Cerebrospinal fluid from Alzheimer’s disease patients as an optimal formulation for therapeutic application of mesenchymal stem cells in Alzheimer’s disease

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Mesenchymal stem cells (MSCs) have emerged as one of the promising treatment options for Alzheimer’s disease (AD). Although many studies have investigated on the efficacy of MSCs in AD, how MSCs actually change following exposure to the AD environment has not been studied extensively. In this study, we investigated on the potential of AD patient-cerebrospinal fluid (CSF) samples to be used as a formulation of MSCs and its application in AD therapeutics. When Wharton’s jelly-derived mesenchymal stem cells (WJ-MSCs) were stored in the CSF of AD patients, the stemness of WJ-MSCs was preserved. Furthermore, several genes were upregulated following storage in AD CSF. This signified the therapeutic potential of CSF formulation for AD therapy. Overall, these findings suggest that CSF from AD patients can be an optimal source for MSC formulation.

Originally found in the stroma of bone marrow¹, mesenchymal stem cells (MSCs) are multipotent progenitor cells which have recently been studied extensively due to their immune modulatory and trophic functions². Alzheimer’s disease (AD) is the most common neurodegenerative disease for which no disease modifying therapy exists so far³–⁴. MSCs have been considered as one of the treatment options for AD since these cells have shown a variety of effects such as reduction of beta amyloid (Aβ) levels, modulation of neuroinflammation, enhancement of endogenous neurogenesis, and also improvement in the behavioral performance of AD-transgenic mice⁶–¹³. A growing body of clinical studies is underway to investigate the safety and efficacy of MSCs when administered to AD patients¹⁴–¹⁶. Several preclinical studies have looked into which routes are more feasible to administer MSCs in AD patients. Animal studies from our group, for instance, showed that intracerebroventricular¹⁷ or parenchymal injections of MSCs⁸ might be more effective than intravenous¹⁸ or intra-arterial injections¹⁹.

While safety, efficacy, and route of administration have been addressed, the clinical grade production of MSCs as a drug product has not been investigated in-depth²⁰. From the isolation of cells to the preparation of the final stem cell therapeutics product, a critical point to consider is whether unintended ingredients could be included²¹. Current formulations for MSCs often include supplements to promote the survival of MSCs. However, some supplements may not exist in the body, thus possibilities of undesirable interactions between excipients and internal factors of recipients following injection cannot be ruled out. Therefore, it is imperative to produce an optimized form of the formulation where the excipients have been well established chemically.

When producing clinical grade MSCs, cerebrospinal fluid (CSF) samples from AD patients may have the potential to be applied as an optimal formulation of MSCs for the following reasons. First, one of the most feasible
**Table 1.** Demographics of Alzheimer’s disease patients and controls. *CSF Aβ42* and tau levels were within normal limits in normal controls except in Control 1 who showed lower CSF Aβ42 according to our norms. On the other hand, AD 1 and 2 showed lower tau levels than expected. However, the ratios of tau to *CSF Aβ42* (***) in all AD samples and controls satisfied the cutoff of AD and normal subjects, respectively. MMSE, Mini-Mental State Examination; CSF, Cerebrospinal fluid; Aβ1–42, Amyloid beta 1–42; t-tau, total tau; p-tau, phosphorylated tau; 18F, Fluorine-18; PET, positron emission tomography.

|                      | Control 1 | Control 2 | Control 3 | AD 1     | AD 2     | AD 3     | AD 4     |
|----------------------|-----------|-----------|-----------|----------|----------|----------|----------|
| Age at sample collection, years | 63        | 72        | 55        | 60       | 50       | 54       |          |
| MMSE at sample collection | 24        | 29        | 27        | 1        | 4        | 12       | 14       |
| Sex                  | Female    | Male      | Male      | Female   | Male     | Female   | Male     |
| CSF Aβ42 level, pg/mL | 307.592   | 844.755   | 794.12    | 331.24   | 370.70   | 415.54   | 394.89   |
| CSF t-tau level, pg/mL| 107.537   | 297.624   | 206.789   | 285.966  | 335.525  | 571.124  | 533.575  |
| CSF p-tau level, pg/mL| 17.75     | 59.59     | 37.87     | 33.13    | 48.64    | 84.83    | 98.97    |
| CSF t-tau/Aβ42**    | 0.350     | 0.352     | 0.260     | 0.863    | 0.911    | 1.374    | 1.351    |
| CSF p-tau/Aβ42**    | 0.058     | 0.071     | 0.048     | 0.100    | 0.131    | 0.204    | 0.251    |
| CSF cell count and chemistry | Negative  | Negative  | Negative  | Negative  | Negative  | Positive  | Negative  |
| 18F Florbetaben (Amyloid PET) | Negative  | Negative  | Negative  | Positive  | Positive  | Positive  | Positive  |

Routes of administration for MSCs is the intracerebroventricular route, where cells have higher chances to penetrate into the parenchyma of the brain. To perform repeated intracerebroventricular injections, insertion of a device such as an Ommaya reservoir has to be preceded prior to the first stem cell injection, which makes the collection of CSF from AD patients easier. MSC formulation using the CSF from the patient to whom the MSCs will be injected into may therefore ensure the safety of the therapeutic agent. Second, since CSF flow is part of the Aβ clearance mechanism in AD, CSF from AD patients might represent a microenvironment for AD. Therefore, MSCs that have been exposed to the AD patient CSF as a formulation would be preconditioned in the AD microenvironment prior to administration. This pre-exposure of MSCs to the AD microenvironment might allow MSCs to better cope with the disease environment than naïve MSCs.

In the present study, we stored human Wharton’s jelly-derived MSCs (WJ-MSCs) in CSF samples obtained from four different AD patients and three normal controls under hypothermic conditions (4°C). We then investigated whether the viability and stemness of WJ-MSCs were compromised following exposure to CSF of AD patients or normal controls. In addition, we explored changes in gene expression levels of WJ-MSCs to assess the therapeutic effects of AD CSF formulation.

**Results**

**CSF biomarkers of AD patients and normal controls.** The four AD patients [male (M): female (F) = 2:2, age range 50–60] who underwent 18F-florbetaben amyloid positron emission tomography (PET) scans were amyloid positive. Negative results were obtained from the three cognitively normal controls (M:F = 2:1, age range 63–81) who also underwent the same amyloid PET scans. Although some samples showed unexpected levels of CSF biomarkers (AD 1, 2: both total and phosphorylated tau lower than AD criteria; Control 1: lower Aβ42 level, marked as ‘*’ in Table 1), tau/Aβ42 ratios (marked as ‘**’ in Table 1), which are known to be more accurate than either of tau and Aβ42 values, were different between the normal control and AD CSF samples (Table 1). Furthermore, these ratios from all AD patients were above the normal range.

**Hypothecomeric storage of WJ-MSCs in AD and normal CSF does not compromise cell viability.** Changes in cell viability of WJ-MSCs stored in normal controls and three normal controls for 72 hours were assessed by performing fluorescence-activated cell-sorting (FACS) analysis after Annexin V/7-AAD staining (Fig. 1a,b). Images of the cells were taken (Fig. 1c) and the CCK-8 cell counting kit assay was also conducted (Fig. 1d). Compared to WJ-MSCs stored in minimum essential medium alpha 1x (MEMα 1x), significant differences in viability were not observed following hypothecomeric storage in both AD CSF and normal CSF (Fig. 1). A small percentage of early but not late apoptotic cells was detected from MEMα 1x and CSF groups (both AD and normal) (Fig. 1a,b). Similar results were obtained from the CCK-8 assays, where differences in viability between the MEMα 1x and the two CSF groups were not detected when the assay was performed every 24 hours for up to 72 hours (Fig. 1d).

**WJ-MSCs maintain stemness following hypothecomeric storage in AD and normal CSF.** Immunophenotype characteristics of human WJ-MSCs were analyzed according to the MSC criteria proposed by the International Society for Cell Therapy (ISCT)26. Like WJ-MSCs stored in MEMα 1x, WJ-MSCs stored in normal and AD CSF expressed the following cell surface markers: CD90, CD73, CD105, CD166 and also did not express the following hematopoietic markers: CD14, CD11b, HLA-DR, CD34, CD45, and CD19 (Fig. 2 and Supplementary Fig. S1). Such results verified that immunophenotypic features were not altered following exposure to both AD and normal CSF samples. WJ-MSCs were also able to differentiate into various mesenchymal lineages (adipogenic, osteogenic, chondrogenic) after exposure to normal and AD CSF (Fig. 3). The differentiation efficiency was also similar to that of WJ-MSCs stored in MEMα 1x, although MSCs stored in AD CSF showed less tendency to differentiate into osteocytes.
mRNA expressions of WJ-MSCs stored in AD CSF were up-regulated compared to WJ-MSCs stored in normal CSF. Expression levels of mRNA for MEMα 1x, normal CSF, and AD CSF groups were analyzed by using the Human Mesenchymal Stem Cell RT² Profiler PCR Array. The expression patterns of
WJ-MSCs exposed to AD CSF and normal CSF were compared to those of WJ-MSCs exposed to MEM α. Based on the PCR array data, scatter plots and heatmaps were analyzed (Fig. 4a,b). The mRNA expression level of WJ-MSCs exposed to AD CSF was variable among the samples (Fig. 4b). Compared to the WJ-MSCs stored in normal CSF (n = 3), significant alterations in mRNA expression levels were exhibited in 47 genes when WJ-MSCs were exposed to AD CSF (n = 4) (Supplementary Table S1). Interestingly, mRNA expression levels of WJ-MSCs exposed to AD CSF were significantly higher than those of WJ-MSCs exposed to normal CSF. Despite variability of expression levels among the patients’ samples, the pattern of upregulated mRNA was consistent.

Functional analyses of altered gene expressions show that exposing WJ-MSCs to AD CSF has potential beneficial effects on AD therapy. Gene expression clustering for upregulated and downregulated genes was performed using the MeV software (Fig. 4c). The functions of altered gene expressions were subsequently classified based on cellular component (Fig. 5a), molecular function (Fig. 5b), biological process (Supplementary Table S2) and functional annotation clusters (Table 2).

The 10 most enriched annotation clusters of significantly changed genes included items related to angiogenesis, signal peptide, negative regulation of programmed cell death, regulation of cell proliferation, extracellular region, tube development, morphogenesis of branching structure, cellular component morphogenesis, regulation of neurogenesis, and sensory organ development.

Discussion
Recent studies have reported on the neuroprotective and neurotrophic features of MSCs. Previous studies also showed that proteins secreted from MSCs induced clearance of Aβ proteins, promoted neurogenesis and also synaptogenesis. These studies indicated that a therapeutic interaction existed between the paracrine factors secreted by the MSCs and the endogenous progenitors present in the brain. While former studies were mainly focused on the therapeutic effects of MSCs, the cellular status of MSCs exposed to the AD brain microenvironment has not been fully elucidated.

Several studies have examined the effects of CSF on stem cells. Growth factors found in the CSF have been reported to affect stem cell proliferation and regulate quiescence and activation of stem cells in the brain. Other studies have used CSF to transdifferentiate MSCs into neural cells. For example, MSCs cultured in vitro in CSF (as a substitute of culture media) transdifferentiated into neural-like cells. However, these studies have focused on elucidating the effects of CSF on stem cells under normal and not disease conditions.

In this study, we applied CSF samples from AD patients as a formulation of WJ-MSCs. For comparison, MEMα 1x, which is conventionally used for MSC formulation was included, and normal control CSF samples were also included to determine whether AD pathology can possibly alter the therapeutic effects of MSCs. According to the results obtained from this study, the viability and stemness of WJ-MSCs were both preserved after exposure to AD CSF under hypothermic conditions. These results confirmed the safety of AD CSF to be used as a potential source of formulation.
Figure 4. Gene expression patterns of WJ-MSCs stored in AD CSF are upregulated compared to WJ-MSCs stored in normal CSF. Gene expression patterns of WJ-MSCs stored in AD and normal CSF samples were compared to those of WJ-MSCs stored in MEMα 1x. (a) Scatter plot and (b) heatmap analysis of differentially expressed genes are shown. Overall, while the gene expression levels of normal CSF samples show decreased patterns, a majority of the genes expressed in AD CSF samples remain unchanged. (c) Euclidean distance clustering of significant genes performed by MeV software is illustrated as a log transformed data. The green and red colors indicate decrease and increase of gene expression, respectively.

Figure 5. Functional annotation of genes upregulated following storage of WJ-MSCs in AD CSF. Functional analysis performed by using the DAVID informatics tool. (a) Cellular component and (b) molecular function of up-regulated genes are illustrated in a bar graph.
We further explored changes in gene expression levels of WJ-MSCs exposed to AD and normal CSF samples. Based on analysis of functional annotation clustering, WJ-MSCs stored in AD CSF expressed genes related to enhancement of extracellular transport and signal peptide, which indicates an increase in paracrine activity. These genes are known to exhibit neuroprotective and neurotrophic features such as negative regulation of apoptosis, regulation of cell proliferation, and regulation of neurogenesis. Furthermore, an increase in the expression of genes involved in cell migration or cell adhesion was also observed, which indicates potential beneficial effects on cell survival following administration. These results suggest that AD CSF may act as an optimal formulation for MSCs that will be injected back to the AD patient from whom the CSF sample was obtained from (Fig. 6).

Our study has several limitations. First, our study was based on a small number of patients. Therefore, further research involving a large number of patients is warranted. Second, although we measured the gene expressions of MSCs to investigate functional changes, we have not yet assessed the efficacy of MSCs in animal disease models (e.g., transgenic AD animal models). Nevertheless, to the best of our knowledge, this is the first study which has investigated the fate of MSCs in AD CSF, the interaction between MSCs and CSF samples, and the potential of AD CSF as a formulation source. Our approach may have several advantages. First, patients will be reinfused with their own CSF, which may contribute to minimizing the side effects of allogeneic MSC administration. Autologous MSCs are also available but not as cost-effective due to the requirement of large scale cell expansion, compared to allogeneic MSCs. Moreover, the surgical process involved in isolating MSCs is difficult to perform in AD patients due to their age. Second, to achieve repeated injections of MSCs into the lateral ventricle of patients, a device such as an Ommaya reservoir has to be surgically implanted. Therefore, collection of CSF from the patient may not be as invasive as expected. In order to ensure patient safety, CSF collection must be performed under closed, sterile procedures. CSF collected under sterile conditions can be used as an optimal formulation of MSCs produced from the GMP facility. Third, using AD CSF as a formulation of MSCs allows MSCs to pre-adapt to the disease environment and to also become pre-activated prior to administration. The use of CSF from AD patients as a source of formulation may enhance the overall efficacy of AD MSC therapy. This approach can also be applied to a wide range of neurological diseases.

### Methods

**CSF collection from AD patients and evaluation of AD biomarkers.** This study was approved by the Institutional Review Board of Samsung Medical Center (IRB approval No. 2015-04-099). In accordance with the guidelines approved by the Institutional Review Board (IRB) of Samsung Medical Center, CSF samples were collected with informed consent from four AD patients and three normal controls. All AD patients fulfilled the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer’s...
Disease and Related Disorders Association (NINCDS-ADRDA) criteria\(^{31}\) for probable AD and also had positive 18F-florbetaben PET scans\(^{34,35}\). Patients with neurological diseases other than AD were excluded from the study. The three normal controls were recruited from orthopedic clinics in our hospital who underwent spinal anesthesia to receive knee surgeries. All of them met the criteria for normal elderly\(^{29}\), had normal Mini-mental State Examination scores defined by age/sex matched cohort\(^{47}\), and also had negative 18F-florbetaben PET scans.

A total of 10–12 mL of CSF was obtained from each of the four AD patients and 3 mL from the three normal controls by lumbar puncture (between the L3/L4 or L4/L5 intervertebral space). Within two hours from collection, CSF samples were centrifuged at 4,000 g for 10 minutes at 4 °C. \(A_\beta_{42}\), P-tau\(^{181P}\), and T-tau concentrations were examined from the CSF samples by using the INOTEST (ELISA) assay. Then, the ratios of tau (both t-tau and p-tau) to \(A_\beta_{42}\) were obtained to validate the difference between normal controls and patients, as these ratios are known to be more accurate than the level of \(A_\beta_{42}\) and tau\(^{25,38}\).

### Hypothermic storage of WJ-MSCs in AD CSF.

According to the Institutional Review Board of Samsung Medical Center, after written informed consent was received by all of the patients, umbilical cord tissue was obtained from healthy patients undergoing childbirth. Wharton's jelly mesenchymal stem cells (WJ-MSCs) were isolated and cultivated according to the standard operation procedures (SOPs) of the Good Manufacture Practice (GMP) facility at Samsung Medical Center.

Passage 5 WJ-MSCs suspended in CSF samples were stored under hypothermic conditions at a concentration of 5.5 × 10^6 cells/1.5 mL, which corresponded to the final product vial concentration used in the GMP facility. The cells were stored in four different AD CSF samples at 4 °C for 72 hours. For the control group, WJ-MSCs were suspended in serum and phenol red free MEM α1x (Gibco, USA) and three normal CSF samples.

### Evaluation of changes in WJ-MSC viability.

Cell proliferation and viability were assessed by using the CCK-8 Cell Counting kit (Dojindo, USA) and Annexin V apoptosis detection kit with 7-AAD (BioLegend, San Diego, CA). MSCs stored for 72 hours under hypothermic conditions from the eight different samples (including the controls) were harvested and analyzed. To determine the number of viable cells in the stocked vials, the cells were stained with Annexin V and 7-AAD antibodies according to the manufacturer's instructions. The cells were resuspended in 500 μL of 1x staining buffer. Then, 5 μL of Annexin V and 7-AAD antibody were added to the binding buffer for 20–30 minutes at room temperature in the dark, prior to being analyzed by flow cytometry (BD Biosciences, USA).

To analyze the viability of the cells at various time points (0, 24, 48, 72 hrs), MSCs stored in CSF were seeded onto 96-well plates (3 × 10^4/well), and after 24 hours, CCK8 assay was performed. CCK-8 solution (10 μL) was added to each well, followed by incubation for 1 hour at 37 °C. The absorbance of CCK-8 was measured at 450 nm by using a microplate reader (x-Mark™, Bio-Rad Laboratories, Inc, USA).

### Flow-cytometric analysis for cell surface markers of WJ-MSCs.

In order to confirm the stemness of WJ-MSCs, cell surface marker analysis was performed. Harvested MSCs were washed in PBS supplemented with 2% FBS in order to block for non-specific binding sites. Immunophenotypic analysis of MSCs was carried out using flow cytometry for the following markers: CD73, CD90, CD105, CD166, CD14, CD11b, HLA-DR (MHCI), CD34, CD45 and CD19 (BD Biosciences, USA). At least 10,000 events were acquired by using the BD FACS Verse flow cytometer, and the results were analyzed by using the BD FACSsuite software version 10. Flow cytometry for appropriate isotype controls were also performed.

### Mesoderm differentiation assays.

Another way to test stemness is to examine the differentiation capabilities of MSCs. WJ-MSCs stored under hypothermic conditions for 72 hours were seeded onto 6-well culture plates at a density of 5000 cells/cm² and expanded until cells reached 80–90% confluency. For osteogenic and adipogenic differentiation and the respective immunostaining experiments, cells were incubated in differentiation media according to manufacturer’s instructions (Gibco, USA). Differentiation medium was replaced every 3 days. After 2 weeks, differentiated cells were stained using the following staining methods: osteogenic: Alizarin Red S, adipogenic; Oil Red O. Osteocytes were fixed with 4% paraformaldehyde (PFA) for one hour and then washed with PBS not including both calcium and magnesium (Gibco, USA). Mineralization of the extracellular matrix was visualized by staining with 40 mM Alizarin Red S (Sigma-Aldrich, USA), pH 4.2, for 5 minutes. Adipocytes were fixed with 4% PFA, washed in 60% isopropanol, and subsequently incubated for 10 minutes with Oil-Red O (Sigma-Aldrich, USA) to visualize the lipid droplets. Cells were then washed in isopropanol and counterstained with hematoxylin.

For chondrogenic differentiation, 2 × 10^6 cells were pelleted in 15 mL conical tubes. Subsequently, the cell pellet was suspended in 500 μL of chondrogenic differentiation medium containing high-glucose Dulbecco Modified Eagle Medium (DMEM; Gibco, USA) supplemented with 500 ng/mL of bone morphogenetic protein-6 (BMP-6) (R&D Systems, U.K.), 10 ng/mL of recombinant human transforming growth factor-β3 (R&D Systems, USA), 1% ITS (insulin 25 μg/mL, transferrin 25 μg/mL, and sodium selenite 25 μg/mL), 50 μg/mL of ascorbic acid-2-phosphate, 0.6 μg/mL of dexamethasone, 40 μg/mL of L-proline, and 100 μg/mL of sodium pyruvate. Differentiation medium was replaced every 3 days. Chondrocytes were fixed with 4% PFA, dehydrated by using ethanol, and embedded in OCT compound (Scigen, USA). Blocks were sectioned and the sections were stained with safranin-O (Sigma-Aldrich, USA), according to the manufacturer’s instructions. Integrity of isolated RNA
Functional analyses of altered genes. Functional analysis was conducted to examine alterations in gene expression following storage of MSCs in AD CSF. For annotation analysis, official gene symbols were uploaded into the DAVID (Database for Annotation, Visualization and Integrated Discovery) informatics tool (DAVID Bioinformatics Resources 6.7[23]). For GO (Gene Ontology) Term analysis, we studied the Cellular Component (CC), Molecular Function (MF) and Biological Process (BP) categories using the GO FAT default settings. For functional annotation searches, we set the following parameters: CC, threshold count 2, EASE 0.01, Benjamini 0.05 (resulting in 15 chart records); MF, threshold count 2, EASE 0.01, Benjamini 0.05 (resulting in 9 chart records); BP, threshold count 10, EASE 0.01, Benjamini 0.05 (resulting in 60 chart records); for functional annotation clusters, medium stringency (resulting in 52 clusters). Enrichment values (GO Terms), enrichment scores (annotation clusters), and statistical determinants (p values and Benjamini coefficients) were calculated by the DAVID software.

Statistical analyses. The results are an average of three independent experiments. Data are presented as mean ± standard error of the mean (SEM). Statistical comparisons of each samples between groups were performed using a one-way ANOVA test (both between groups and within groups). Differences were considered statistically significant when P < 0.05. All the statistical analyses were performed using SigmaPlot, version 12.5 and SPSS software, ver 19.0 for Windows.

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Author Contributions
J.L. designed, performed the experiments, analyzed the data, and wrote the manuscript. S.J.K. performed the experiments. J.H.K. received permission to use the hWJ-MSCs. H.J. analyzed the data and received permission to use the AD-CSF. N.K.L. and J.W.H. revised the manuscript. J.H.K. helped provide the mesenchymal stem cell source. J.W.C. and D.L.N. supervised the project, designed the experiments, and also wrote the manuscript.

Additional Information
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