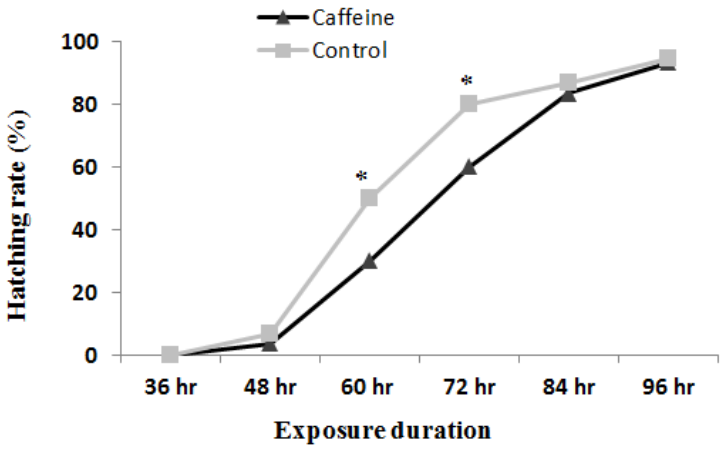


Figure 1. **Hatching rate of zebrafish embryos exposed to 100μM caffeine for different durations.** Hatching rate was observed at 48hpf, and the overall hatching was calculated at 96hpf. Effect of caffeine treatments appeared at 60 and 72hpf by a delay of hatching rates with a significant difference with control ($P<0.05$). Data are presented as means ±SD (n=30 individuals in each replicate in each treatment). The asterisk mean a significant difference ($P<0.05$) with control.

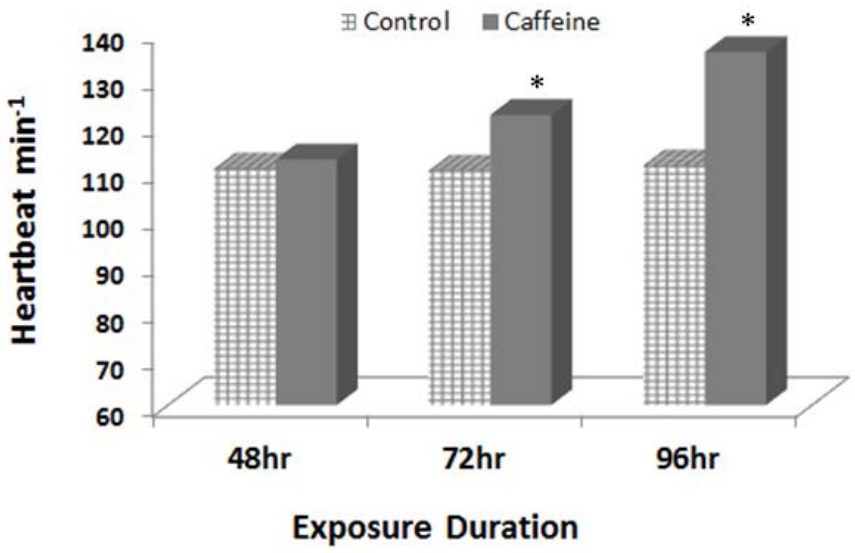
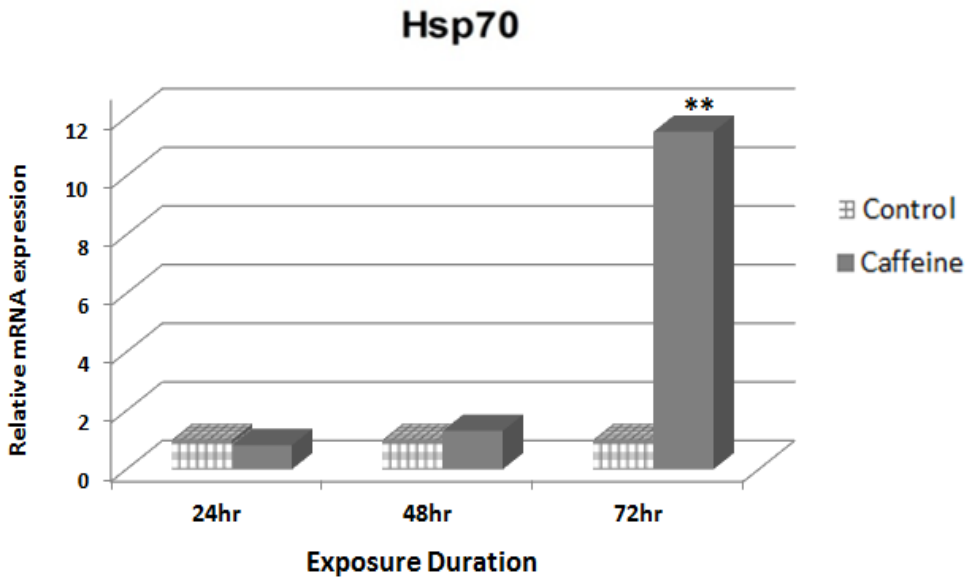


Figure 2. Heartbeat min^{-1} in zebrafish embryos exposed to 100µM caffeine at different durations; 48, 72 and 96hpf. I counted the heartbeat per 1 minute for the treatment and control at each point. The averages heartbeat in the control groups were 110, 110 and 112 at 48, 72 and 96hpf, respectively with no significant difference (P>0.05). Significant high heartbeat was found in caffeine treatment 122 and 126 Heartbeat min^{-1} at 72hpf and 96hpf, respectively. Values are relative to the average of control group at each point and expressed as means +SD (n=10 individuals in each each replicate in each treatment). * means the values are significantly different with control (P <0.05).

A


Hsp70
Relative mRNA expression
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24hr
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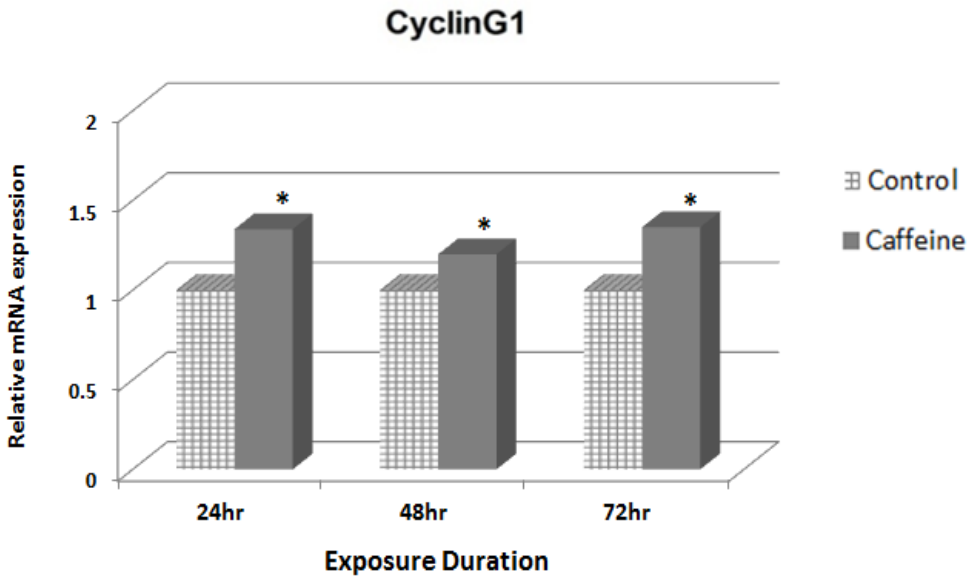
72hr
Exposure Duration
Control
Caffeine

B


CyclinG1
Relative mRNA expression
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Exposure Duration
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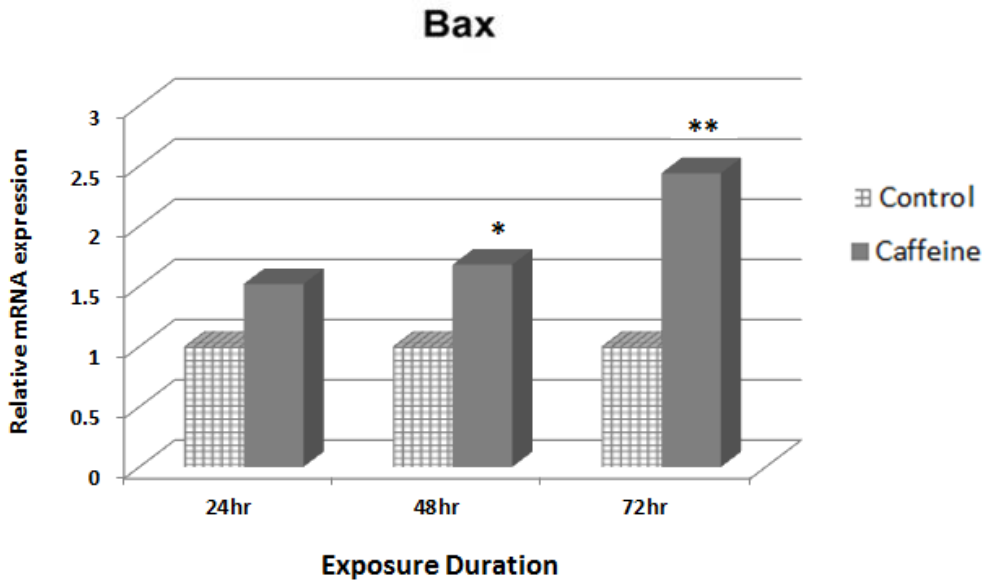
Caffeine

C


Bax
Relative mRNA expression
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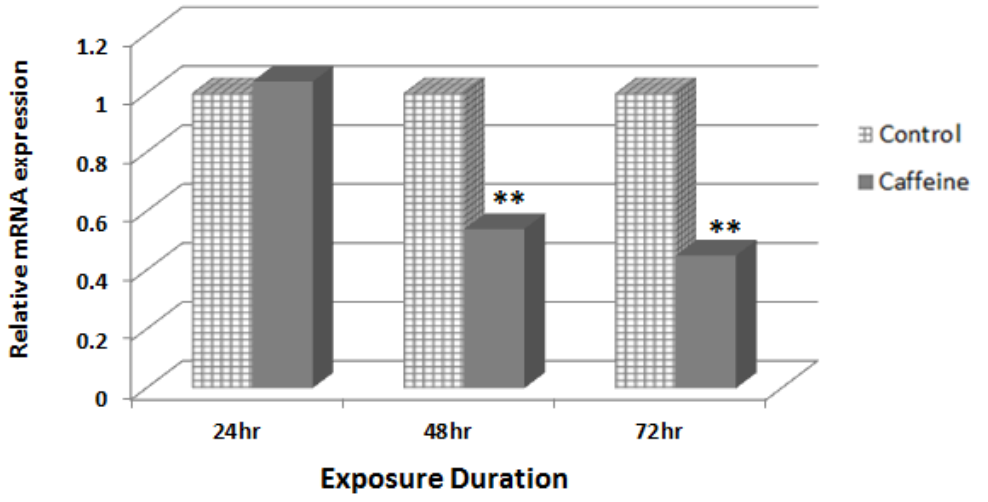
Bcl-2
Relative mRNA expression
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1
0.8
0.6
0.4
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0
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24hr
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Control
Caffeine

Figure 3. Relative quantitative mRNA of cell damage-related genes. I analysed the expression of oxidative stress Hsp70 (A), mitochondrial metabolism Cyclin G1 (B), apoptosis Bax(C) and Bcl-2 (D) of the zebrafish embryos after exposure to 100 μM caffeine solution for different durations at early developmental phases; 24, 48 and 72 hpf. The control values in each point were adjusted to equal 1, and the treatments values were multiplied in the same factor of control. Values are relative to the control group and expressed as a fold-change means +SD (n=15 individuals from each replicate from each treatment). * means values significantly different with the control ($P<0.05$), **mean values significantly different with the control ($P<0.01$).

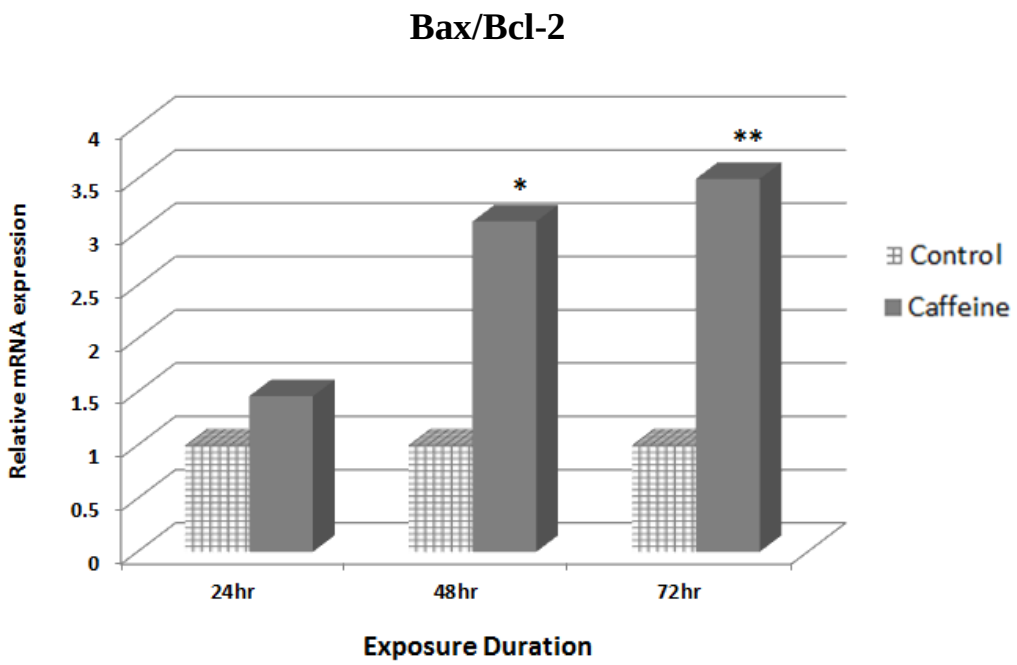

Figure 4. Bax/Bcl-2 ratio of zebrafish embryos exposed to 100 µM caffeine at different durations. mRNA isolated from zebrafish embryos exposed to 100 µM caffeine solution for different durations 24, 48 and 72hpf. Values are relative to the control group and expressed as means +SD. * Mean values significantly different with the control group ($P<0.05$), ** mean values are significantly different with the control ($P<0.01$).

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CHAPTER II

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

2.1. Abstract

Epidemiological studies suggested that caffeine/coffee could be an effective therapeutic agent against Alzheimer disease (AD). The study was conducted to test the effect of caffeine on Alzheimer's molecular factors and two cell communication systems involved in AD, 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) genes. There was inverse relation between AR and DR. This study demonstrated that caffeine has effect on the tested genes but 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.

2.2. Introduction

Epidemiologic studies have increasingly suggested that caffeine/coffee could be an effective therapeutic against AD (Arendash and Cao, 2010) AD is the most common form of neurodegenerative disease with more than 20 million cases worldwide (Goedert and Spillantini, 2006). 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 (Mark, 2004).

AD generally featured by presence of neurofibrillary tangles and amyloid deposits that form plaques and cerebrovascular accumulations (Xiong et al, 2005). Extensive research on understanding mechanisms that underlie the disease process has led to the identification of a number of genetic, environmental, and lifestyle factors that significantly contribute to the increased risk of developing AD and play major roles in the disease pathogenesis (Verdile and Martins, 2008). However those genes may increase the risk of developing the disease, the most well-established link between AD and genetics is in familial early-onset AD. The discoveries of genetic aberrancies that either cause or increase the risk of AD heralded a rapid increase in knowledge of the molecular and cellular alterations responsible for neuronal degeneration and cognitive dysfunction in AD (Selkoe

and Schenk, 2003). One of the most intensively studied molecules is APP (Goldgaber et al, 1987; Tanzi et al, 1987). It is known as main controller of senile plaques which 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 (Guo et al, 2012). Two other genes linked to early-onset familial Alzheimer Disease 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 mis-folded proteins in the ER, and cleavage of APP (Tedde et al, 2003).

Also one of the most widely studied risk factor for sporadic AD is apolipoprotein E (apoe). This gene is an allele of which (apoe4) has been associated with increased risk for late-onset AD. People who carry one or two copies of the ApoE ε4 allele carry an increased risk of developing AD, however the ε4 allele is not necessary or sufficient to cause AD (Prasanthi et al, 2010). ApoE associates with lipoprotein particles and facilitates their interaction with lipoprotein receptors (Suh and Checler, 2006). The gene coded by Sorl1 is a neuronal ApoE receptor. Lack of the ApoE receptor is suspected to be a

contributory factor to Alzheimer's (Scherzer et al, 2004).

For decades now, zebrafish have become a promising model in many research areas, including neuroscience, developmental biology and toxicology (Lele and Krone, 1996; Vascotto et al, 1997; Ivetac et al, 2000; Bowman and Zon, 2010) . Three distinct zebrafish adenosine receptors A_{2aa}, A_{2ab} and A_{2b} were discovered (Wendy et al., 2009). Adenosines are biological endogenous purine nucleosides that modulate a variety of physiological processes with an important role in signal transduction which increases drastically when brain ischemia is caused by a stroke. They are also potent anti-inflammatory agents that play an important role in tissue protection and repair (Jacobson and Gao, 2006). In the central nervous system CNS, adenosine is involved in regulating neurotransmitter release as well as postsynaptic neuronal responses (Cunha, 2001).

The A_{2a} has emerged as a potentially attractive therapeutic target for the treatment of Parkinson’s disease (PD), based in part on its unique distribution in the CNS (Wendy et al., 2009). The effects of the modulation of gene expression on normal aging and in pathological conditions as Alzheimer’s disease are still unclear, but the use of non-selective antagonists like caffeine to treat AD-related cognitive deficits is showing promising results (Fredholm et al, 2005; Anisur, 2009). In our present work, we tested the effect of caffeine on mRNA expression

of a package of confirmed AD-involved genes in two concentrations, 10 and 100μM, and also analyzed the correlation with 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- mRNAs expression in order to understand the mechanisms of the disease in this model and pursuit to improving the pharmaceuticals for this disease.

2.3. Materials and Methods

Zebrafish maintaining

Zebrafish (*Dario rerio*) were obtained locally and raised under our established laboratory housing system. Adult zebrafish were kept in glass aquaria under recirculation system with a photo period cycle of 14 and 10 hours, light and dark, respectively and temperature of 28° Celsius. 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 hours in a 28℃ incubator. In the next day, the barriers were removed at the beginning of light period to allow mating and spawning. We followed the Care and Treatment of the Animals guidelines by Institutional Animal Care and Use Committee, Seoul National University (Approval no. SNU-050418-2). The technical procedures were according to the zebrafish guidelines book (Westerfield, 1995).

Caffeine solutions and Exposure

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

Experiment design

The fertilized eggs were collected and washed twice with E3 medium. About 2hpf (cleavage stage 32–64 cells), eggs were distributed randomly into three 12-well plates to start exposure, one plate for each check point. The plates were filled with 3ml of exposure solution 10, 100 μM or distilled H_2O in three biological replicates for each treatment. At 24hpf, the embryos from the first plate were

prepared for molecular analysis. 1.5 ml exposure solution of the other plates was exchanged with fresh one daily until the end of the experiment.

Monitoring assay

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

Molecular analysis

Embryos from each treatment were pooled and prepared for tRNA extraction using the protocol provided by Chen Laboratories, Department of Chemical and Systems Biology, Stanford University (Abdelkader et al, 2012). cDNAs were synthesized using TOPscript™ cDNA Synthesis kit (CAT EZ005S). Polymerase chain reaction was performed according to the kit provider guidelines (AccuPower® PCR PreMix, Cat: K-2016). The reactions were done by mixing (1 µl cDNA, 1 µl forward primer, 1 µl reverse primers and complete the mix to 20 µl with Distilled water). The thermal cycling protocol was (95^{o}C for 3 min, followed by 40 cycles of 95° C for 10 s, 60° C for 30s and 72° C for 40s, then one cycle of 72°

C for 2min. Optical density of the bands was measured by ImageJ program. (http://imagej.nih.gov/ij/index.html). Real-Time RT-PCR (qPCR) was performed according to the Takara Bio Inc. guidelines. A 25µl RT-PCR reaction was done by adding 1 µl cDNA, 1 µl forward primer, 1µl reverse primer, 12.5 µl SYBR Premix Ex Taq (Takara Bio Inc. Shiga, Japan) and 9.5 µl of Nuclease-free water (Ambion Inc., Austin, TX, USA) to give the final volume of the reaction, 25µl. 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 analyzing data) according to the company's instructions. The thermal profile for real-time RT-PCR was 95°C for 3 min, followed by 40 cycles of 95° C for 10 s, 60° C for 30 s and 72° C for 40 s. We used βactin as a housekeeping gene to normalize the results by eliminating variations in mRNA and cDNA quantity and quality. Each mRNA level was expressed as a ratio to βactin 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 $2^{\Delta\Delta ct}$ formula method. All primers used in this experiment are described in Table 1.

Statistical analysis

Relative mRNA expression results were shown as the mean ± SEM of relative normalized expression of RT-PCR values. We performed two-way analysis of variance (ANOVA) to analyze the data followed by Tukey's multiple comparison tests. Results of optical density of mRNA expression were also analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni posttests. * Means significant difference occurred for a given parameters when $P<0.05$ and ** means high significance when $P<0.01$. The entire statistical analysis was carried out using Graphpad prism (Version: 4.03).

2.4. Results

Monitoring assay

100µM concentration of caffeine has been approved that it has no effect on the locomotor activity. Here, we did not observe any morphological change during the experiment in both 100 µM and 10 µM concentration or control. Hatching started at about 48hpf in all treatments and control. There was no difference in overall hatching rate between treatments and control (data not shown). The overall survival rate (Fig. 1) and were 84%, 78% and 76% of control, 10 µM and 100 µM,

respectively with no significant difference (P>0.05).

Molecular analysis

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

Optical density of adenosine receptors expression

We analyzed the expression of each adenosine receptor by measuring the optical density of mRNA expression using ImageJ software. Caffeine altered the mRNA expression of all tested receptors (Fig. 2). At 24hpf, 10µM of caffeine significantly up regulated the expression of A_{2aa} and A_{2ab} genes (P<0.05) and high significantly up regulated the expression at 100 µM (P<0.02). The changes of expression at 96 and 168hpf had no significant difference with control except at 100 µM (P<0.05). Effect of caffeine on Adenosine A_{2b} receptor was controversial since the expression at 24hpf was significantly lower than control even though it was higher than control with no significant difference (P>0.05). The vice versa was occurred at 96hpf. The expression was significantly lower than control at 168hpf under both concentrations.

Optical density of dopamine receptors mRNA expression

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

Relative quantitative of AD genes expression

All selected genes involved in AD pathway; APP, psen1, psen2, apoe and sorl1 are already expressed also at early developmental stages of zebrafish embryos. First, control was adjusted to 1 value and the expression changes of all tested genes were calculated as a fold change compared to the control. Caffeine altered the genes expression at all check points (Fig.4). A down regulation was occurred to the expression at 24hpf. 10 µM of caffeine significantly ($P<0.05$) down-regulated all tested genes except APP. A high significant ($P<0.01$) down-regulation was occurred in 100 µM caffeine treatment. The highest down-

regulation was found in APP gene, about 0.4 fold compared to control. Thus, at 24hpf, caffeine down-regulated all tested genes expression and the effect increases at the higher concentration, 100 µM. At 96hpf, continuous exposure to caffeine showed different effect on the expression of all the tested genes. A down-regulation of Sorl1, ApoE and Psen2 genes at 10 µM treatment was 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 about 0.9 fold changes with control. Caffeine 10 µM concentration up-regulated the expression of APP and psen1 by 1.2 and 1.9 fold changes significantly with control however the 100 µM treatment up-regulated the expression of APP and psen2 by 2.6 and 1.2 fold changes respectively, significantly with control and non-significantly in apoe by 1.1 fold. To identifying expression pattern after completion the neurodevelopment. mRNAs from developing zebrafish larvae at 168hpf were analyzed. Caffeine significantly down regulated sorl1 and APP at 10 µM and 100 µM but in psen1 was only at 100 µM treatments. Down-regulation of apoe was at 10 µM treatment and 100 µM with no significant difference with control. Also the down regulation of psen1 was non-significant at 10 µM treatment and in psen2 at 100 µM treatment.

2.5. Discussion

It was suggested that caffeine could be an effective protective and therapeutic against AD. The confirmed genetic susceptibility factors for AD were selected and studied in this present work. I analyzed the expression of APP, psen1, psen2, apoe and sorl1 genes in developing zebrafish embryos at 24, 96 and 168hpf after exposure to two different concentrations of caffeine, 10 and 100 µM. Also I studied the effect on the expression of two major neurotransmitters in cell communication systems associated to AD; Adenosinergic and dopaminergic systems by analyzing the expression of their receptors.

I found that all tested genes already expressed at early phases of development with a pattern of fluctuation during the development and caffeine treatment.

First, it was reasonable to using 100 µM concentration of caffeine that it was studied previously in zebrafish experiments and approved with no effect on the locomotor activity and also unable to promote significant embriotoxicity and phenotypic features (Chen et al, 2008; Selderslaghs et al, 2009).

To study the direct effect of caffeine on AD genes expression, I choose a package of genes have already confirmed of their involved role in AD. APP gene provides instructions for making a protein called amyloid precursor protein. This

protein is found in many cells including brain and 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 (Mattson, 1997). 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 in the brain of neurotoxic forms of Aβ. The evidence supporting the "amyloid hypothesis" of AD is extensive and has been reviewed (Hardy, 1997). 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 (Zhang et al., 2006). Those genes are considered the involved genes of amyloid pathway that the central to the disease is altered proteolytic processing of the APP as mentioned above resulting in the production and aggregation of neurotoxic forms of Aβ. In our study we found that caffeine has direct effect on those genes since it down-regulated the expression at early developmental stages of zebrafish embryos. The significant up-regulation in APP and psen1 expression was occurred at 96hpf but after completion the neurodevelopment, caffeine significantly down-regulated their expression compared with control.

I also found that caffeine regulates the expression of psen2 gene. This gene is known for its role in processing amyloid precursor protein (Gerrish et al., 2012). Research suggests that psen2 works with other enzymes to cut amyloid precursor protein into smaller segments (peptides), amyloid beta peptide and soluble amyloid precursor protein (sAPP), the later 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 amyloid-β peptide are under investigation (Das, 2008; Melnik and Plewig, 2013). The up-regulation of this gene after caffeine treatment may increase the formation of neurons and / or help for generation of substantial numbers of new neurons in human (Wade, 1999; Nowakowski, 2006).

The most widely studied risk factor for sporadic AD is apolipoprotein E (apoe) (Prasanthi et al., 2010). Apoe gene mutation of the gene for apoe is predictive of Alzheimer's disease (Wade, 2007). The significant reduction in sorl1 expression , the receptor of apoe gene, has been found in brain tissue of AD patients (Anderson et al., 2005). The apoe receptor has also been linked to the regulation of APP, faulty processing of which is implicated in AD (Scherzer et al., 2004). A more recent study by a group of international researchers supports the proposition that sorl1 plays a part in seniors developing Alzheimer's disease, the findings being significant across racial and ethnic strata (Rogaeva et al., 2007;

Hall, 2007). In addition to the link between Sorl1 and beta amyloid, there's a connection between sorl1 and the apoe gene already known to influence the risk of developing Alzheimer's (Dodson et al., 2008). The protein sorl1 is a receptor which binds itself to nearby molecule and then causes a reaction within a cell. The specific molecule binds to apoe.

The interactions between sorl11 and apoe have been surprisingly elusive (Dodson et al., 2008). Actually, it is difficult to believe the functional links between those two genes are merely coincidental. Here, we observed the relation between both genes after exposure to caffeine, down-regulation of Sorl1 did not cause down-regulation of its target gene, apoe. Accordingly, the continuous exposure to caffeine may protect and maintain the apoe gene expression from a reduction of its receptor expression after neurodevelopment completed and this may support the idea of that long term of caffeine reception in moderate levels may protect against neuro system inflammation (Ross et al., 2000; Thompson and Keene, 2004).

Accordingly, caffeine has positive affect against AD by a reduction in the expression of the genes associated to the disease. Also, continues exposure to caffeine may give positive impact against the expression behavior of APP and the psen1 genes.

In order to study the correlation between caffeine and cell communication systems, I first studied the appeal of caffeine for AR. I analyzed the optical density of three zebrafish adenosines A_{2aa}, A_{2ab} and A_{2b} mRNA expression. I found that caffeine up-regulated the expression of A_{2aa} and A_{2ab} only at 24hpf. 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 (Aden et al., 2000; Leon et al., 2002). In zebrafish embryos, the effects of caffeine on the studied genes appear to be selective to A_1 and A_{2aa} adenosine receptors probably by the high affinity of these receptors for caffeine (Fredholm et al., 1999). My results demonstrated that caffeine has regulatory effect on all adenosine receptors especially A_{2aa} and A_{2ab} and this may indicate that those two receptors have higher affinity for caffeine than other receptor.

A_{2a} is the highest affinity receptors 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 A2a adenosine receptor (Boehmler et al., 2009).

The interaction between adenosine receptors and 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 (Antoniou et al., 2005; Ferre et al., 1997; Fuxe et al., 2007). The tested concentrations in this experiment have no effect on the locomotor activity of developing zebrafish but I found that A2aa and A2ab up-regulation at 24hpf was accompanied by a reduction of both dopamine receptors expression especially at the higher concentration of Caffeine and almost blocked the signal. But continues exposure to caffeine up regulated the expression of both dopamine receptors at 96hpf and 168hpf whereas the adenosine receptors expression significantly down regulated at the 100 μM caffeine concentration. This phenomenon declares the fact of the ability of adenosine A2a antagonists to reverse the locomotor suppression that results from interference with DA transmission. So, caffeine can cause its stimulating effect on the dopamine receptors genes expression at later stages of zebrafish development and will be reasonable therapeutic target for protection against AD. Hence, the stimulation of dopamine receptors is also an important event associated to AD that the Dopamine is particularly involved in the regulation of cognitive processes associated with Alzheimer's disease. 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, the level of dopamine may be low in people with Alzheimer's disease (Lifen et al., 2004).

Hence, caffeine may help AD patients in directly by maintaining or raising the expression of their dopamine and make them feel better.

This study demonstrated that caffeine regulates the expression of Amyloid pathway involved genes. The direct positive effect of caffeine is a partially up-regulation of psen1 and psen2 genes expression. However caffeine down regulated the expression of sorl1 gene, it also down regulated the expression of APP gene expression and this interaction may play a role in the protective effect of caffeine against AD in the long term treatments. Caffeine also may play indirect role in the protection against AD via stimulations the expression of DR across unknown mechanism, although there is no evidence up to date demonstrates the role of DR in AD. .

Zebrafish is promising organism to study AD at the molecular level since all tested genes already expressed at early phases of development. AR; A_{2aa} and A_{2ab} have higher affinity for caffeine than A_{2b} under our experiment conditions. Caffeine has direct regulatory effect on amyloid pathway involved genes and we suggest it may help AD patients via stimulation of DR expression.

Table 1. Primers used in PCR and RT-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	A_{2aa}	NM_001039815.1	ATCATCGTTGGTTTGTTCGCC	CCACTGAGTTTGCGTGTGAGA	139
8	A_{2ab}	NM_001040036.1	CCGAGAGGAAGTCTCCTCCA	CCAGCCACATTCGGGTCAT	197
9	A_{2b}	NM_001039813.2	GGATTCGCTCTACATCGCCA	AGTGATGGCAAAGGGGATGG	181
10	drd_{2a}	AY183456.1	ACATCTTCGTCACCCTGGAC	CGCAATCACACAGAGAGCAT	242
11	drd_{2c}	AY333792.1	TTATGCCCTGGGTGGTGTAT	CCCGTCTCTTGGAGCTGTAG	195

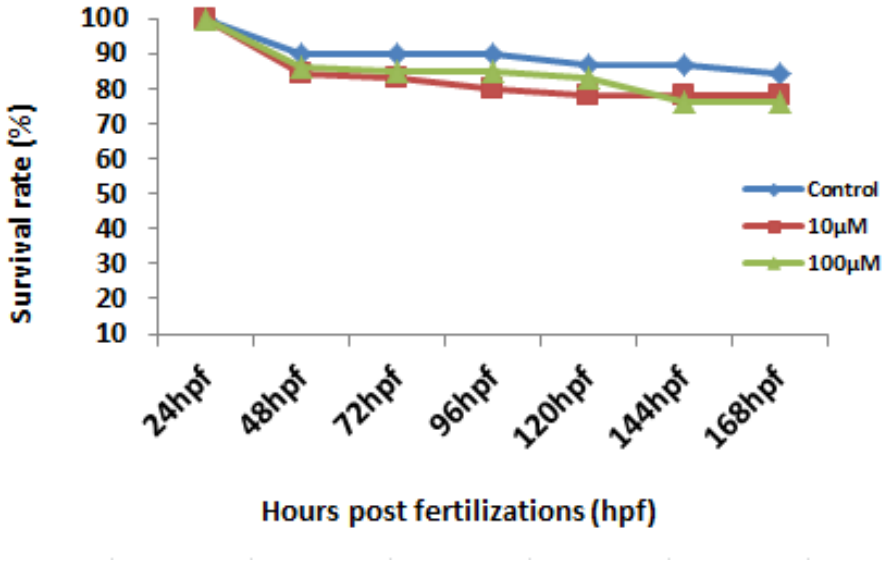
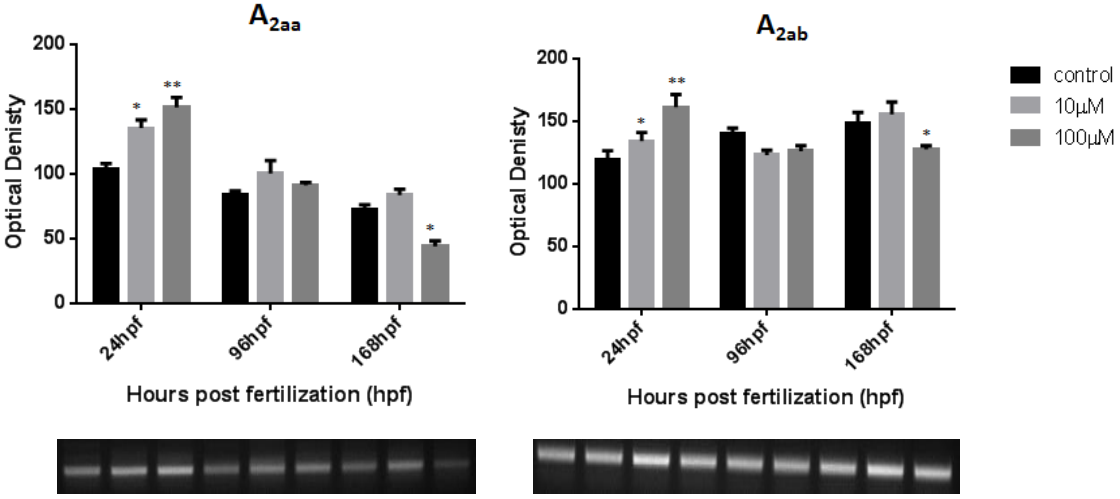


Figure 1. Survival rate of developing zebrafish after exposure to Caffeine solutions. We checked the survival rate every 24 hours from 24 to 168hpf. Values represent the means ±SEM. No significant difference between caffeine treatments and control P>0.05)


A2aa
Optical Denisty
200
150
100
50
0
*
**
*
24hpf
96hpf
168hpf
Hours post fertilization (hpf)
A2ab
Optical Denisty
200
150
100
50
0
*
**
*
24hpf
96hpf
168hpf
Hours post fertilization (hpf)
control
10μM
100μM

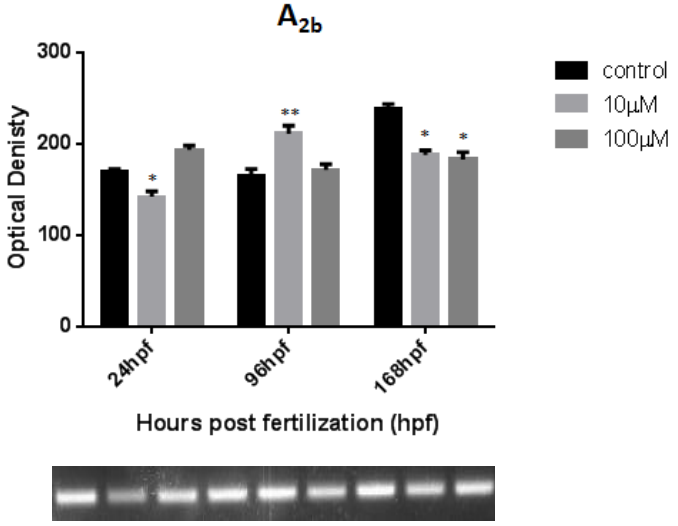

A2b
Optical Denisty
300
200
100
0
*
**
*
*
24hpf
96hpf
168hpf
Hours post fertilization (hpf)
control
10μM
100μM

Figure 2. Effect of caffeine on the gene expression of adenosine receptors at 24, 96 and 168hpf. Embryos from each treatment were pooled and prepared for total RNA extraction. cDNAs were synthesized from extracted total RNA. Polymerase chain reaction was performed according to the kit provider guidelines. The reactions were done by mixing (1 µl cDNA, 1µl forward primer, 1 µl reverse primer and complete the mix to 20µl with Distilled water). The thermal cycling protocol was (95°C for 3 min, followed by 40 cycles of 95° C for 10 s, 60° C for 30s and 72° C for 40s, then one cycle of 72° C for 2min. Bands intensity was measured by ImageJ program. A_{2aa} is Adenosine receptor 1aa., A_{2ab} is Adenosine receptors2ab. And A_{2b} is adenosine receptor 2b. * means significant difference with control ($P<0.05$), ** means high significant difference with control ($P<0.02$)

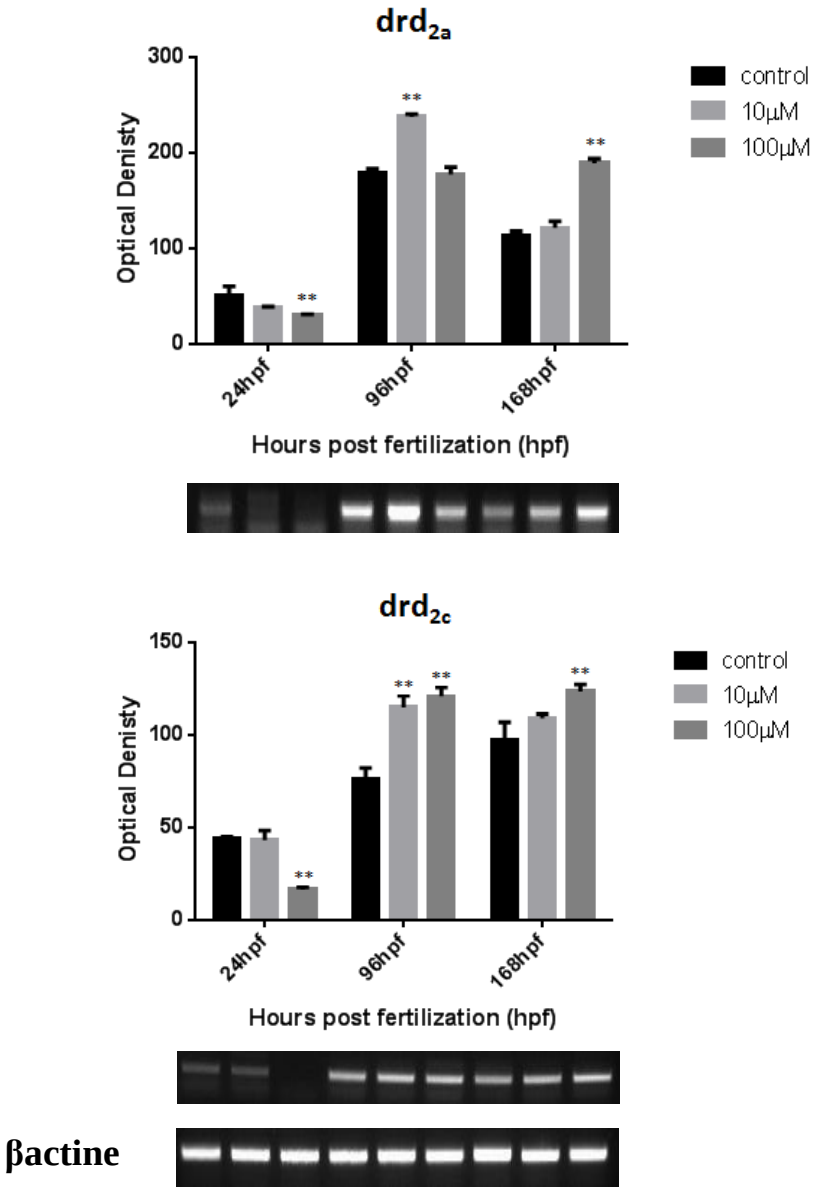
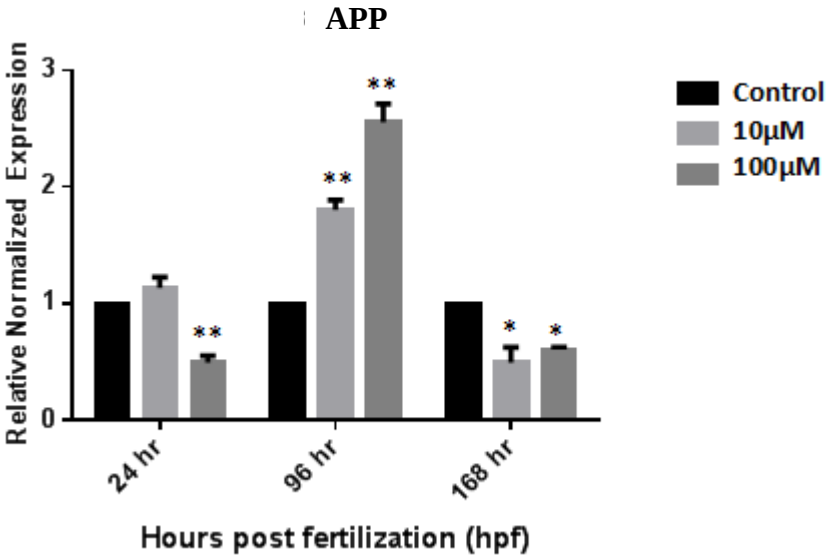
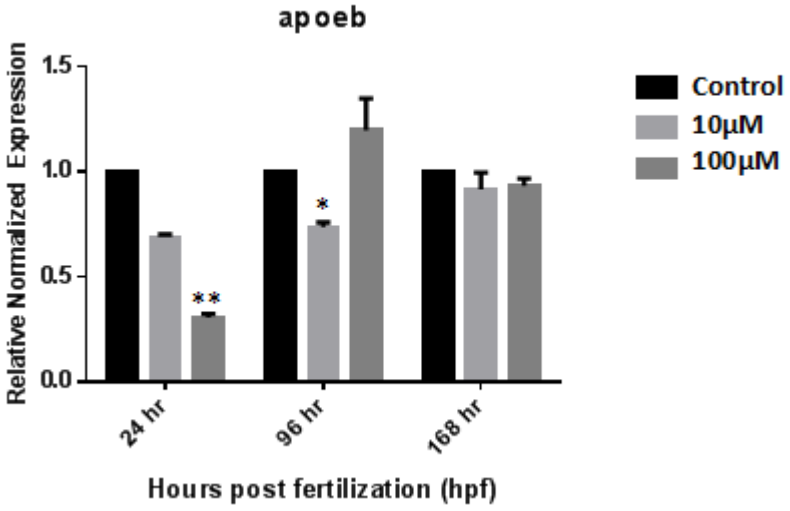
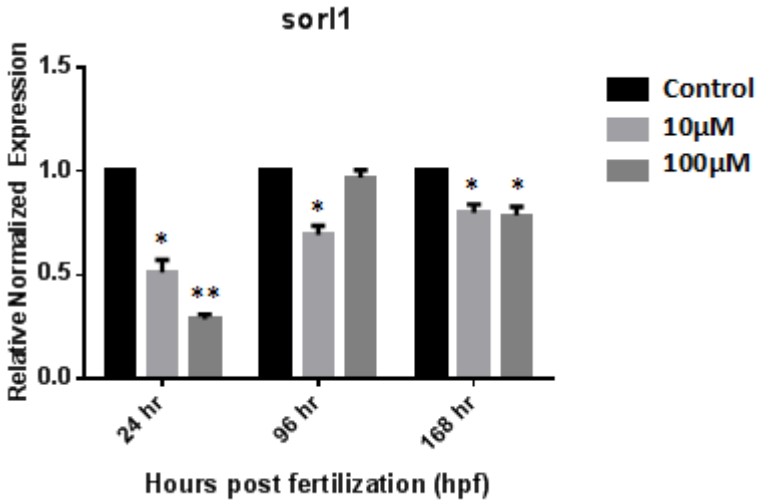
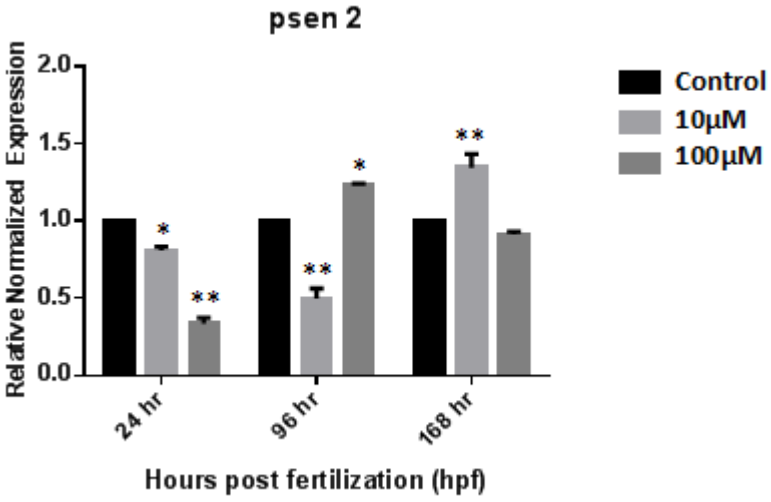

drd2a
300
200
100
0
Optical Denisty
**
**
**
24hpf
96hpf
168hpf
Hours post fertilization (hpf)
control
10μM
100μM
drd2c
150
100
50
0
Optical Denisty
**
**
**
**
24hpf
96hpf
168hpf
Hours post fertilization (hpf)
control
10μM
100μM
βactine

Figure 3. Effect of Caffeine on the gene expression of dopamine receptors at early developmental stages. Embryos from each treatment were pooled and prepared for total RNA extraction. cDNAs were synthesized and PCR was performed according to the kit provider guidelines. The reactions were done by mixing (1µl cDNA, 1µl forward primer, 1µl reverse primer and complete the mix to 20µl with Distilled water). The thermal cycling protocol was (95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30s and 72°C for 40s, then one cycle of 72°C for 2min. Bands intensity was measured by ImageJ program. drd_{2a} is dopamine receptor 2a. and drd_{2c} is dopamine receptor2c. * means significant difference with control ($P<0.05$), ** means high significant difference with control $P<0.02$)


APP
Relative Normalized Expression
3
2
1
0
**
**
**
*
*
24 hr
96 hr
168 hr
Hours post fertilization (hpf)
Control
10μM
100μM


apoeb
Relative Normalized Expression
1.5
1.0
0.5
0.0
**
*
24 hr
96 hr
168 hr
Hours post fertilization (hpf)
Control
10μM
100μM


sorl1
Relative Normalized Expression
1.5
1.0
0.5
0.0
*
**
*
*
*
24 hr
96 hr
168 hr
Hours post fertilization (hpf)
Control
10µM
100µM


psen 2
Relative Normalized Expression
2.0
1.5
1.0
0.5
0.0
*
**
**
*
**
24 hr
96 hr
168 hr
Hours post fertilization (hpf)
Control
10µM
100µM

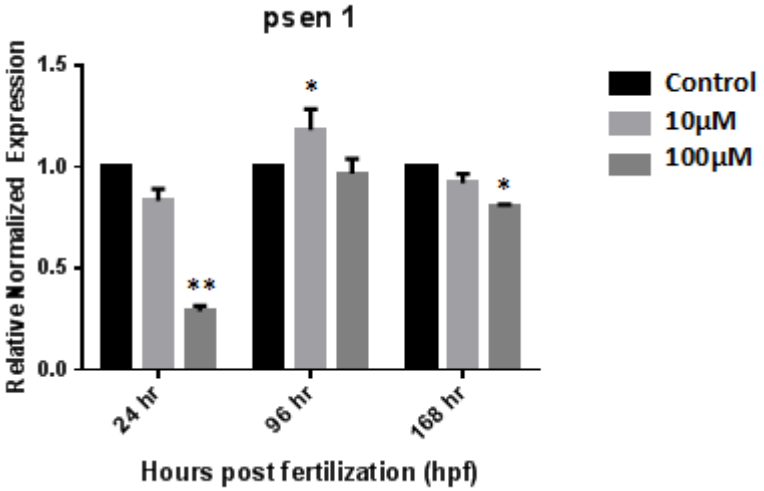

psen 1
Relative Normalized Expression
1.5
1.0
0.5
0.0
*
**
*
24 hr
96 hr
168 hr
Hours post fertilization (hpf)
Control
10µM
100µM

Figure 4. Effect of Caffeine on the gene expression of AD involved genes at early developmental stages. Bars represent the means ±SEM. Real-Time RT-PCR (qPCR) was performed according to the Takara Bio Inc. guidelines. A 25µl RT-PCR reaction was done by adding 1µl cDNA, 1µl forward primer, 1µl reverse primer, 12.5µl SYBR Premix Ex Taq and 9.5µl of Nuclease-free water to give the final volume of the reaction, 25µl. The thermal profile for real-time RT-PCR was 95°C for 3 min, followed by 40 cycles of 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 βactin 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 $2^{\Delta\Delta ct}$ formula method. * means significant difference with control ($P<0.05$), ** means high significant difference with control $P<0.01$). Two ways ANOVA was used followed by Tukey's test. The represented values are the fold change of mRNA expression compared to control

2.5. References

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CHAPTER III

Construction and characterization of TALEN Knockout zebrafish *Danio rerio* of Sortilin-related receptor (sorl1) gene: Model of neurodegenerative disorders

3.1. Abstract

This study utilized the TALENs technology to study 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. Three mutations were found in TALEN's spacer sequence in genomic DNA extracted from malformed embryos compared to controls. There were apoptotic cells under green fluorescence light in the TALEN microinjected embryos with bent tail shape at 24hpf around over the body stained with acridine orange. This study demonstrates that 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.

3.2. Introduction

The importance of functional genomics using model organisms has been recognized as an important prerequisite for interpretation of the impact of sequence variants on the function of gene(s), leading to initiatives such as the zebrafish phenome project (Sood et al, 2013).

Zebrafish have been widely used as a model system for studying developmental processes, but in the last decade, they have also emerged as a valuable system for modeling human disease. The development and function of zebrafish organs are strikingly similar to those of humans, and the ease of creating mutant or transgenic fish has facilitated the generation of disease models. Zebrafish share a high genetic similarity to humans, and approximately 70% of all human disease genes have functional homologs in zebrafish (Langheinrich, 2003). Also zebrafish considered a powerful vertebrate model organism for functional genomics due to the ease of microinjections of genetic material into embryos. Microinjections of morpholinos or mRNA leading to transient loss or gain of function phenotypes, respectively, can be used to assess roles of genes during embryonic development (Ekker, 2000; Bedell et al, 2011). The early embryos of zebrafish are numerous, large, easily accessible, and easy to manipulate

genetically. They represent an excellent system for analysis of early biochemical and molecular biological events during embryogenesis that may be more difficult to investigate in mammalian systems (Amatruda et al, 2002). One more advantage of the zebrafish model system is the ability to use forward genetics to reveal critical gene functions by their mutant phenotype.

Reverse genetic tools are available, although it was more challenging and time-consuming to identify mutations in specific genes of interest and virtually impossible to induce mutations in a targeted manner (Sharon, 2008). Currently, there are a variety of genetic or genomic manipulation technologies available like Zinc finger endonucleases (ZFNs). ZFNs are chimeric fusions between DNA-binding zinc finger proteins and the nonspecific cleavage domain of the FokI endonuclease. They can induce double-strand breaks in a specific genomic target sequence, which are imprecisely repaired by nonhomologous end joining (NHEJ) (Lieber, 2010). ZFN mRNA is injected into one-cell–stage embryos to generate zebrafish carrying the desired genetic lesions. It has been shown that germ cell mosaicism in the identified ZFN allele–bearing founder fish is up to 50% (Urnov et al, 2011). The bottleneck of this approach was the generation of ZFNs with high and specific activity in vivo (Zhu C, 2011).

In a similar strategy, researchers have recently fused transcription

activator-like (TAL) effectors to a FokI cleavage domain generating custom-made transcription activator-like effector nucleases (TALENs) to disrupt target genes in zebrafish (Li, 2011). TALENs are precise and efficient genomic engineering tools, which have been successfully used in many fields of scientific research (Joung and Sender, 2013). TALENs are fusion proteins and work in pairs, consisting of a modular DNA-binding domain and a FokI endonuclease monomer (Moscou and Bogdanova, 2009; Boch et al, 2009). When two TALENs bind to their DNA targets, the FokI monomers will dimerize and introduce a DNA double-strand break within the specific binding site (Boch and Bonas, 2010). The DNA break can either be repaired by NHEJ or homologous recombination (HR), which results in deletion/insertion mutations and specific site mutations or specific sequence additions, respectively. However, the conventional TALENs screening systems are mainly based on b-galactosidase or a single fluorescence reporter (Huang et al, 2011; Zhang et al, 2011) which functions in an indirect manner and limits the assessment of the transfection efficiency.

In this study, I designed TALENs plasmids to disrupt the first exon of zebrafish Sorl1 gene , a neuronal apolipoprotein E receptor (apoe), the gene for which is predominantly expressed in the central nervous system (Scherzer et al, 2004) (Accession number: XM_005157550.1). Recently, it was confirmed that

lack of this gene is suspected to be a contributory factor to Alzheimer's. A significant reduction in sorl1 expression has been found in brain tissue of Alzheimer's disease patients (Wade, 2007). The apoe receptor has also been linked with regulation of amyloid precursor protein, faulty processing of which is implicated in Alzheimer's (Anderson et al, 2005).

TALEN_sorl1 mRNAs injection into cytoplasm of 1cell stage of zebrafish aimed to demonstrate the effect of lack of this gene on the development of zebrafish embryos and to introduce a new model that may help for following studies in diagnostics, understanding the mechanisms of action, involved pathways and drug screens of development disorders including AD.

3.3. Materials and Methods

Zebrafish

Danio rerio were maintained locally in our lab facility at the Department of laboratory animal medicine, College of veterinary medicine (Seoul National University). After microinjection, Embryos were collected and grown in E3 media (5 mM NaCl, 0.17 mM KCl, 0.4 mM CaCl2, and 0.16 mM MgSO4) at 28℃ incubator for further analysis.

Construction of TALEN-sorl1 Plasmids

TALENs were constructed in a two-step assembly system (Addgene, Golden Gate TALEN Kit) according to (Cermak et al., 2011). In the first assembly (Figure 1A and B) , the pFUS vector (pFUS-A2A, pFUS-A2B or pFUS-B1-B6; 150 ng/ml) and the RVD plasmids (150 ng/ml) were incubated at 37℃ for 30 minutes with BsaI-HF (10 U, New England Biolabs) and 10 mM ATP (Invitrogen) in a 10 ml reaction volume (Sakuma et al., 2013). Subsequently, T7 ligase (1,500 U, Enzymatics) was added, and the reaction mixture was incubated at 25℃ for 1 hour. 0.5 ml of 10 mM ATP and 0.5 ml of Plasmid-Safe DNase (10 U/ml, Epicentre) were added, and the mixture was incubated at 37℃ for 1 hour. After the enzymes were inactivated by heating at 70℃ for 30 minutes, the reaction solution was used to transform JM109 cells on an LB plate containing 50 mg/ml spectinomycin, X-gal (5-Bromo-4-Chloro-3-Indolyl-b-D-Galactoside) and IPTG (Isopropyl-b-D-thiogalactopyranoside). The RVD ordering in the assembly vector (pFUS vector) was confirmed by sequencing. In the second assembly, 100 ng each of the pFUS-A2A, pFUS-A2B and pFUSB1- B6 plasmids containing the first assembly RVDs; 150 ng of the appropriate last repeat plasmid (pLR-HD, pLR-NI, pLR-NG, or pLR-NN) and 150 ng of pCS2PTAF were incubated with 0.5 ml of BsmBI (10 U/ml, New England Biolabs) in a 10 ml reaction volume. The ligation

reaction and Plasmid-Safe DNase treatment were performed as described for the first assembly. The ligation solution was used to transform JM109 cells on an LB plate containing 50 mg/ml ampicillin, X-gal and IPTG. The TALEN construct was confirmed by sequencing.

Solution Preparation and Microinjection

Constructed TALEN plasmids were linearized with NotI, and mRNAs were synthesized using the SP6 mMESSAGE mMACHINE Kit (Ambion) according to the manufacturer's protocol. Synthesized TALEN mRNAs were mixed together in equal quantity with phenol red and injected into cytoplasm of 1-cell stage embryos. We mix equal volume of mRNAs and 2x buffer containing 0.05% phenol red, 250 mM KCl and 40 mM HEPES (pH 7.4), so final phenol red concentration is 0.025%. We inject 1nl of the prepared mixture containing about 100-300 ng/ul mRNAs. Fertilized eggs were transferred to agarose ramps and microinjected with TALEN mRNAs then maintained in E3 medium at 28℃ incubator for further analysis.

Genomic DNA extraction and genotyping

Genomic DNA was prepared from non-injected and TALEN-injected embryos at

1dpf using the Gentra Puregene Tissue Kit (QIAGEN) according to the manufacturer's protocol. Targeted genomic loci were amplified using primers designed to anneal approximately 150–200 base pairs upstream and downstream from the expected cut site. F_ CCCTGTCACACCTTATCGTCA and R_TAAACAGAGCTGGTTTTGGGG. And following this protocol: 95° C for I min followed by 30 cycles of 95℃ for 20sec, 60℃ for 30 sec and 72℃ for 30 sec and final extension step is 72° for 1 min. The amplified samples were run into gel electrophoresis and the target bands with same size were eluted using QIAquick Gel Extraction Kit (Cat: 28704) according to the manufacture's protocol then were sequenced.

Acridine orange fluorescence test

In this experiment, we used acridine orange staining to detect the apoptotic cells in the TALEN microinjected embryos. Acridine orange staining can identify apoptotic cells in live embryos. Live dechorionated embryos are incubated in a 2 µg/mL solution of acridine orange in 1X E3 for 30 min. at room temperature. Embryos were washed quickly in E3 and then visualized on a stereo dissecting microscope equipped for epifluorescence.

3.4. Results

Evaluation of zebrafish sorl1 TALENs activity.

Two sets of TALENs plasmids, A and B, were constructed and tested for their activity by reporter-based assay, data shown in (Table 1). The test analysis showed high activity of generated TALEN plasmids than controls. The fold change of sorl1_A TALENs with sorl1_A reporter recorded value was about 4 times, whereas the negative control was only 0.02. Also the fold activation was over 32 compared to the negative control without TALEN. Similar records were obtained almost with both TALENs constructed sets which indicates a high activity of the constructed TALENS.

Lack of sorl1 induces various malformations of zebrafish phenotype

We found also that the TALEN microinjected embryos did not develop normally since early stages and developed several malformations like stunted structure, bent axis, bent tail, cardiac edema and yolk sac edema (Figure 2A), compared to the control (Figure 2B) which had no any defects and developed normally.

Lack of sorl1 induces bent tail “Hooked tail”

At 24hpf, normal zebrafish tail should be grown and separated from the body. We observed that almost all survived embryos that microinjected with TALENs induced bent tail phenomenon and were not able to hatch (Figure3 A, B and C). On the other hand, control embryos developed normally and hatched within 48h (Figure3D, E and F). After artificial hatching of TALEN injected embryos, we let them to grow, however they were not able to swim. Bent tail shape became sharper at 48h and took Perpendicular angle to the longitudinal axis of the body at 72h giving that hooked shape (Figure 2C).

Lack of sorl1 induces severe cardiac edema

Since early stages of development, we found cardiac sac edema in TALEN_sorl1 microinjected embryos which were obvious and determinable when compared with control (Figure 4 and 5). This edema increased more at the following stages until 7dpf and attached to the yolk sac in some individuals causing severe shape, we called it “Like-balloon shape”Balloon-shape (Figure 5 B), when the larvae were totally not be able to swim. Also there were multiple malformations in one individual of some larvae like bent tail and cardiac edema (Figure 5 C).

Mutation detection of TALENs microinjected embryos

Genomic DNA was prepared from non-injected or TALENs-injected embryos using the Gentra Puregene Tissue Kit (QIAGEN) according to the manufacturer's protocol. The TALEN target site was amplified using PCR with specific primers. After electrophoresis, the target bands with expected size were determined and extracted from gel and purified then sequenced. The alignment results of sequenced DNAs compared with control and reference showed alteration and addition of nucleotides in TALEN target site of microinjected embryos whereas no changes in the control. TALEN injection caused 3 mutations in its target site. The first mutation is alteration of cytosine nucleotide (C) base to guanine nucleotide (G) base. The other mutations are addition of two thymine nucleotides (T) bases on different locations of the target sequence (Figure 6).

TALEN-sorl1 induced Apoptotic cells

I applied Acridine orange fluorescence test to detect the apoptosis in TALEN sorl1 microinjected embryos. I found that the tested embryos developed apoptotic cells around the body (Figure 7), however there were no obvious fluorescence as indicator of apoptosis in the control.

3.5. Discussion

I used transcription activator-like effector nucleases (TALENs) technology to generate TALEN plasmids to disrupt the first exon of zebrafish Sorl1 gene. mRNAs were synthesized from linearized plasmids and microinjected into cytoplasm of 1-cell stage zebrafish. I found that the lack of Sorl1 gene resulted severe malformations in the microinjected embryos like bent tail, stunted structure and cardiac edema.

A number of different methods have been developed for the design and assembly of gene specific ZFNs and TALENs, making them easily available to most zebrafish researchers (Sood et al, 2013). TALEN technology is highly efficient and has been widely used for targeted gene disruption in vivo in recent years (Li, 2011; Joung and Sender, 2013). TALENs have been shown to be capable of generating mutations in endogenous zebrafish genes (Sander et al, 2011 and Huang et al, 2011).

Targeted mutagenesis using TALENs can be used to generate knockout zebrafish lines for analysis of their function and/or developing disease models. Recent publications using zebrafish to study the pathology of human neurodegenerative diseases including Parkinson’s, Huntington’s, and Alzheimer’s

indicate that zebrafish genes and their human homologues have conserved functions with respect to the etiology of neurodegenerative diseases. The characteristics of the zebrafish and the experimental approaches to which it is amenable make this species a useful complement to other animal models for the study of pathologic mechanisms of neurodegenerative diseases and for the screening of compounds with therapeutic potential (Yanwei et al, 2011).

Previously, I studied a package of confirmed molecular factors associated to AD. I found that all of them already expressed at early developmental stages of zebrafish (Abdelkader et al, 2015). Among those molecular factors is sorl1 gene. Variants in the gene encoding sorl1 are associated with AD, as well as its neuroimaging markers. To date this gene was not being knocked out or knocked down in zebrafish or in another model. The pathologic hallmarks of AD are extracellular Aβ protein-containing neuritic plaques and intracellular hyperphosphorylated tau-containing neurofibrillary tangles. Early-onset AD is associated with mutations in three genes involved in Aβ proteolysis displaying autosomal-dominant inheritance patterns in humans: amyloid-β precursor protein (AβPP), Presenilin 1 (psen1), and Presenilin 2 (psen2). Late-onset AD is linked to a number of genetic risk factors including the apolipoprotein E (apoe), which is a cholesterol transport protein, and the neuronal sorl1, which acts as a sorting

receptor for APP. Sorl1 gene is a neuronal receptor that interacts with the amyloid precursor protein to regulate amyloidogenesis.

According to the amyloid hypothesis, deposits of Aβ outside the neuron are the underlying cause of AD. The competing tau hypothesis states that the hyper phosphorylated tau protein, which forms neurofibrillary tangles inside neurons, is the catalyst for AD progression. This leads to the disassembly of microtubules essential for neuronal transport, disrupting neurotransmitter communication between neurons, and ultimately resulting in cell death.

In my experiment, I found that the most obvious and consistent phenotype observed in the Sorl1-lacked embryos was a stunted structure and development of bent or hooked tail at about 24hpf and increased with the time. This phenotype mimics that of the ‘captain hook’ mutants first described in the large-scale genetic screen for embryonic lethal mutations in zebrafish (Mullins et al., 1996).

I suggest that the reason of malformation caused by knocking out Sorl1 gene is due to the interaction between Sorl1 gene and APP. To demonstrate this phenomenon and to understand why TALEN-Sorl1 microinjected embryos did not develop well and/or developed stunted structure and axial malformations, I must illustrate that interaction between sorl1 gene and APP. According to this hypothesis, sorl1 binds to the APP. It functions as an intracellular sorting receptor

as APP is being trafficked between the secretory pathway, the cell surface, and, subsequently, endosomes. Sorl1 is localized primarily to the trans-Golgi network and early endosomes, shuttling between these two membrane compartments. Sorl1 interaction with APP in endosomal compartments limits the amyloidogenic proteolysis of APP. Reduced brain levels of sorl1 are thought to alter the transport and processing of APP to increase generation of Aβ peptides in early or late endosomes. (Haass et al, 2012; Miller and Zheng, 2012; O'Brien and Wong, 2011). Our suggestion is supported by the finding of Yanwei et al, 2011, who found that the loss of zebrafish Appa and Appb function by MO knockdown resulted in reduced body length and defective convergent-extension movements during gastrulation. Interestingly, these defects are rescued by wild-type human APP mRNA (Yanwei et al, 2011; Joshi et al, 2009).

I also checked the apoptosis in live microinjected and non-microinjected embryos. I used acridine orange fluorescence stain in this experiment. The base of acridine orange fluorescence stain method is that the ATP-dependent lysosomal proton pump is preserved in apoptotic but not necrotic cells therefore apoptotic cells will take up the acridine orange dye whereas living or necrotic cells will not (Darzynkiewicz, Bruno et al. 1992). Also, this method is useful for identifying mutants based on an apoptotic phenotype in order to further characterize them in

living assays (Daniel and games, 2013). I found that all tested individuals of microinjected embryos with TALEN mRNAs developed apoptotic cells around over the body especially at the twisted tail area, under green fluorescent light in a dissection stereoscope.

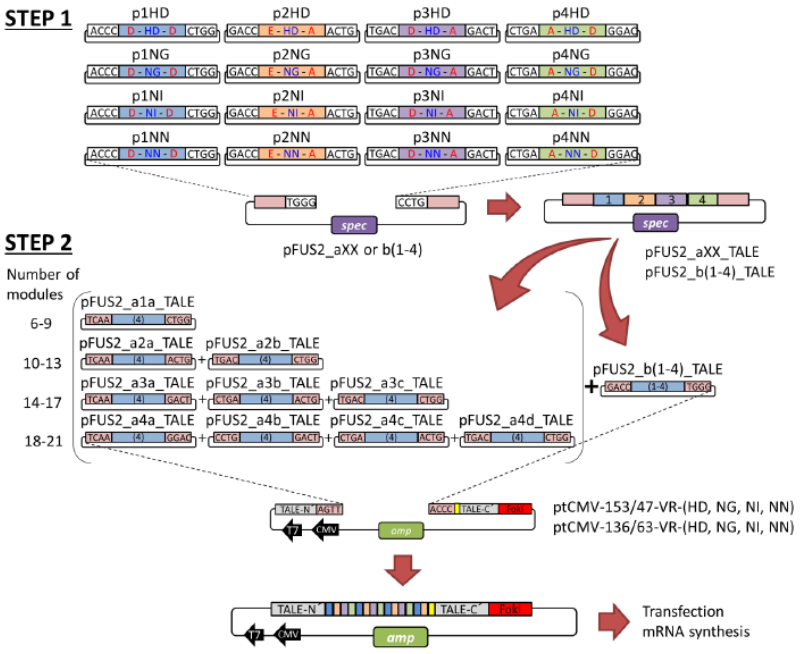
I conclude that disruption of the first exon of sorl1 gene by TALENs mRNAs causes a reduction of App function and accordingly to this reduction, treated embryos had stunted structure, bent tail and apoptosis cells. Due to their versatile and unique features, I believe that zebrafish will play an increasingly prominent role in the identification and study of AD molecular factors and therapeutic discovery.

Table 1. Evaluation of zebrafish TALENs sorl1 activity

	Fluc	Rluc	Fluc/Rluc	Relative activity
ZFN reporter with positive control ZFN	596002	109672	5.4344044	1
Negative control (sorl1_A reporter without TALENs)	46050	118368	0.389041	0.071588518
Negative control (unrelated reporter with sorl1_A TALENs)	39407	58422	0.6745233	0.124120924
sorl1_A reporter with sorl1_A TALENs	1288717	58555	22.008659	**4.049874997**
Negative control (sorl1_B reporter without TALENs)	31312	134738	0.2323918	0.042763058
Negative control (unrelated reporter with sorl1_B TALENs)	68658	47608	1.4421526	0.265374542
sorl1_B reporter with sorl1_B TALENs	1005923	59203	16.991082	**3.126576578**

Two sets of TALENs plasmids, A and B, were constructed and tested for their activity by reporter-based assay. The fold change of sorl1_A TALENs with sorl1_A reporter recorded value was about 4 times, whereas the negative control was only 0.02. The fold activation was over 32 compared to the negative control without TALEN. Similar records were obtained almost with both TALENs constructed sets. Fluc is Firefly luciferase; Rluc is Renilla luciferase.

Figure 1A. Scheme of Platinum gate assembly used to construct TALENs.



In the step 1, assemble RVD repeats for the chosen target sequences. In this method TALENs bearing 6-21 repeats. It is necessary that we choose appropriate combinations of vectors for the desired module numbers. To construct each pFUS2_axx_TALE plasmid harboring four modules, perform Golden Gate assembly. Transform 0.5-1 µl of the reaction product directly to chemical competent *E. coli* such as XL1-Blue and streak the transformant on Spectinomycin/X-gal/IPTG LB plate. Culture overnight (O/N) at 37℃. Select the desired clones using colony PCR. Culture the desired clones with 100 ng/µl Spectinomycin/LB media. Purify the plasmids using Miniprep kit.

In the step 2, Perform using the 4-module ligated constructed plasmids. Transform the reaction product to XL1-Blue and streak the transformant on Ampicillin/X-gal/IPTG LB plate. Culture O/N at 37℃. Screen for correctly assembled clones by colony PCR. We use TALE-F/TALE-R primers both for ptCMV-153/47-VR and ptCMV-136/63-VR vectors. Culture the desired clones and purify the plasmids using Miniprep kit.

(Source: Sakuma, T. and Yamamato, T., 2014: DOI: 10.1038/srep03379)

Figure 1B. First exon of zebrafish sorl1 gene and TALEN primers design.

sorl1_A_Left sorl1_A_Right

ATGGCGTCAGGGCAGACACGCAAGATGCTGGCGCTCAGTCGCTGTGCCATATATCTAT

sorl1_B_Left sorl1_B_Right

TATTATTACTAGTTCCCGCTGCGATCTCCTCCACACTTCGACTCCACCACGATCAGAG

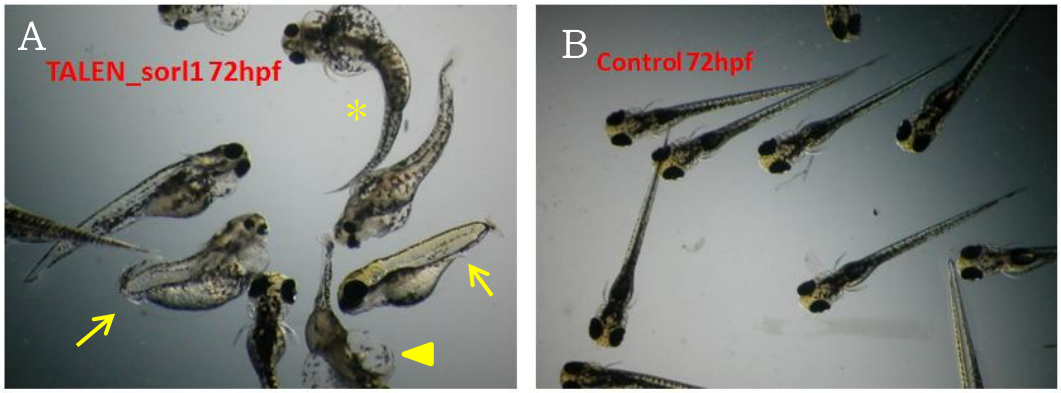
ATTCGTACTACCACAGGACAGAGGTTTTTCCCTTGTGAGCGCACACCTGGAGCCTGCA

GAAAGTCGCGTCGTGCGCCTGGAGAGAGAAGTCAGGGAGGCTTCAGCCGCGCATCAGC

TTCGCGTTCGGCGGAATGCTGCTGGAGCGCCCGTGCCAAATGTTTATGGAATG

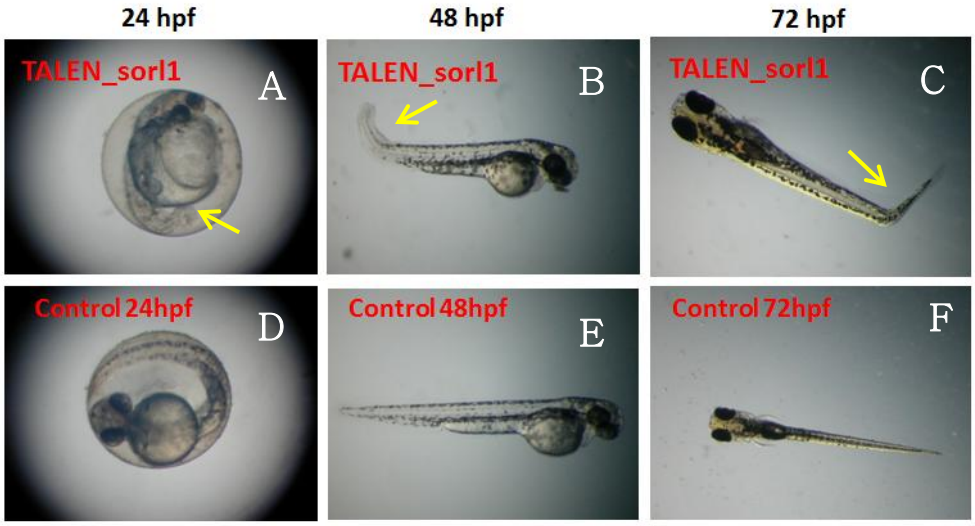
Two pairs of TALENs, sorl1_A and sorl1_B, were designed in the first exon (highlighted in red and blue letters, respectively). Underline indicates start codon.

Figure 2. Phenotype Abnormalities in the TALEN_sorl1 microinjected embryos at 72hpf.



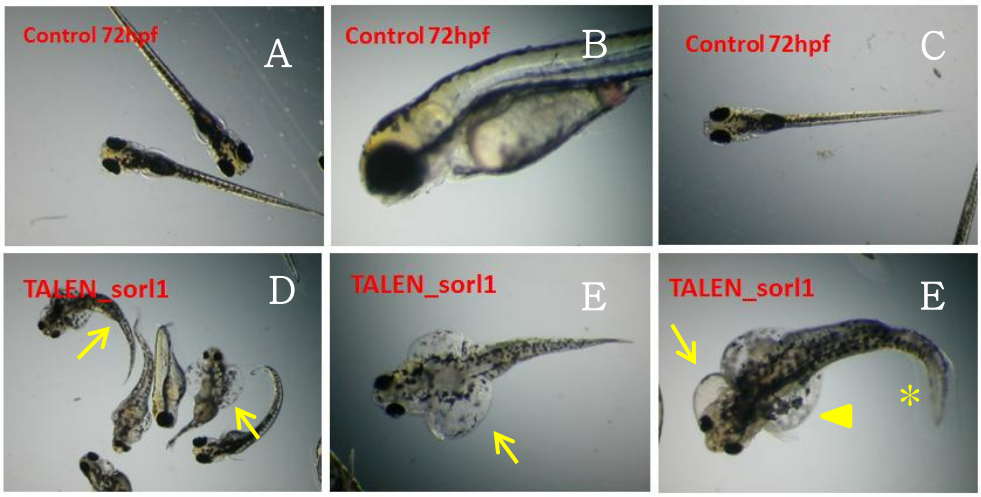
The left image (A) shows various malformations on the TALEN_sorl1 microinjected embryos at 72hpf. The right image (B) shows control at the same age with normal development. The yellow arrows in image (A) show abnormal body shape and stunted structure. Arrow head shows cardiac edema. Star shows bent axis of the body.

Figure 3. Malformed tail structure in microinjected embryos.



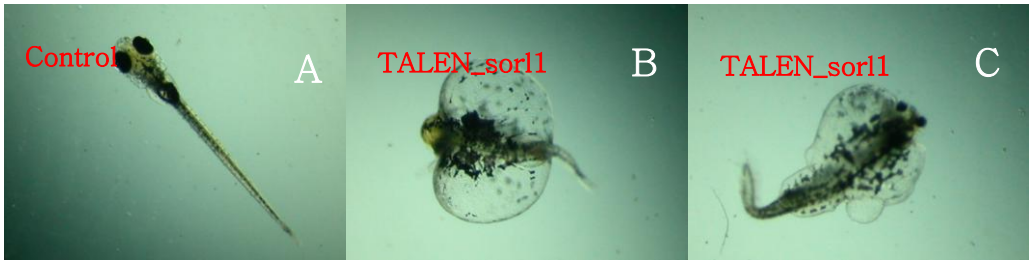
Three checkpoints of zebrafish development stages were checked. The upper images A, B and C show the TALEN_sorl1 microinjected embryos. The lower images D, E and F show controls at the same checkpoint. The yellow arrows show malformed tail structure. At 24hpf (Image A), there was malformed shape on the unhatched embryos tail caused difficulty of hatch. At 48hpf (Image B) after the eggs were Dechorionated, the tail has hooked-like shape. At 72hpf (Image C), the tail took a perpendicular angle to the longitudinal axis of the body and caused difficulty to move.

Figure 4. Cardiac edema in the microinjected embryos at 72hpf.



The upper images A, B and C show control at 72hpf. The lower images D, E and F show severe cardiac edema in the TALEN_sorl1 microinjected embryos at the same age. The yellow arrows in image D, E and F show the cardiac edema. The arrow head in image F shows yolk sac edema, star shows the hooked tail.

Figure 5. Severe cardiac edema in microinjected embryos at 7dpf

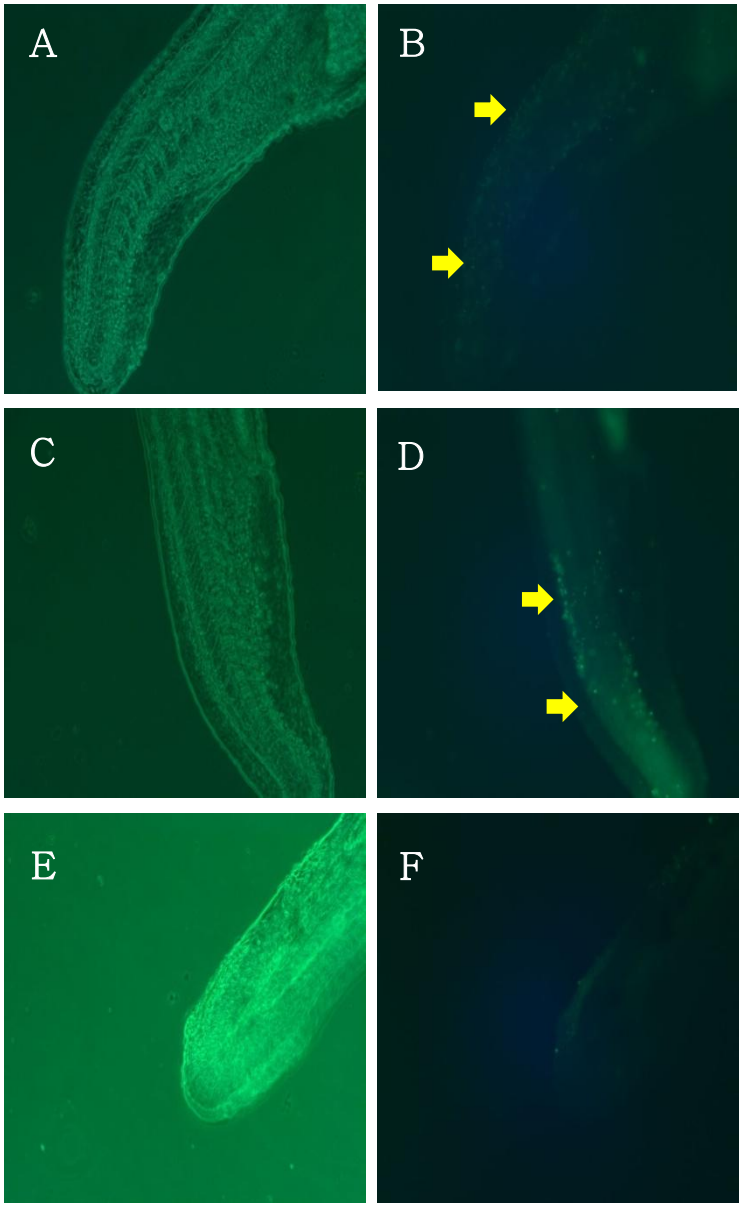


The left image (A) shows the control with normal development structure at 7dpf. In image B and C, the arrows show severe cardiac edema in the TALEN_sorl1 microinjected embryos. The head arrow in C shows bent tail as multiple malformations in the same individual of some larvae.

Figure 6. Mutation detection in genomic DNA of TALENs microinjected embryos.

Nucleotides with green color topped with arrows demonstrate the TALEN-L and TALEN-R primers sequences. Nucleotides with red color are the spacer sequences of TALEN target. Nucleotides with black color are the control sequence of sorl1_A TALEN target site. Three mutations at the TALEN spacer sequence. Nucleotides with blue color are G, T and T alteration and insertion, respectively.

Figure 7. Acridine orange fluorescence staining to detect the apoptotic cells of TALEN microinjected embryos.



24hpf embryos were Dechorionated to apply acridine staining experiment. Acridine orange stain the apoptotic cells which seen under green fluorescence light in green color. A, C and E are the bright field. B, D and F are fluorescence field. Yellow arrows in B and D show the apoptotic cells with. Image F is the control.

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General Conclusion

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 positive 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.

국문초록

제브라피쉬를모델에서 아밀로이드전구단백질과 소르틸린수용체에 대한

카페인의 효과

서울대학교 대학원

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생명의학연구에서는 질병의 병리학적인 과정을 세포 및 분자생물학적 수준에서 이해하고 새로운 치료법을 개발하기 위해 다양한 동물모델을 적용한다. 병리학 분야에서는 주로 랫드와 마우스와 같은 고등포유류 시스템을 이용한 모델이 이용되었다. 랫드와 마우스 모델은 다양한 장점을 가지고 있지만, 유지비용이 많이 들고, 배아를 다루기가

어려우며, 대규모 유전학적 연구에는 한계가 있다는 단점을 지니고 있다. 더욱이, 실험동물의 윤리적 이용이라는 측면에서 제브라피쉬가 생명의학연구에서 유용한 모델로 주목받고 있다.

제브라피쉬 (*Danio rerio*)는 분자유전학과 발생생물학에서 주로 활용되었다. 현재는 새로운 약물을 개발하거나 다양한 생리학적 및 병리학적 과정을 모델링하는 연구에서 주목을 받고 있으며, 최근에는 유전자 변형이나 특정 유전자의 결손 유도와 같은 최신 기술을 이용한 유전자 기능연구에 많이 활용되고 있다. 제브라피쉬의 배아는 빠르게 성장하는데, 배아가 투명하기 때문에 현미경으로 관찰이 가능하다. 제브라피쉬 치어는 배지에 용해되어 있는 작은 크기의 분자 물질을 피부와 아가미를 통해 쉽게 흡수한다. 발생 7일 후에는 피부보다 경구를 이용한 흡수가 이루어진다. 제브라피쉬는 뛰어난 번식력, 배아의 투명성, 경제성의 장점을 가지고 있고, 상대적으로 짧은 기간 내에 다양한 화학물질을 평가할 수 있다. 약물독성평가에서 종료시점은 단순히 생존률을 통해 평가할 수 있다.

카페인은 식품 중에서 가장 포괄적으로 연구 되고 있는 물질이며, 음식이나 음료를 통해 섭취를 해왔지만, 카페인을 지속적으로 섭취하는

것이 잠재적으로 건강에 어떤 영향을 미치는지에 대해 연구된 바가 없다. 다른 연구에서는 카페인 섭취가 알츠하이머와 같은 신경계 질환에서 치료효과를 나타낸다는 보고가 있다.

본 연구에서는 심박률 및 세포손상과 관련된 유전자 발현 변화를 통하여 카페인 노출시간의 영향을 평가하였다. 세포손상과 관련된 유전자로서 산화스트레스 유전자 (HSP70), 미토콘드리아 대사 (Cyclin G1)과 세포자멸사 (Bax and Bcl-2)를 배아초기 단계에서 평가하였다. 100mM 농도에서 수정 후 48시간에서 96시간까지 모니터링 한 결과, 동일한 사망률 및 부화율을 보였고 형태학적으로 유의적인 변화가 없었다. 그러나 심박률을 증가하는데 영향을 미쳤다. 정량적 RT-PCR 결과 HSP70은 수정 후 72시간에서, Cyclin G1은 수정후 24, 48, 72시간에서 Bax는 48, 72시간에서 유의적인 증가 양상을 보였다. Bcl-2는 수정 후 48, 72 시간에서 유의적은 감소를 보였다. Bax/Bcl-2 비율은 수정 후 48, 72 시간에서 유의적으로 증가하였다. Bax 또는 Bcl-2 관련 단백질은 미토콘드리아의 전압-의존성 음이온 채널의 개폐에 관여하면서 이를 증가시키며, 막전위를 소실 시키고 cytochrome C 의 분비를 유도한다. Bax 발현이 증가되고 Bcl-2 발현이 감소되면서 부정적인 영향을 미치게 된다. 따라서, 본 연구결과를 통하여 카페인이 미토콘드리아 의

존성 경로를 통하여 Bax 및 Bcl-2 발현을 조절함으로써 세포자멸사를 유도한다는 것을 알 수 있다.

두번째 연구에서는 제브라피쉬에서 카페인이 알츠하이머 질환의 분자적 요소 및 알츠하이머 질환의 아데노신 수용체와 도파민 수용체에 미치는 영향을 연구하였다. 모든 유전자가 초기 발생과정에서 이미 발현하고 있음을 확인하였다. 대조군 뿐만 아니라 10μM, 100μM의 카페인 처치군에서도 형태학적 변화는 관찰되지 않았다. 수정 후 24시간에는 알츠하이머 관련 유전자들의 발현이 카페인에 의해 감소된 것을 확인할 수 있었으며, 다른 시간대에서는 분포되는 양상으로 관찰되었다. 수정 후 7일에는 amyloid precursor protein (APP) 및 presenillin1 (psen1)와 같은 아밀로이드 경로 관련 유전자들이 카페인에 의하여 전체적으로 감소되는 경향을 보였으며, presenillin2 (psen2) 유전자는 증가되었다. APP 및 psen1의 발현이 감소되는 것은 뇌에서 아밀로이드반의 축적을 감소시키므로 알츠하이머 환자들에 유익한 것이다. Psen2 유전자는 다른 효소들과 함께 APP를 더 작은 분절조각들로 잘라내는 역할을 하기 때문에, 이 유전자가 증가되는 것 또한 유익하다고 할 수 있다. 또한 신경세포 형성의 증가에서도 중요한 역할을 하는 것으로 생각된다. 아데노신 수용체 2aa (A2aa)와 2ab (A2ab)는 아데노신 수용체 2b (A2b)

보다 카페인에 강한 반응을 보이는 것을 확인할 수 있었고, 아데노신 수용체의 전체적인 발현은 감소되는 것으로 나타났다. 아데노신 수용체와 도파민 수용체는 상반되는 관계를 가지고 있다. 카페인에 의하여 도파민 수용체 d2a (drd2a)와 d2c (drd2c)의 발현이 유의적으로 감소하였다. 수정 24시간 후에는 이러한 발현이 거의 억제되었으나, 수정 96시간 및 168시간 후에는 발현을 유의적으로 자극하였고, 이러한 현상은 알츠하이머 환자들이 카페인을 유지하는데 유익한 것이라고 할 수 있다. 본 연구에서 확인한 유전자들에서는 카페인이 역설적인 효과를 보였으나, 이는 아밀로이드 경로 관련 유전자인 APP와 psen1 발현은 감소시키고 psen2 발현은 부분적으로 증가시키면서 알츠하이머에서 긍정적인 역할을 할 것으로 생각된다. 이는 아데노신 수용체의 길항작용과 더불어 도파민이 발현되는, 아데노신 수용체와 도파민 수용체 간의 상호 관계를 통하여 이해할 수 있다.

세 번째 연구에서는 Transcription activator-like effector nucleases (TALENs) 기술을 활용하여 제브라피쉬에서 sorl1 유전자의 첫번째 엑손이 억제된 플라스미드를 제작하였다. Sorl 유전자는 유전적으로 알츠하이머 질환과 연관이 있다. APP 처리 경로에서 sorl1이 스위치와 같은 중요한 역할을 한다고 알려져 있다. 알츠하이머 질환에서

sorl1 유전자는 감소되어 있는데, sorl1은 Aβ (알츠하이머반의 직접적인 전구체)를 증가시키는 beta-site amyloid precursor protein cleaving enzyme 1 (BACE1)에 APP를 도입하는 역할을 한다. 본 연구의 목적은 제브라피쉬 발달 과정에서 이 유전자의 역할을 탐색하고 제브라피쉬 유전자결손 모델에서 APP와의 연관성을 규명하고자 하였다. 제브라피쉬 배아 1세포기의 세포질에 TALENs' messenger Ribonucleic acids (TALEN mRNAs)를 주입한 결과, 제브라피쉬 발달 과정에서 몇몇 이상형성을 관찰할 수 있었고, 주로 비정상적인 성장, 구부러진 꼬리 및 몸체, 심장 부종, 성장 저해된 구조가 관찰되었다. 일부 배아에서는 한 개체 내에서 하나 이상의 이상이 관찰되기도 하였다. TALENs mRNAs를 주입한 뒤 이상 형성을 보인 배아로부터 획득한 genomic DNA의 TALEN 간격 서열에서 세 곳의 변이를 관찰하였고, 이러한 변이는 시토신 염기 (C)에서 구아닌 염기(G)로의 변화와 두 개의 티민 염기(T)의 추가를 보였다. 또한 이상 형성을 보인 배아에서 세포자멸사를 확인하기 위해 수정 24시간 후의 아크리미딘 오렌지 염색을 수행하여 형광 하에서 관찰하였고, 꼬리가 구부러진 배아에서 몸체 전체적으로 세포자멸사가 나타나는 세포를 확인할 수 있었다. 본 연구는 sorl1 유전자가 제브라피쉬 발달에서 중요한 역할을 하며 첫 번째 엑손에서 억제를 유도한 경우 심한 이상 형성을 유도함을 보여주었다.

이전 연구에 비추어보면, TALENs를 미세주입함으로써 sorl1을 억제하는 것이 APP의 기능을 감소 혹은 소실 시키며, 이는 후기 엔도좀 경로로 유입되는 APP의 비율을 증가시킴으로써, 성장 저해 및 구부러진 몸체와 같은 이상 형성을 유도하는 것임을 시사한다.

본 연구는 카페인이 실험 조건하에서 제브라피쉬에 분자적 수준으로 역설적인 효과를 갖는다는 것을 보였다. 그러나 카페인은 표현형의 결함은 가져오지 않았으며, 발달되는 제브라피쉬에서 복잡한 분자적인 영향을 보였다. 카페인은 미토콘드리아 의존성 경로를 통하여 세포자멸사를 유도할 수 있다. 카페인은 아밀로이드 경로에 직접적인 영향을 미치며, 아밀로이드 경로에 관련된 유전자들을 조절함으로써 알츠하이머 질환에서 긍정적인 역할을 할 수 있는 것으로 보인다. 아데노신 수용체와 도파민 수용체간에는 상반된 관계를 가지면서 알츠하이머 질환에 보호효과를 보인다. Sorl1 유전자의 감소는 APP의 감소를 유도하여 성장저해된 구조를 가져옴으로써 제브라피쉬 발달에서 중요한 역할을 하며, 다른 이상 발생에서도 역할을 하는 것으로 보인다.

제브라피쉬는 사람의 신경변성 질환의 분자적 기초를 도출하는데 유용한 대체 모델이 될 수 있으며, 고유의 성질을 이용함으로써, 고속 약

물 검색에 필요한 척추동물 모델로서 활용 가능하다.

후속 연구를 통하여 신경변성 이상에 대해 연구하고 치료제를 개발하기 위한 유용한 모델로서 안정적인 Sorl1 유전자 결손 제브라피쉬 모델을 구축하는데 초첨을 맞추고, 이러한 모델을 이용하여 APP 및 sorl1 간의 연관성을 연구함으로써 새로운 치료제를 탐색 및 개발할 수 있을 것이다.

주요어: 카페인, 심박률, 세포손상 관련 유전자, 아밀로이드 경로, APP, sorl1, TALEN, 제브라피쉬

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