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AN OVERVIEW OF IN VITRO RESEARCH MODELS FOR ALZHEIMER'S DISEASE (AD)

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ABSTRACT

Alzheimer's disease (AD) is the most common form of age-related dementia. It is a neurodegenerative disease characterized by two aberrant features, the amyloid plaques and the neurofibrillary tangles which result in progressive memory loss and cognitive disturbances. This has led to devastating suffering to the patient, caregivers, family and economy of the country. As a result, scientists are putting efforts in understanding the mechanisms underlying the development of the disease as well as treatment for the disease. To do so, an ideal model is required that can mimic the development of AD, demonstrating the progressive degeneration of the neurons and formation of amyloid plaques and neurofibrillary tangles. In this review paper, currently available *in vitro* models for AD will be discussed, which include the cancer, primary culture and stem cell lines, highlighting on the benefits and limitations of each. More attention will be focused on the latest established disease-specific induced pluripotent stem cells (iPSCs) isolated from familial AD patients and Down syndrome patients. These models have their own advantages and limitations, therefore, more research needs to be done to come up with a model that is suitable not only for fundamental understanding of the disease but also for drug discovery and development.

1.0 Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by production of β-amyloid proteins (Aβ) and hyperphosphorylated Tau protein leading to extensive loss of synaptic connections and neurons in the hippocampus and cerebral cortex [1], leading to cognitive decline, hence dementia [2]. According to World Health Organization (WHO) 2012, 35.6 million people worldwide have dementia, and out of this population, 60-70% are AD patients. The number of people with dementia is predicted to nearly double by the year 2030 to 65.7 million, and triple to 115.3 million by 2050, whereby with the rise of dementia patients, the AD patients' cases is also expected to increase.

The pathogenesis of AD has been linked to genetic and environmental factors, with aging as the single greatest risk factor [3]. The symptoms manifest at the age of 65, where one out of eight people have AD, and as the age increases to 85, half of the population has AD (WHO 2012). There are two types of AD namely; early onset Alzheimer's disease (EOAD) and late onset Alzheimer's disease (LOAD). EOAD is familial and accounts for 10% of the people with AD, where the symptoms manifest before the age of 65 [4]. Genes associated with EOAD include Amyloid precursor protein (*APP*) gene which is located on chromosome 21, and it codes the amyloid precursor protein [5]. Under normal conditions, Amyloid precursor protein is digested to form smaller fragments called amyloid beta (Aβ) peptides; Aβ40 and Aβ42, respectively [6]. However, mutation of *APP* gene results in buildup of Aβ peptides which then lead to the formation of amyloid plaques, a hallmark for AD that is associated with pathogenesis of AD [7]. This gene increases the chances of down syndrome patients to develop AD because of the extra copy of APP gene the patients have [5]. Presenilin 1 (*PSEN1*, on chromosome14) and presenilin 2 (*PSEN2*, on chromosome1) which encode for γ-secretase are also involved in EOAD. Mutations in these genes (*APP*, *PSEN1* and *PSEN2)* lead to production of $A\beta_{40}$ and insoluble $A\beta_{42}$. The insoluble $A\beta_{42}$ accumulates to form amyloid plaques which are toxic to the neurons resulting to autosomal dominant familiar Alzheimer's disease (FAD). Tau is another gene associated with EOAD. Tau is a phosphoprotein associated with axoplasmic transport whereby it promotes microtubule binding and assembly of axons to stabilize it [8]. Mutation of *Tau* gene reduces the interaction of microtubules with Tau and also increased production of Tau resulting in buildup of Tau in the brain [9].

On the other hand, there are several genes associated with late-onset AD (LOAD). LOAD is the most common type of AD affecting 90% of the people with AD. It is sporadic and it begins at the age of 65years and above [4]. The most common gene associated with LOAD is apoliprotein E (APOE). This gene has 3 alleles (APOE e2, APOE e3, and APOE e4). People with allele APOE e4 have increased risk of developing AD [10]. APOE-e4 promotes the formation of insoluble amyloid as compared to soluble amyloid.

Much effort and progress has been made to create a better understanding of AD pathogenesis. This has been accomplished using both *in vivo* and *in vitro* models. The *in vitro* models used are either the transgenic models, cancer cell lines (neuroblastoma and pheochromocytoma cells) or primary neuronal cultures. Vital proteins are critical for development of AD where these proteins demonstrate involved in AD) in rodents has led to increased interest of using human induced pluripotent stem cells (iPSCs) and human embryonic stem cells (hESC) [5,11,12,13] a models to mimic AD development. This is because amyloid β-peptide (Aβ) formed by the rodents differ from that of human whereby it does not form fibrils involved in the disease to elucidate the disease development and provide a cell culture-based system for drug screening for prevention and treatment [11].

This cognitive and memory decline age-related disease causes suffering to the patient and their caregivers. These difficulties are gradually increasing in magnitude as the mean population age is rising. Getting treatment or prevention of AD has attracted extensive attention worldwide [2]. That is why there is a need for an appropriate *in vitro* model that can mimic the pathogenesis and progression of AD. In this review, much attention will be paid to *in vitro* models for AD, which are represented by the cell cultures that are used to study AD pathogenesis and drug discovery and also some of the chemicals and genetic manipulations applied on these cell culture to produce changes that are associated with AD. The benefits and limitations of each will also be given attention.

2.0 Methods of creating Alzheimer's Disease model *in vitro*

There are several methods of creating Alzheimer's disease model *in vitro* based on the causes and the mechanisms underlying the disease. This involves synthetic compounds such as amyloid beta peptide, a major component of amyloid plaques found in the brain of Alzheimer's disease patient, or through insertions of gene mutants associated with AD via gene delivery (Table 1).

2.1 β-amyloid

Progressive degeneration of neurons is mainly observed in the hippocampus and cerebral cortex (areas responsible for cognition and memory) among other parts of the brain due to

Table1: Summary on the methods of creating AD model *in vitro*

Method of creating model	Common use and benefits	Limitations	References
Misfolded or mutated Amyloid beta peptide	Induces oxidative damage	Expressed extracellularly There is no progressive pathogenesis	17, 1, 18, 28
Gene modifications	Expressed intracellular and extracellularly Progressive neurodegeneration	Risk of non-disease mutations	5, 12, 21

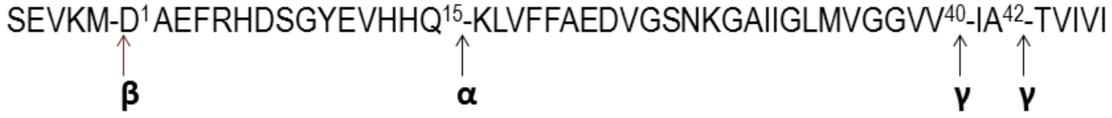

Fig.1 shows the sequence of Aβ within the *APP* proteolytic cleavage sites. β, α- and γ-secretase show the main cleavages site while numbering relates to conversions of Aβ (30).

accumulation of beta amyloid peptide, a well-known neuropathogenic hallmark of AD. The buildup of Aβ leads to oxidative stress (an imbalance between free radicals and antioxidants) in the neurons which play a key role in the development of AD [14]. Aβ results after the *APP* is cleaved by the proteolytic enzymes β-secretase and γ-secretase (Figure 1) [15].

Aβ has several isoforms, Aβ_{1-42} is the component found in the amyloid plaques, and it has 42 amino acids. Other isoforms includes Aβ_{1-40}, Aβ_{25-35}. Several researchers have used Aβ_{1-42} and Aβ_{25-35} to induce oxidative stress and also study neuroprotection of natural products using different cell lines. Aβ_{1-42} has been used to study the neuroprotective effects of *Camellia Sinensis* [16]. On the other hand, Aβ_{25-35} has been used to study neuroprotective effects of natural products such as Spanish red wine [17]; the effects of salidroside in SH-SY5Y human neuroblastoma cells [1] and the effects of tanshinone IIA in rat cortical neurons [18]. When matured neurons are exposed to Aβ, there is increased reactive oxygen species (ROS) production (which attacks the neuronal lipids, proteins and nucleic acids), mitochondria dysfunction, apoptosis and down regulation of antioxidant genes inevitably leading to neuronal dysfunction [19], these are features associated with AD. However, these models that use the synthetic amyloid peptide do not demonstrate the progressive nature of neurodegeneration in AD.

2.2 Genetic modifications

There are a number of genes linked with AD pathogenesis. Mutants of these genes are used to create an AD model *in vitro*. Presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) mutations are contributing factors for autosomal-dominant early-onset familial AD [11], by enhancing the production of A$\beta_{(1-42)}$ which forms amyloid deposits intracellulary and extracellulary [20]. A cell line that is capable of differentiating into neurons is transfected with these mutated genes. Insertion of mutated *APP* gene into the cells has also been used to recapitulate the AD model *in vitro*. Fibroblast cells isolated from the skin of Down syndrome patients are reprogrammed to induced pluripotent stem cell [5]. These cells contain triplicate copy of *APP* which results to progressive accumulation of Aβ due to misprocessing of *APP* and neurofibrillary tangles due hyperphosphorylated Tau proteins; the two hallmarks of AD.

APP and *Tau* genes have also been transduced using adenovirus vector for gene delivery in rat hippocampal neurons and dorsal root ganglions [21]. The results showed varying pattern of cell death by apoptosis for APP and clusters formation for tau positive cells over 2 to 5 days. Also, the neurites outgrowth was reduced in both transgenes in dorsal root ganglions. These models are better than the use of synthetic amyloid peptide which is only expressed extracellularly. But still the models do not mimic the pathogenesis of AD as there could be high chances of mutations leading to production of Aβ.

3.0 *In vitro* models used to study Alzheimer's Disease

In order to create an Alzheimer's disease model *in vitro,* a cell line is required. Some of the cell lines used to study AD includes primary culture cell lines derived from rodents, cells derived from cancer cells such as neuroblastoma and pheochromocytoma cells and recently, researchers have started utilizing induced pluripotent stem cells (iPSCs) to create an Alzheimer's disease model *in vitro* by either taking the skin cells of down syndrome patients or by transfecting cells with genes associated with AD (*PSEN1, PSEN2* or *APP*) (Table 2).

3.1 Neuroblastoma

Human neuroblastoma (SH-SY5Y) has been used to generate an *in vitro* model for AD and other neurodegenerative disease by directing the SH-SY5Y cells into neuronal lineage using several differentiating factors. These cells have synaptic structures, functional axonal vesicle transport, and express neurospecific proteins including nuclear protein NeuN, neuron specific class III β-tubulin and synaptic protein Sv2 [22]. This is highly important when investigating the role of tau and microtubule function in Alzheimer´s disease [23]. This cell line has been used to understand the mechanisms underlying the progression of AD and drug discovery. Once mature neurons are obtained, they are exposed to toxic Aβ leading to neurodegeneration [1]. However, this model does not recapitulate the real scenario of the AD patient due to interaction between different cancer genes.

Table 2: Cell lines used in AD study

Cell type	Benefits	Limitations	References
Cancer derived cells Neuroblastoma (SH-SY5Y)	• Express neuronal markers when differentiated • Easily available	Does not recapitulate real scenario of AD due to different cell signaling by cancer genes	(1)(22) (23)
Primary cultures	• Easily available • Can obtain specific neuron subtype	Mostly from rodents, does not mimic AD, as they lack receptors that allow the human Aβ peptide	(25)(29)(24)
Stem cells (iPSCs)	• Disease-specific neurons • Patient-specific neurons	High risks of mutations Time consuming	(21)(5)(11)(12).

3.2 Immortal rat hippocampal cell lines

This cell line is created from embryonic rat hippocampus, due to a need for cell lines derived from a known brain region origin that express phenotypes of particular subsets of cells, unlike the cancer cell lines. The cells are immortalized by retroviral mediated oncogene transduction using tsA58 and U19tsa alleles of simian virus 40 large tumor antigen [24]. When immortalized, this cell line has two special characteristics; conditional proliferation and ability to differentiate after cessation of division to neurons. These neurons express morphological and phenotypical markers of neurons and glial; NFP (Neurofilament protein) and GFAP (glial fibrillary acid protein) positive cells. This cell line is important for understanding pathogenesis of AD as the hippocampal neurons are responsible for cognition and memory, an area that is affected in AD patient. This cell line is of more significance as compared to tumor cell lines (derived from tumor but expressing neuronal phenotypes), which are limited by their malignant nature and lack of cell lineage specificity. This cell lines have been used to understand the neuroprotection mechanism [25,26].

3.3 Human Induced pluripotent stem cells (iPSCs)

An AD model is established using primary human fibroblast cells isolated from Familial Alzheimer's disease (FAD) patient [11]. These cells are reprogrammed using OCT4, SOX2, KLF4, LIN28 and NANOG transcription factors to induced pluripotent stem cells (iPSCs). From the iPSCs, two clones are established by retroviral transduction using presenilin1 mutations A246E (PS1-2 iPSC and PS1-4 iPSC) and with PS2 mutations, N141I (PS2-1 iPSC and PS2-2 iPSC). FAD patient specific iPSCs underwent neural differentiation to model AD pathogenesis *in vitro*, this aimed to determine the effect of presenilin mutations during neural differentiation. Increased ratio of $A\beta_{42}$ to $A\beta_{40}$ in iPSCs with mutated PS1 and PS2 was observed as compared to non-AD control iPSCs. Increase of $A\beta_{42}$ secretion by living human neurons derived from AD patient directly supports the amyloid cascade pathogenesis. These cells were also tested for possibility of using the iPSCs for drug screening using γ-secratase inhibitor and modulator. The results showed that Aβ secretion by adding agents against γ-secretase were inhibited and modulated as expected. Therefore, living human neurons from patients (FAD-iPSCs-derived neurons) are suitable for drug development and validation of new drugs [11].

Another *in vitro* human cellular model for AD pathogenesis derived from down syndrome (a disease that results due to trisomy 21) patient has been reported [5]. A Down syndrome patient was chosen as the disease has high incidence of AD due to triplicate of *APP* in chromosome 21 which results in autosomal dominant EOAD [27]. This model was created by differentiating iPSC lines and ES cells lines derived from patient with Down syndrome to cortical neurons. The differentiated cortical neurons from Down syndrome ES cells (DS-ES cells) and the control does not exhibit differences in the expression and localization of full-length APP protein. During the early stage of neuronal culture of the control group and the DS cortical neurons, pathogenic $A\beta_{42}$ peptide accumulation was not detected. However, production of $A\beta_{40}$ and $A\beta_{42}$ was increased after 70 days of neuronal culture (later stage). In both DS-ES cell and DS-iPS cell-derived cortical neurons, there was similar distribution of intracellular and extracellular aggregates of $A\beta_{42}$ peptides. Later stages of AD pathogenesis, which is marked by the two hallmarks of AD were also represented by the presence of hyperphosphorylated Tau protein in the dendrites and cell bodies of DS-iPSC–derived cortical neurons. This model is appropriate to help us understand the pathogenesis of AD in Down syndrome patient at an early and late stages. It can also be used as a good model for drug screening as the disease is progressive and the two hallmarks of AD are observed with time, therefore recapitulating AD pathogenesis in human.

4.0 Conclusion

Much effort has been put by the researchers in order to mimic some features of AD in non-neuronal human cells (PC12, neuroblastoma cells, rat- and mouse-derived hippocampal neurons) or by using a number of animal models, but none of the approaches is really satisfactory. Human tissues can only be used after the post mortem, in this case we are limited to understand the pathogenesis of AD during the early stage because the damage has already occurred. This has led to increased interest in human induced pluripotent stem cells (iPSCs) and embryonic stem cells (ES cells) as compared to rodents. Rodent could not develop a good model that is able to recapitulate the development of AD, due to different formation of Aβ both biochemically and biophysically from that of human (13). However, iPSC-derived model from human samples have high tendency of mutations. Therefore more research needs to be done to come up with a model that would mimic the pathogenesis of AD thus helping reduce the devastating effect of AD. Establishing an *in vitro* AD model from neurons differentiated from specific type of stem cells, with less risk of mutations, might be a hope in developing a model that will mimic the development, progressive and pathogenesis of AD.

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