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Original investigation

Effect of Mesenchymal Stem Cells on Cochlear Cell Viability After Cisplatin Induced Ototoxicity

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ABSTRACT

Aims: Ototoxicity is one of the main side effects of the chemotherapeutic agent Cisplatin (CDDP). CDDP ototoxicity is caused by damage of the organ of Corti, spiral ganglion cells or lateral wall (stria vascularis and spiral ligament). Mesenchymal stem cells (MSCs) were shown to differentiate into neurogenic and auditory hair cells in vitro. In this study, effect of MSCs in CDDP ototoxicity model of HEI-OC1 cochlear cells was evaluated.

Method: The cochlear cells were exposed to 50 and 100 microM CDDP for 24, 48, 72 hours with and without MSCs as coculture. The viability of the cells was analyzed with trypan blue dye and the percentage of apoptosis with Annexin-V by flow cytometer. The differentiation of MSCs to immature cochlear cells were shown by Math1, Calretinin and Myosin IIa immunohistochemistry.

Results: At 100 microM dose, CDDP caused cytotoxicity on cochlear cells predominantly via necrosis. In co-culture, MSCs decreased cochlear cell damage of CDDP. In co-culture the ratio of Math1 and calretinin positive cells were increased supporting the idea of differentiation of MSCs into immature hair cells.

Conclusion: In this in vitro study, our data support that MSCs protects cochlear cells from CDDP cytotoxicity. MSC therapy might be a candidate cellular therapy approach to overcome CDDP ototoxicity. The mechanism seems to be via differentiation of MSCs into immature hair cells. Our next step is to plan in vivo nude mice neuroblastoma animal model comparing CDDP therapy with and without systemic MSC administration and check ototoxicity.

Keywords: mesenchymal stem cells, cochlear cells, cisplatin ototoxicity

INTRODUCTION

Cisplatin (CDDP) is one of the mostly used primary treatment chemotherapy agent for many types of cancer among human solid tumors, such as ovarian, prostate, cervix, head and neck, lung and bladder (1). It causes damage to the DNA of the tumor cells, besides normal cells; creating reactive oxygen derivatives, that cause the cells to undergo apoptosis. With the use of higher doses of CDDP, the cell can also go to necrosis. However, ototoxicity, nephrotoxicity, neurotoxicity and bone marrow toxicity, which occur especially with the use of high doses of CDDP, constitutes an important problem considering the therapeutic profile and usefulness of the drug (2, 3).

Chemotherapy induced side effects, can be eliminated or decreased with supportive treatment agents such as antioxidants. CDDP ototoxicity has been shown to have an effect on at least three major target tissues in the cochlea which are; the Corti organ, spiral ganglion cells, the lateral wall (stria vascularis and the spiral ligament) (4, 5). Some of these mechanisms are known to cause DNA damage by covalent binding with guanine base, and reactive oxygen derivatives (ROS) destroy antioxidant enzymes in the cochlea (6, 7).

In addition, the aldehyde 4-hydroxyninal released due to ROS are important ways that are thought to play a role in the hairy cells, leading to an intense calcium intake and apoptosis (8). There are many protective agent studies including chemical agents, natural extracts against CDDP ototoxicity in cochlea cells. In the recent

literature, there are various dose and/or time dependent activities of Korean red ginseng (9), dexamethasone (10), resveratrol (11), silymarin (12), metformin (13), selenium (14) and many more. With the emergence of many effective agents to prevent CDDP ototoxicity, the issue of cellular therapy has been brought up to renew the deceased cochlea cells as a new approach.

Mesenchymal stem cells (MSCs) have become a current subject of research as a promising therapeutic approach based on cell transplant because of its multipotent abilities and differentiation to mesenchymal cell series such as osteoblast, adipocyte and chondroblast (15). The main sources of MSCs in adults are bone marrow, adipose tissue and lung tissue. In addition to these sources, when MSC isolation from placenta its derivatives was evaluated, conversion to chondrogenic, osteogenic, adipogenic, myegenic and neurogenic series was observed after obtaining from mucosal connective tissue in umbilical cord.

In this study, it is aimed to co-cultivate cochlear cells (HEI-OC1) with or without MSCs (C57BL/6 MSC line) and CDDP exposure to search for possible protective or regenerative role of MSCs against CDDP induced cochlear cell damage as an in vitro representative form of ototoxicity.

MATERIALS AND METHODS

This study was constructed by cell lines. It does not include investigations involving human subjects. Ethics Review Committee approval is not required.

Cell Culture

The HEI-OC1 (cochlea) cell line which was kindly supplied from Dr Frederick Kalinec; and C57BL/6 mesenchymal stem cell lines was used in this study for in vitro experiments. The HEI-OC1 cell line was cultured with 10% Fetal bovine serum, at 33° C, 10% CO2, 95% humidity, in Dulbecco's modified (Biochrom) medium. 5000 cells were added in each 96 well. C57BL/6 MSCs were cultured in 37% C, 5% CO2, 95% humidity in MSC culture medium (OriCellTM, Cyagen) containing 10% FBS, 1% L-Glutamine, 1% Penicillin/ Streptomycin. MSCs were removed from flasks by tripsinisation. The coculture was conducted by adding 3000 MSCs per each 96 well after HEI-OC1 cells become confluent (approximately 40000 cells per well) as adherent cells in wells.

Cisplatin Administration

CDDP (Koçak Farma) stock solution was used. After preparing CDDP at doses of 25, 50, 100, 250 and 500 µM, it was incubated in 96-well plates with HEI-OC1 and MSCs for 24, 48, 72 hours 6 wells for each condition. At the end of the period, cell viability was evaluated with trypan blue and WST-1 assay.

Cell Viability Tests

WST-1 reagent was incubated at 37° C for 2 hours after adding 10 µl per 100 µl sample. At the end of incubation, absorbance were measured at 480 nm with an ELISA plate reader. For viability assessment with trypan blue, cells were removed with trypsin and 20 µl trypan blue was added to 10 µl sample to determine the viability percentage in the cell count device. The viabilities of all groups were calculated relative to the viability of the control group.

Flow Cytometric Apoptosis Determination with Annexin-V

According to the WST-1 results, the LD50 dose of CDDP-induced apoptosis was determined in cochlear cells, MSCs and co-culture of cochlear and MSCs. After incubating all groups with LD50 CDDP dose for 24 hours, cells were detached with trypsin and centrifuged. 5 µl FITC conjugated Annexin-V and 10 µl PI were added to each 100 µl sample (approximately $1x10^6$ cells). After incubating at +4°C for 15 minutes, 400 µl of 1x Annexin-V binding buffer was added. The analysis was performed with BD Accuri C6 flow cytometry device and software.

Evaluation of Mesenchymal Stem Cell Differentiation

In order to evaluate differentiation situation of MSCs, HEI-OC1 specific biomarkers were determined by immunocytochemical staining in the experimental groups. HEI-OC1 cells were evaluated as Myosin IIA (Bioss), Math1 (Bioss) and Calretinin (Bioss) positive cells. Cells were collected and spreaded onto the slides. After fixation with methanol and 4% paraformaldehyde solutions, the cells were permeabilized for intracellular staining. Blocking for non-specific staining, incubations with primary antibodies and secondary antibodies and hematoxylin staining steps were performed. Myosin IIA, math1 and calretinin positivity were evaluated under a light microscope.

Statistical Analysis

Using the SPSS 20.0 software, the test for comparing the means of viability, apoptosis, and differentiation markers (non-parametric Mann Whitney U test) for each condition, 6 wells for each condition were analyzed simultaneously. $P<0.05$ was considered statistically significant.

RESULTS

Cell Viability Results

The viability percentages of HEI-OC1 cells after 48 hours of 25 uM, 50 uM, 100 uM, 250 uM and 500 uM CDDP treatment were 42.9%, 34.2%, 22.6%, 16.2% and 11.2% respectively. The viability percentages of HEI-OC1 cells after 72 hours of 25 uM, 50 uM, 100 uM, 250 uM and 500 uM CDDP treatment were 92.3%, 91.3%, 77.8%, 38.5% and 19.2% respectively. Accordingly, for in vitro ototoxicity modelling 50 uM and 100uM doses of CDDP and 48 hours of incubation were selected. After MSCs were treated with 25 uM, 50 uM, 100 uM, 250 uM and 500 uM of CDDP, cell viability percentages were 61%, 70%, 48.8%, 31.1% and 17.5% respectively. When HEI-OC1 cells and MSCs were co-cultured and treated with CDDP, total cell viabilities in each dose were increased as shown in Figure 1.

Accordingly, when HEI-OC1 was cultured alone, the cell viability rate was high. When MSC was cultured alone, cell viability rate was high. The lethal effect on CDDP, HEI-OC1 cells at 50uM dose was not sufficient. It was observed that the cytotoxic effect of CDDP on HEI-OC1 cells at 100uM dose was appropriate for

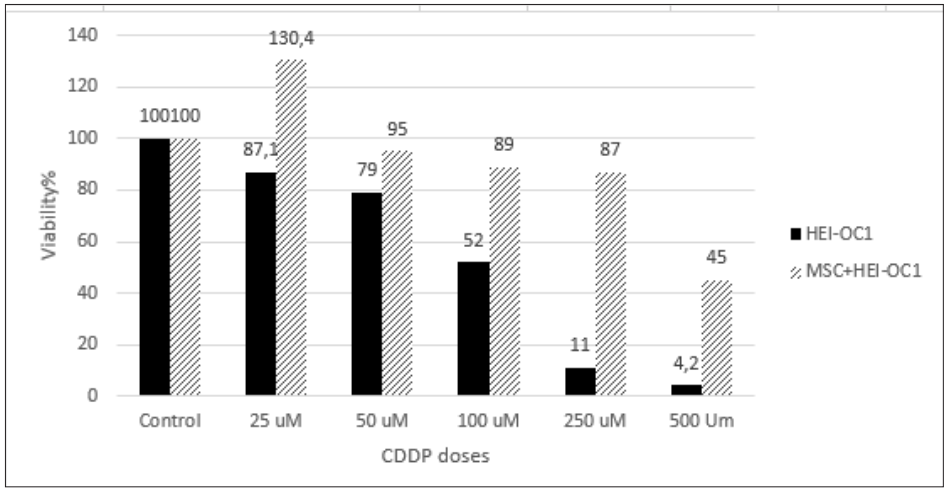

Figure 1. CDDP cytotoxic effect on HEI-OC1 cells with or without MSCs. MSCs decreased cell death at all doses.

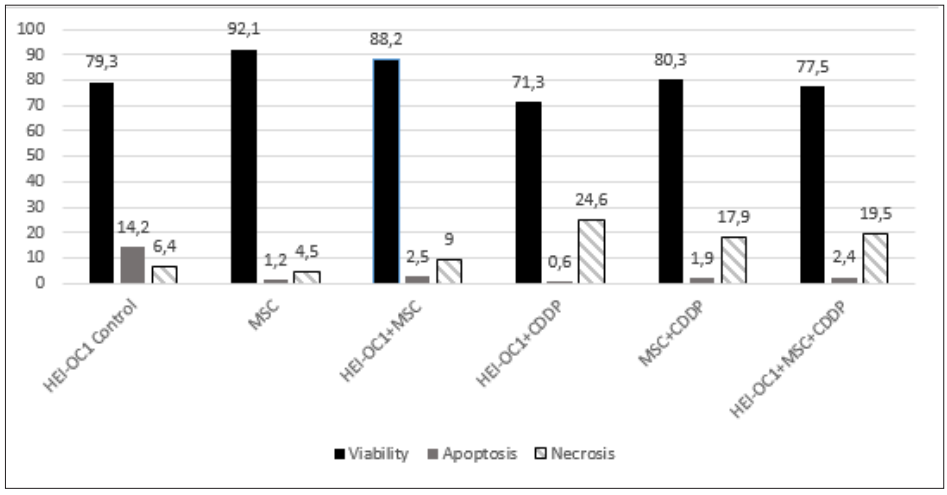

Figure 2. Viability, Apoptosis and Necrosis ratios of conditions tested. In co-culture, MSCs decreased cochlear cell damage of CDDP compared with cochlear cells CDDP only.

Table 1. Mean values of apoptosis stages percentages of data is given. Lower apoptosis and necrosis is observed in MSC co-cultured CDDp administered cochlear cells

Experimental group	Early apoptosis	Secondary apoptosis	Necrosis
HEI-OC1	14.2	6.4	0
HEI-OC1 + CDDP 50 uM	0.6	24.6	22.1
HEI-OC1 + CDDP 100	0.5	60.0	57.0
HEI-OC1 + MSC	2.5	9.0	0.2
MSC	1.2	0.3	4.2
MSC+ CDDP 50 uM	1.9	4.1	17.9
MSC + CDDP 100 uM	2.3	4.9	14.5
HEI-OC1 + MSC + CDDP 50 uM	2.4	5.9	19.5
HEI-OC1 + MSC + CDDP 100 uM	2.4	7.3	20.9

ototoxicity modelling. It was found that the viability of HEI-OC1 cells co-cultured with MSC cells were increased, so that MSCs was thought to protect HEI-OC1 cells. It was observed that both doses of CDDP had a cytotoxic effect on MSC cells.

Apoptosis Results Determined By Annexin-V Staining

In non-treated control HEI-OC1 group, early apoptosis was 14.2%, secondary apoptosis was 6.4% and necrosis was 0%. When HEI-OC1 cells were treated with 50 uM and 100 uM of CDDP, early apoptosis were 0.6% and 0.5%, secondary apoptosis were 24.6% and 60.0%, necrosis were 22.1% and 57.0% respectively. In non-treated control MSC group early apoptosis was 1.2%, in secondary apoptosis 0.3% and 4.2% in necrosis. When MSCs were treated with 50 uM and 100 uM of CDDP, early apoptosis were 1.9% and 2.3%, secondary apoptosis 4.1% and 4.9%, necrosis were 17.9% and 14.5% respectively. After HEI-OC1 and MSCs were co-cultured for 48 hours, early apoptosis was 2.5%, secondary apoptosis was 9.0% and necrosis was 0.2%. When this co-cultured cells were treated with 50 uM and 100 uM of CDDP, early apoptosis were 2.4% and 2.4%, secondary apoptosis 5.9% and 7.3%, necrosis were 19.5% and 20.9% respectively (Figure 2). Apoptosis data is shown at Table 1.

Differentiation Findings of MSCs Among HEI-OC1

As a result of the immunocytochemical staining related to differentiation; math-1, calretinin and myosine 2A were negative in the HEI-OC1 control group. Nuclear stained math1 was seen to be cytoplasmic positive in places. In the MSC control group, calretinin was negative, myosine 2A was highly positive (100%) and math1 was 60% positive. In the triple combination of HEI-OC1 + MSC and CDDP 50 µM, 60–70% necrotic-apoptotic of

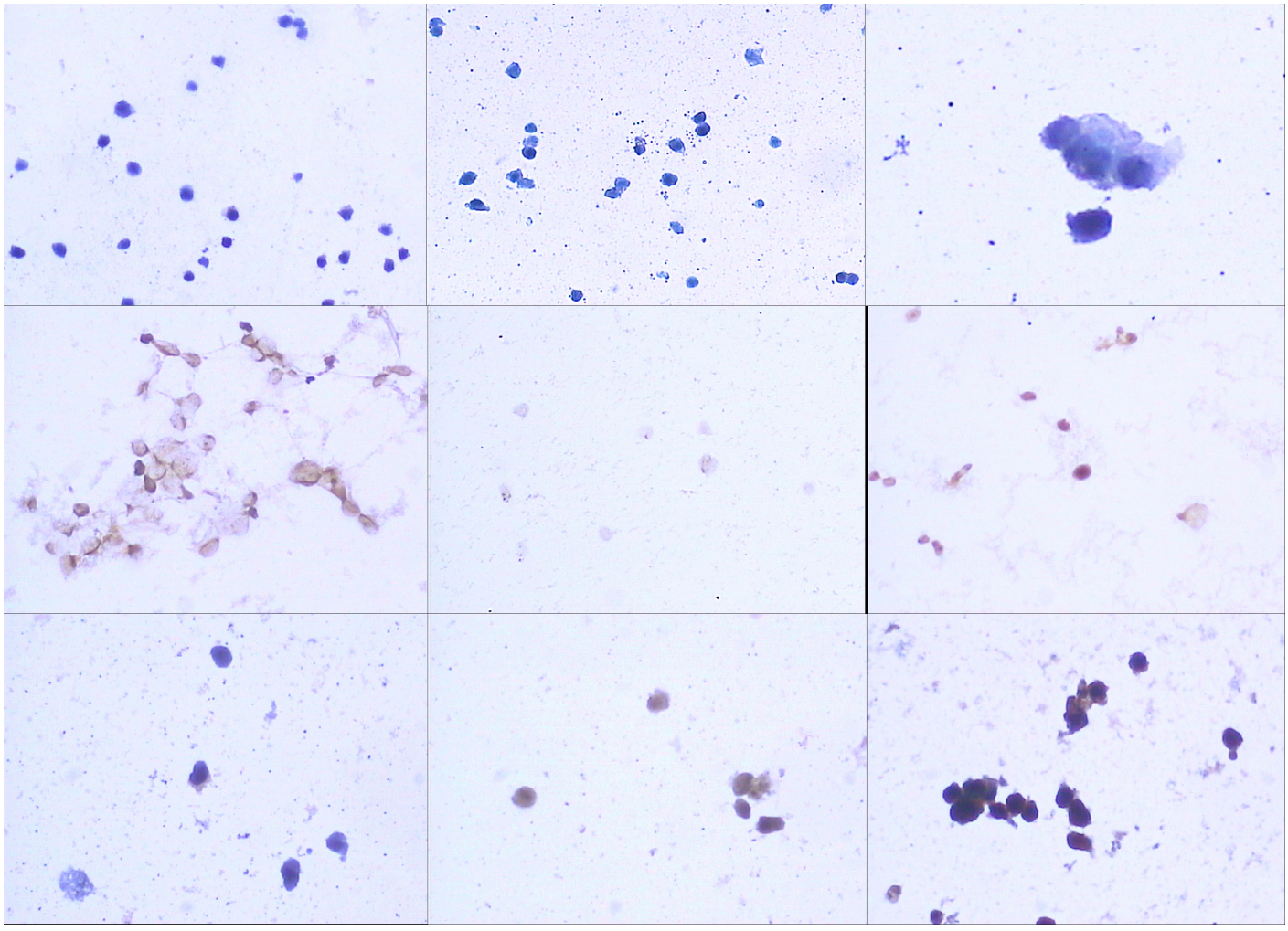

Figure 3. Immunocytochemistry expression patterns of HEI-OC1 cells, MSC cells and their combination with CDDP (ICC, DABx100). Calretinin, Myosin IIA and Math1 is known to be highly expressed in stem cells and immature cochlear cells. CDDP did not caused negativity in stem cell properties of the cells.

calretin was detected. In the remaining 30–40% live cells 4% were positive. While myosine 2A was 70% positive, math1 was 40% positive. In HEI-OC1, MSC and CDDP 100 µM triple combination, 20% was calretinin positive, 30% was myosine positive and 100% was math1 positive. In HEI-OC1 and MSC co-culture, 20% was calretinin positive, 70% was myosine 2A positive and 40% was math-1 cytoplasmic positive. All three markers are negative in the HEI-OC1 + CDDP 50 and 100 µM group. Similarly, three markers were negative in MSC + CDDP 50 and 100 µM groups (Figure 3).

Statistical analysis supported that MSCs reduced CDDP cytotoxicity on HEI-OC1 cells significantly both in viability and annexin-V analysis ($p<0.05$).

DISCUSSION

In this in vitro study, we demonstrated that MSCs decreased cochlear cell damage of CDDP in co-culture. Besides the antitumoral cytotoxic effect of CDDP was not found to be changed with MSCs co-culture on neuroblastoma cells. Besides we showed that Math1 and calretinin positive cells were increased supporting the idea of differentiation of MSCs into immature hair cells. Our findings supported the idea that MSCs might be a valuable cellular treatment candidate for CDDP ototoxicity.

MSCs as a cellular treatment strategy, has been widely used in daily practice. It might be a candidate treatment strategy to overcome ototoxicity side effect. This study had significant in vitro data supporting our hypothesis that MSC administration might protect from CDDP ototoxicity side effect during chemotherapy. The ototoxic effect of CDDP to the cochlear cells is thought to be eliminated by differentiation of MSCs into the cochlea cells.

CDDP ototoxicity, is one of the important side effects of chemotherapy in many cancers. One of the important research area of our team is neuroblastoma. New treatment stratejies for neuroblastoma and to overcome side effects of chemotherapy is current important topics. This study in vitro supported that MSCs treatment strategies might overcome ototoxic effect. The cochlear cells only did not express proteins calretinin, myosin IIa and Math 1 which are found to be expressed in immature cochlear cells.

Math-1 gene has been identified as the earliest hairy cell specific gene in developmental process. Loss of Math-1 gene expression was found to cause complete failure of hairy cell differentiation in mouse cochlea. The overexpression of Math-1 was found to generate new hairy cells. In additionally, overexpression of Math-1 led to differentiation of bone marrow derived mesenchymal stem cells to hairy cells. According to these findings, Math-1 is considered as a key transcription factor in hairy cell development (16). Similarly, we also found that Math-1 expression was increased when HEI-OC1 cochlear cells were cultivated with bone marrow derived MSCs and treated with CDDP.

Non-muscle myosin 2A which plays significant role in convergent extension, was shown to change cochlear length and cellular patterning. In addition, inhibition of myosin 2A pharmacologically resulted in inhibition of extension of sensory epithelium and disrupted hairy cell generation. Accordingly, non-muscle miyosin 2A is known to have crucial role in early stages of hairy cell development (17). Our data in which myosin 2A expression was found to be increased in CDDP treated HEI-OC1 and MSCs co-cultivated group, supported literature findings.

Calretinin which is a calcium binding protein, is commonly used to characterize cell specifications in sensory system. Calretinin expression was found to be highest in type I neurons in the mid-cochlear region and assumed significant in the development of new hairy cells and their functions (18). Similarly, our study showed no calretinin expression in HEI-OC1 cells while its expression was increased after CDDP treatment in HEI-OC1 and MSC co-cultivated cells. These data suggested that increased calretinin expression was associated with new generating cochlear cells.

This study is the first one studying HEI-OC1 cochlear cells as co-culture with MSCs. This point of view is one of the strengths of our study. Cultivation of HEI-OC1 cells and MSCs is a difficult technique and laboratory procedure. One of the weaknesses of this study was that the cultivation Centigrate degree is not the same of the two different cells. To overcome this conflict we first cultivated MSCs at 37°C, % 5 CO2 level and kept them at 33°C, % 10 CO2 and observed that cultivation continued.

Our next step is to plan in vivo nude mice neuroblastoma animal model comparing CDDP therapy with and without systemic MSC administration and check ototoxicity.

In conclusion, MSCs is found to protect cochlear cells from CDDP cytotoxicity, besides MSCs did not change antitumor effect of CDDP on neuroblastoma cells in vitro. MSC therapy might be a candidate cellular therapy approach to overcome CDDP ototoxicity. The mechanism seems to be via differentiation of MSCs into immature hair cells.

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