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Immuno-Histochemical Study of the Potential Impact Effect of a Single Intra-Peritoneal Injection Dose of Cisplatin on Stem Cells of Dental Pulp of Albino Rats

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Abstract

Background: Dental pulp stem cells are group of mesenchymal stem cells (MSCs) which have the ability to self-renew, high proliferative potential and capability to produce different cell types. MSCs appear in dental pulp after pulp injury or damage.

Aim of the study: Evaluate the response of dental pulp stem cells to the DNA-damaging cytostatic cisplatin treatment.

Material and Methods: The current study used a sample consisted of 30 healthy adult male albino rats (each weighed between 250 to 300 g) .The rats were distributed into two groups (15 rats in each group) as follows: Control Group (Group I): In this group, rats were injected with isotonic saline. Cisplatin group (Group II): In this group, a single intraperitoneal injection of Cisplatin 7 mg/kg of body weight was given to rats. One week later rats were anesthetized and sacrificed and their jaws examined and rapidly eliminated from the adherent connective tissue. Specimens were collected and prepared with haematoxylin and eosin for histological examination. Streptavidin-biotin immunohistochemical method for Vimentin and CD44 localization for stem cells characterization and homing detection respectively.

Results: The light microscopic results showed degeneration in the pulpal tissue of group II animals (Cisplatin Group) represented by cytoplasmic vacuolization, idiopathic calcification, hyaline and fatty degeneration. The results of the present study showed significant increase (P value <0.01) in the expression of Vimentin antibody on cells walls after Cisplatin treatment. In our results, significant P value (P<0.01) was detected between the anti-CD44 antibody expression on the outer cellular membranes of epithelial, odontoblasts, lymphocytes and stem cells in group II more than cells of group I.

Conclusion: Cisplatin treatment affects both Vimentin and CD44 expression on outer walls of dental pulp stem cells (DPSCs) via triggering apoptosis. Apoptosis increases TNF-α which upregulates Vimentin, and apoptosis downregulates CD44 by activating wild type (functioning) P53.

Keywords: MSCs; Mesenchymal Stem Cells; Cisplatin; Vimentin; CD44; Apoptosis; P53

Introduction

Dental pulp, a soft connective tissue containing blood vessels, nerves, and mesenchymal tissue, has a central role in primary and secondary tooth development and ongoing maintenance for instance in reaction to caries [1]. MSCs are delivered from neural crest cells and from epithelium during early embryogenesis. The possibility that tooth pulp might contain mesenchymal stem cells was first suggested by the observation of severe tooth damage that affects both enamel and dentine and forces the pulp to stimulate a limited natural repair process, during which new odontoblasts are formed to produce new dentine to repair the lesion [2]. Putative stem cells from the tooth pulp and several other dental tissues have now been identified [3]. Dental pulp stem cells are a population of mesenchymal stem cells (MSCs) with many unique properties, such as the ability to self-renew, high proliferative potential and capability to produce different cell types [4]. Dental pulp is an interesting source of mesenchymal stem cells, due to the large abundance of cells from one tooth and the noninvasive isolation methods compared to other adult tissue sources [5].

First trail to isolate stem cells from dental pulp of third molar but later on multiple successful isolation were done from permanent, primary teeth and supernumerary teeth in a non-harmful procedures from the donors [6]. There are currently no specific biomarkers that uniquely define (Dental Pulp Stem Cells) DPSCs. They express MSC-like phenotypic markers such as CD27, CD29, CD44, CD73, CD90, CD105, CD146, CD166, CD271, and STRO-1. Yet they do not express CD34, CD45, CD14, or CD19 and HLA-DR surface molecules [7]. Similar to embryonic stem cells, DPSCs express stemness-related markers such as Oct-4, Nanog, and Sox-2, as well as the cytoskeleton-related markers (Nestin and Vimentin) [8]. DPSCs are described as multipotent which can differentiate into cells of osteogenesis [9], chondrogenesis, adipogenesis, dentinogenesis [10], odontogenesis [11], neurogenesis [12], and myogenic lineages [5]. DPSC are highly proliferative and retain their stem cell characteristics after prolonged culture [13]. They could therefore be used as a generic allogeneic source of mesenchymal stem cells. Their use as autologous cells, however, is currently restricted to children who have not yet lost all their deciduous teeth [14].

Cisplatin-based chemotherapeutic regimens are the most frequently used (neo) adjuvant treatments for the majority of solid tumors [15]. It has become a cornerstone of antineoplastic chemotherapy to treat many types of cancer, including ovarian, cervical, stomach, bladder and head& neck cancer [16]. Cisplatin is especially efficacious for testicular cancer, which has an overall cure rate greater than 90% and nearly 100% at stage I [17].Cisplatin is clinically proven to combat different types of cancers including sarcomas, cancers of soft tissue, bones, muscles, and blood vessels. Although such cancers have recently received better prognosis and therefore have become less life threatening [18] significant challenges remain with regard to their cure. Also, because of drug resistance and considerable side effects [19], so the dosage (single and cumulative) and administration (schedule and means), and the systemic and individual conditions such as skin pigmentation, age, diet, blood pH all affect the interactions with radiotherapy [20]. Certain effects are dose-dependent; thus, they can be controlled but not prevented [21]. Among the adverse effects that may develop, the most frequent ones are gastrointestinal symptoms. More than 90% of patients experience nausea and vomiting; these symptoms are counteracted by the administration of antiemetic drugs such as the antagonist of serotonin receptor 3 (5-HT3) and dexamethasone. In a smaller number of cases, general symptoms were detected, such as fever, hyposthenia, altered sleep-wake cycle, myelo-suppression and alteration in the liver, skin and respiratory apparatus. Among the negative side effects with a more or less severe involvement of tissues include neurotoxicity, nephrotoxicity and ototoxicity [22].

Cisplatin (CIS) is a strong genotoxic and mutagenic agent [23]. It is able to induce DNA damage in a broad range of eukaryotic cells, from Drosophila melanogaster (DM) to humans, either *in vitro* and *in vivo*. Because of its capacity to cause DNA adducts, CIS can induce DNA strand breaks in DM somatic cells in vivo, evaluated by the Comet assay [24] and SMART test [25]. A large panel of mammalian cells have already been exposed to CIS, and its genotoxic potential has been confirmed on cells from hamster [26], mice [27], rats [28] and human normal and cancer cells [29]. Exposure of cells to cisplatin triggers cellular pathways involved in DNA repair, cell cycle arrest and apoptosis [30]. Thus far, nothing is known about the behaviour of DPSCs after treatment with cisplatin. Characterization of these cells under genotoxic stress conditions is necessary to assess their possible use.

Material and Methods

The current study used a sample consisted of 30 healthy adult male albino rats (each weighed between 250 to 300 g) which were purchased from Tiedor Bilhars Research Institute, Cairo, Egypt. The experiment was held at the animal house in Faculty of Dentistry, Suez Canal University, Ismailia, Egypt. Animals were housed, five per cage, with controlled temperature. The room was exposed to 12 hours of light and dark cycles. All animals were given pellets formed of seeds, grain, cracked corn and tap water. The rats were distributed into 2 groups (15 rats in each group) as follows:

1. Control Group (Group I): In this group, rats were injected with isotonic saline.
2. Cisplatin group (Group II): In this group, a single intraperitoneal injection of Cisplatin 7 mg/kg of body weight was given to rats. Cisplatin® (MERCK generiques-France) 19&30.

One week later, in a closed vessel made of glass with cotton soaked in a lethal dose of diethyl ether, rats were sacrificed and their jaws examined and rapidly eliminated from the adherent connective tissue. Specimens were collected and immersed in 10% formalin after 24-48 hours they were put in 10% EDTA (pH 7.4) - for bone decalcification- and the solution was changed every week for 3-5 weeks. The specimens were washed in phosphate buffered saline (PBS) and then embedded in paraffin. The embedding process was carried out by immersion in 70%, 80%, 96% ethanol (90 minutes each), three immersions in absolute ethanol (60 minutes each), two immersions in xylol (90 minutes each) and two immersions in liquid paraffin at 60°C (120 minutes each).

a. The Sections of 5μm were obtained and applied to clean glass slides and stained with hematoxylin and eosin stains in order to be examined under a light microscope.
b. The Sections of 5μm were obtained and applied to clean glass slides and stained with Streptavidin-biotin immunohistochemical method for Vimentin localization for stem cells characterization.
c. The Sections of 5μm were obtained and applied to clean glass slides and stained with Streptavidin-biotin immunohistochemical method for CD44 localization for stem cells homing detection.

Immunohistochemical Method used for Detection of (Vimentin and CD44)

The immunohistochemical detection system used is ultravision mouse tissue detection system: Antimouse HRP/DAB which is brought together with primary antibody; mouse monoclonal antibody of Vimentin [sc-373717 Santa Cruz, Biotechnology, USA], and mouse polyclonal anti-CD44 [Anti-CD44 , Sigma-Aldrich, Germany]. The reagents in the kit constitute a labelled streptavidin-biotin immune-enzymatic antigen detection system. This technique involves the sequential incubation of the section with an unconjugated primary antibody specific to target antigen, a biotinylated secondary antibody that reacts with the primary antibody enzyme labelled streptavidin and DAB chromogen. To evaluate the proliferation of positive immunoreactivity for Vimentin, and CD44 in the dental pulp, an ordinary light microscope was used. Then, the optical density of Vimentin and CD44 positive cells and the intensity of the immunostaining were assessed using the J Image analyser computerized system.

Results

Light Microscopic Picture of

Group I: (Control)

Light microscopic examination of pulp tissues of rats of group I (control group) revealed normal stratification of odontoblasts with normal connective tissue stroma and normal sized blood vessels (Figure 1).

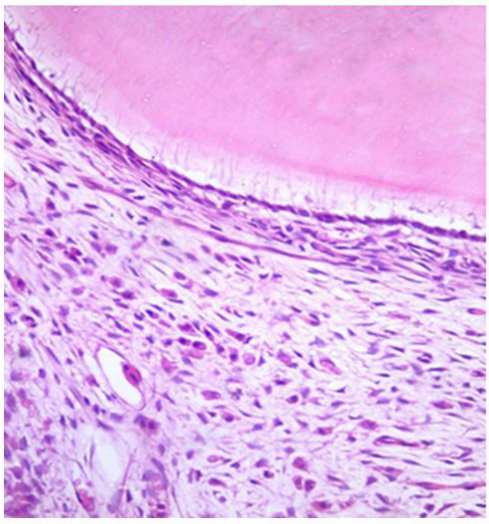

Figure 1: A photomicrograph of normal pulp tissue (control group). (*H&E Orig.mag.X20*)

Group II: (Cisplatin)

Examination of group II received single intraperitoneal dose of 7mg/ Kg body weight. Cisplatin, showed areas of vacuolization and fatty degeneration of some odontoblasts and in other areas where odontoblasts were overcrowding. Osteodentine appeared as idiopathic calcifications inside pulp tissues, fibrosis of pulpal connective tissue appeared with chronic inflammatory cells. Several dilated blood vessels of different sizes appeared filled with blood (Figure 2).

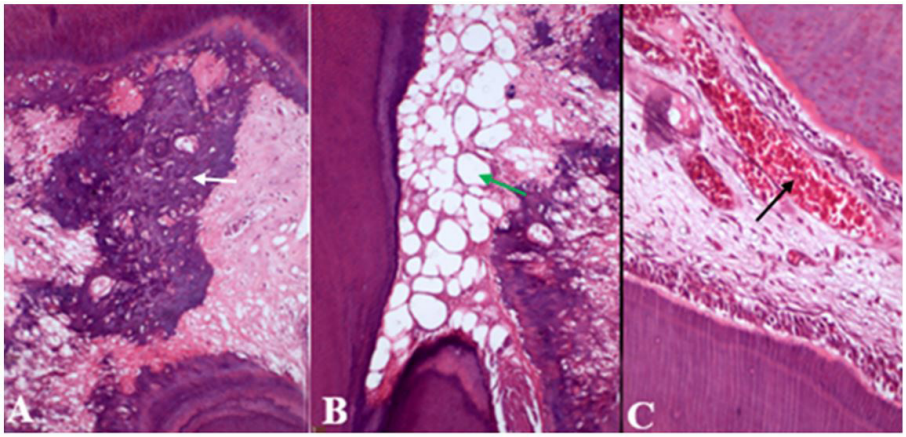

Figure 2: Photomicrographs of pulp tissue of group II received Cisplatin are showing (A) Vacuolization and idiopathic calcifications "osteodentin"(arrow) in areas of odontblastic layer and pulp core (B) Vacuolization and fatty degeneration of some odontoblasts (arrow) and the underlying C.T. which also showed areas of fibrosis (C) Crowding in odontoblastic layer and dilated blood vessels engorged with blood (arrow) (*H&E Orig.mag.X20*)

Immunohistochemical Examination

Vimentin expression is usually obvious around the cytoplasmic membrane and perinuclear membranes. Many cells (mesenchymal cells) may express Vimentin in connective tissue such as monocytes, macrophages, multinucleated giant cells, nerve cells, and stem cells. It was found severe overexpression of Vimentin antibody in group II (after receiving Cisplatin) (Figures 3 & 4) in comparison with that was expressed in group I (without Cisplatin treatment) (Figures 5 & 6).

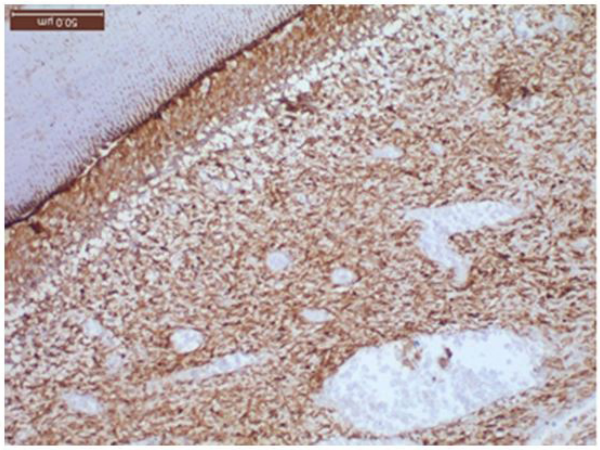

Figure 3: A photomicrograph is showing severe Vimentin anti-body expression of monocytes, macrophages, nerve cells and stem cells in group II. (*Vimentin anti-body, Orig.mag .X20*)

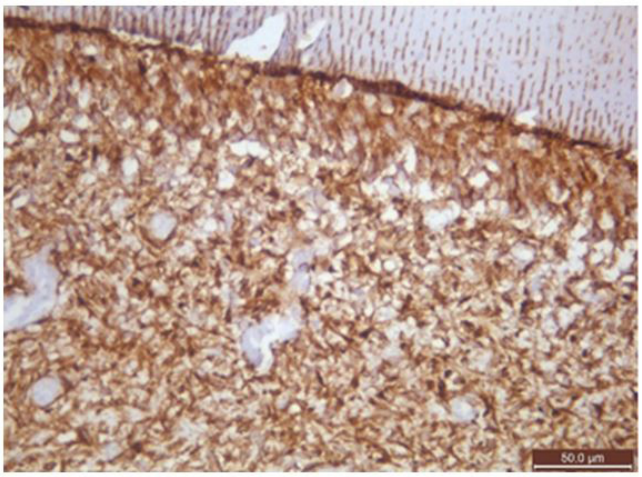

Figure 4: A photomicrograph is showing severe Vimentin anti-body expression of monocytes, macrophages, nerve cells and stem cells in group II. (*Vimentin anti-body, Orig.mag. X20*)

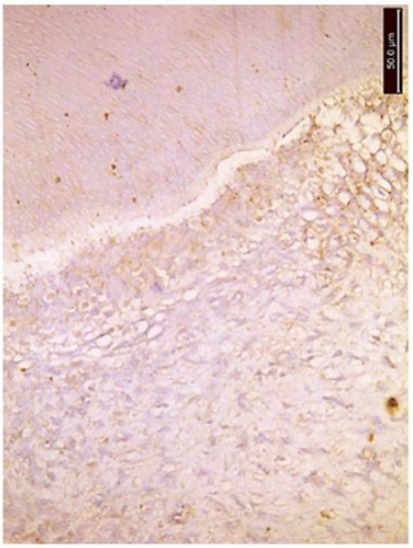

Figure 5: A photomicrograph is showing mild vimentin anti-body expression of some sporadic monocytes, macrophages, nerve cells and stem cells in group I. (*Vimentin anti-body, Orig.mag. X20*)

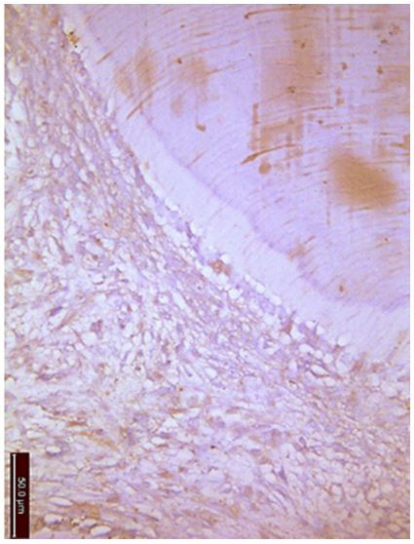

Figure 6: A photomicrograph is showing mild vimentin anti-body expression of some mesenchymal cells, notice its expression in cell membranes and perinuclear in group I. (*Vimentin anti-body, Orig.mag. X20*)

On the other hand, Anti-CD44 antibody expression appeared as brownish membranous (outer cell membranes). The anti-CD44 antibody expression appeared severe without Cisplatin treatment (group I) (Figure 7) and mild after Cisplatin treatment (group II) (Figure 8).

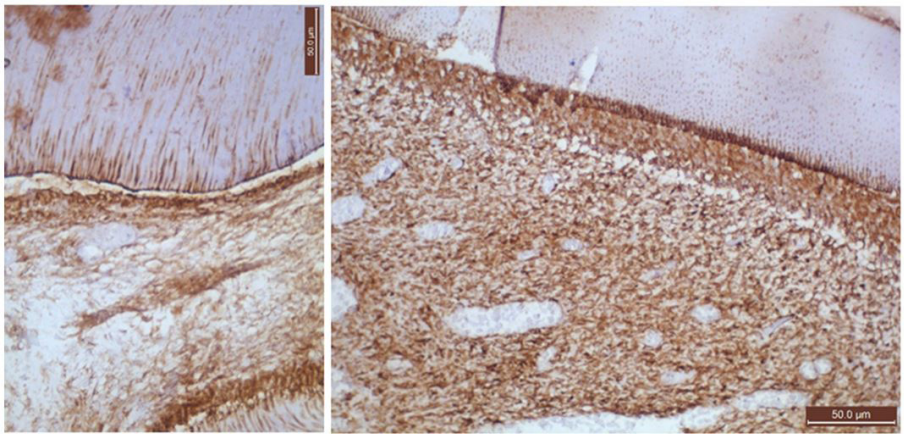

Figure 7: Photomicrographs are showing severe anti-CD44 anti-body expression of odontoblasts, monocytes, macrophages and stem cells in group I (without Cisplatin treatment). (*Anti-CD44 anti-body, Orig.mag .X20*)

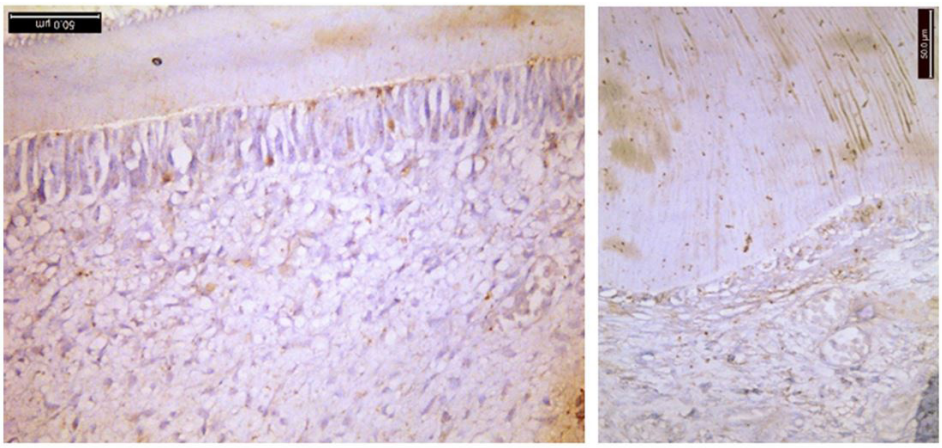

Figure 8: Photomicrographs are showing mild anti-CD44 anti-body expression of odontoblasts, monocytes, macrophages and stem cells in group II (with Cisplatin treatment). (*Anti-CD44 anti-body, Orig.mag .X20*)

Statistical Analysis

Results were statistically analyzed by SPSS version 22(SPSS Inc., Chikago, IL, USA). Mann-Whitney tests were used to compare between control and Cisplatin Group. Results in Table 1 showed highly significant difference between the two groups using Mann-Whitney (U)

test at P value <0.01), the Cisplatin group had the highest value for optical density of cells to Vimentin (268.805± 6.224) comparing with Control group (65.840±7.7.554), these indicated that with Cisplatin treatment Vimentin expression increased on the cell walls (Chart 1).

	ControlGroup	Cisplatin Group	Mann-Whitney Test	
			U	p- value
Mean	65.84	268.805	-3.780-	0.000*
SD (±)	7.554	6.224		

**, means significant between groups at (P<0.01)

-Mann-Whitney (U) test was used to compare between two groups at significant 0.01level.

Difference in mean Vimentin antibody optical density between Control group and Cisplatin group

Table 1: Anti-Vimentin antibody

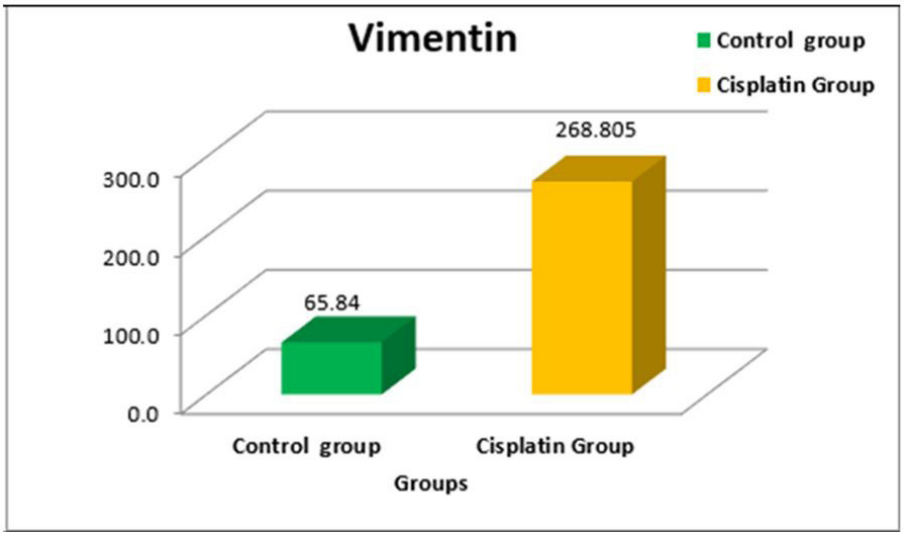

Chart 1: Optical density between Control group and Cisplatin group

Results in Table 2 showed highly significant difference between the two groups using Mann-Whitney (U) test at P value <0.01) , the control group had the highest value for optical density of cells to anti-CD44 antibody (257.922± 9.950) comparing with Cisplatin Group (93.153±7.349), these indicated that Cisplatin treatment decreased the expression of CD44 on cell membranes (Chart 2).

	Control Group	Cisplatin Group	Mann-Whitney Test	
			U	p- value
Mean	257.922	93.153	-3.576	0.000**
SD (±)	9.95	7.349		

**, means significant between groups at (P<0.01)

-Mann-Whitney (U) test was used to compare between two groups at significant 0.01 level.

Difference in mean anti-CD44 antibody optical density between Control group and Cisplatin group

Table 2: Anti-CD44 antibody

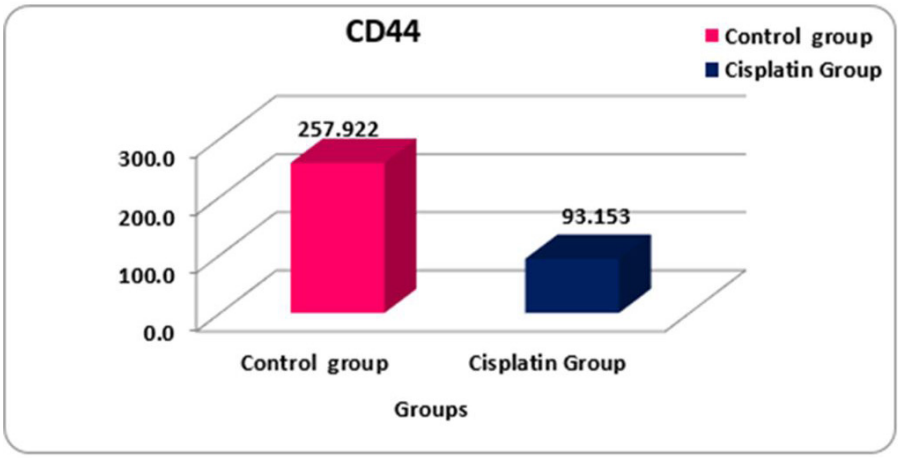

Chart 2: Optical density between Control group and Cisplatin group

Discussion

Cisplatin is one of the most commonly utilized anti-neoplastic agents in multiple solid tumours including ovarian carcinoma, non-small cell lung carcinoma and head and neck squamous cell carcinoma (HNSCC) [31]. In this study, the sensitivity of dental pulp stem cells (DPSCs) to DNA-damaging cytostatic Cisplatin was evaluated. The histological examination of this study showed massive degenerative changes in the dental pulp represented by osteodentin formation, vacuolization and areas of odontoblast loss. Fibrosis, areas of fatty degeneration, and blood vessels dilatation also were noticed. Atari, *et al.* [32] reported that dental pulp stem cells show self-renewal ability and multilineage potential [32]. The pulp tissue also contains a wide variety of undifferentiated cells that can differentiate into odontoblast-like cells. Many morphological studies have suggested that the dental pulp is capable of forming hard tissues including dentin and bone [33].

DPSCs have large capacity for proliferation, the ability to maintain their cellular phenotype for a long time period, and the sensitivity of response to toxins [34]. Seifrtova, *et al.* [35] in their study stated that when dental pulp stem cells were exposed to 5, 10, 20, or 40 mmol/L cisplatin they had a greater genotoxic stress response compared with normal human dermal fibroblasts. Cisplatin in high concentrations activates mitogen-activated protein kinases and apoptosis in dental pulp stem cells and not human dermal fibroblasts. Gene p53, being a tumor suppressor protein, is an essential regulator of the cell cycle and apoptosis and plays an important role in cisplatin activity [35]. The use of chemotherapeutic agents such as cisplatin can lead to increased inflammatory response in the arterial vessel wall [36]. The previous studies may explain the histological results of this investigation, as the cytotoxicity of the DPSCs caused loss of regenerative capacity and physiological maintenance of the dental pulp. This explanation is also confirmed by immunohistochemical results of this research.

Vimentin is a protein that is expressed in mesenchymal cells. It is well known as type III intermediate filaments which are (tubulin, microtubules and actin). Vimentin is found in animal cells as in bacteria [37]. So, it is used as a marker for all mesenchymally derived cells. Expression of Vimentin intermediate filaments (VIFs) correlates with mesenchymal cell shape and motile behaviour. Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition, which is obvious in transformation of epithelial cells into cancer cells [38]. Vimentin is well accepted as the component that gives cells their integrity as it is attached to nucleus, mitochondria and endoplasmic reticulum. It is believed to be secreted from Golgi apparatus. Moreover, it is responsible for the movement and migration of cells as occur in case of migration of cancer cells and in non-Hodgkin's lymphoma as it gives cells some resiliency [39].

Vimentin is upregulated by the tumour necrosis factor alpha (TNF-α). It is believed that Vimentin may have a role in immune system as it is secreted on the outer membranes of macrophages during inflammatory reactions40. Cisplatin increases TNF-α which induces apoptosis and apoptosis by its turn upregulates Vimentin [41,42]. The results of the present study showed significant increase (P value <0.01) in the expression of Vimentin antibody on cells walls after Cisplatin treatment. That may be explained, as mentioned above, that Cisplatin increases TNF-α which triggers apoptosis and causes upregulation of Vimentin. The encoded protein by CD-44 gene is a cell surface glycoprotein which is involved in cell-cell interaction, cell migration and cell adhesion. CD-44 is a receptor for hyaluronic acid (HA) that can mediate cell-matrix interactions. According to the degree of affinity of cells to HA the expression of anti-CD44 antibody appears as a membranous brownish staining [43]. CD44 is essential in homing and stem cell properties of leukemic stem cells [44].

A study by Seifrtova, *et al.* [35] was performed to study the effect of Cisplatin on the dental pulp stem cells (DPSCs). They found that Cisplatin stimulated tumour suppressor gene P53 (apoptosis) in DPSCs even in small doses. As well known that protein P53 is essential in maintenance of genomic stability that means in case of DNA damage, P53 is activated to cause cell cycle arrest (apoptosis) [35]. It was confirmed by Pestell, *et al.* [45] that the cell sensitivity to Cisplatin is connected to the presence of wild type P53 [45]. In our results, significant P value (P<0.01) was detected between the anti-CD44 antibody expression on the outer cellular membranes of epithelial, odontoblasts, lymphocytes and stem cells in group II more than cells of group I. Meaning, downregulation and decrease in anti-CD44 expression after Cisplatin treatment than before Cisplatin treatment.

That may be explained as Cisplatin induced apoptosis via stimulation of wild type P53 inside DPSCs. These results are in consistent with the results from a study by Seifrtova, *et al.* [35] they reported increase in the expression of protein P53 and its post-translational modification by phosphorylation of serine 15 after treatment with Cisplatin inside DPSCs. These results indicate that the P53 pathways modulate the fate of DPSCs [35]. In a study by Godar, *et al.* [46] they found that increased wild type P53 (apoptosis) inhibited expression of the CD44 cell-surface molecule via binding to a noncanonical P53-binding sequence in the CD44 promoter. This interaction enabled an untransformed cell to respond to induced stress. They also reported increase in expression of CD44 on cell walls in tumour-genesis in absence of wild type P53 (meaning predominate mutant P53) [46].

Conclusion

Cisplatin treatment affects both Vimentin and CD44 expression on outer walls of dental pulp stem cells (DPSCs) via triggering apoptosis. Apoptosis increases TNF-α which upregulates Vimentin. On the other hand apoptosis downregulates CD44 by activating wild type (functioning) P53.

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