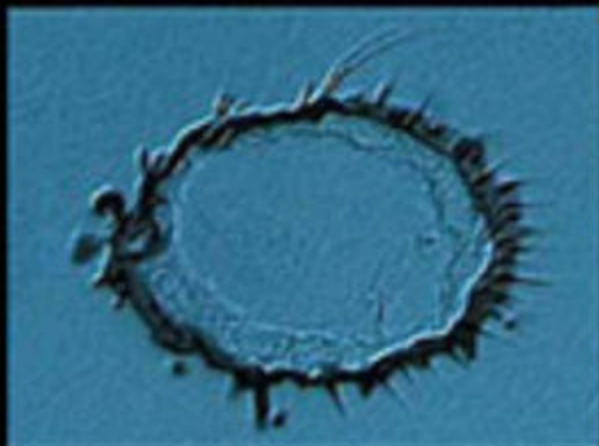
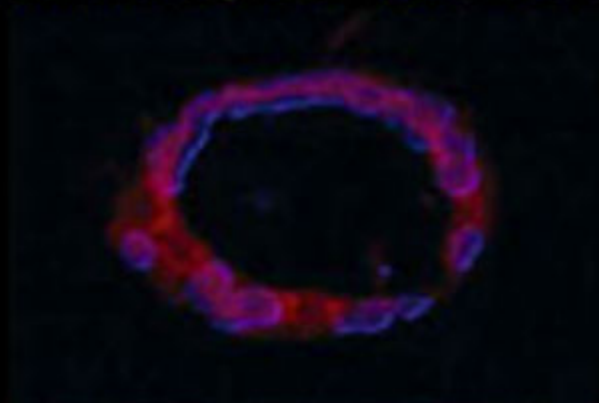


# Archives

of Biological Sciences



Official journal of the  
Sethian Biological Society

ISSN 0034-4664 (printed)  
ISSN 1821-4339 (online)



Vol. 47 No. 4  
August 2015

# CYTOTOXICITY OF GLASS IONOMER CEMENT ON HUMAN EXFOLIATED DECIDUOUS TEETH STEM CELLS CORRELATES WITH RELEASED FLUORIDE, STRONTIUM AND ALUMINUM ION CONCENTRATIONS

Tatjana V. Kanjevac<sup>1</sup>, Marija Z. Milovanović<sup>2,\*</sup>, Olivera Milošević-Djordjević<sup>3</sup>, Živoslav Tešić<sup>4</sup>, Mirjana Ivanović<sup>5</sup> and Aleksandra Lukić<sup>6</sup>

<sup>1</sup> *Department for Preventive and Pediatric Dentistry, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia*

<sup>2</sup> *Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia*

<sup>3</sup> *Faculty of Science, University of Kragujevac, Kragujevac, Serbia*

<sup>4</sup> *Faculty of Chemistry, University of Belgrade, Belgrade, Serbia*

<sup>5</sup> *Clinic for Pediatric and Preventive Dentistry, School of Dental Medicine, University of Belgrade, Belgrade, Serbia*

<sup>6</sup> *Department of Endodontics, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia*

\*Corresponding author: marijaposta@gmail.com

---

**Abstract:** Stem cells from human exfoliated deciduous teeth (SHED) can be used as a cell-based therapy in regenerative medicine and in immunomodulation. Pulp from human deciduous teeth can be stored as a source of SHED. Glass ionomer cements (GICs) are commonly used in restorative dentistry and in cavity lining. GICs have lower biocompatibility and are cytotoxic for dental pulp cells. In this study, seven commonly used GICs were tested for their cytotoxic effects on SHED, for their potential to arrest mitosis in cells and induce chromosome aberrations, and were compared with the effects of composite. Fuji II, Fuji VIII, Fuji IX, Fuji plus and Vitrebond had significantly higher cytotoxic effects on SHED than composite. Only SHEDs that have been treated with Fuji I, Fuji IX, Fuji plus and composite recovered the potential for proliferation, but no chromosome aberrations were found after treatment with GICs. The cytotoxic effects of GICs on SHEDs were in strong correlation with combined concentrations of released fluoride, aluminum and strontium ions. Fuji I exhibited the lowest activity towards SHEDs; it did not interrupt mitosis and did not induce chromosome aberrations, and was accompanied by the lowest levels of released F, Al and Sr ions.

**Key words:** Glass ionomer cements; cytotoxicity; fluoride; aluminum; strontium; SHED.

**Received** October 21, 2014; **Revised** October 30, 2014; **Accepted** November 3, 2014

---

## INTRODUCTION

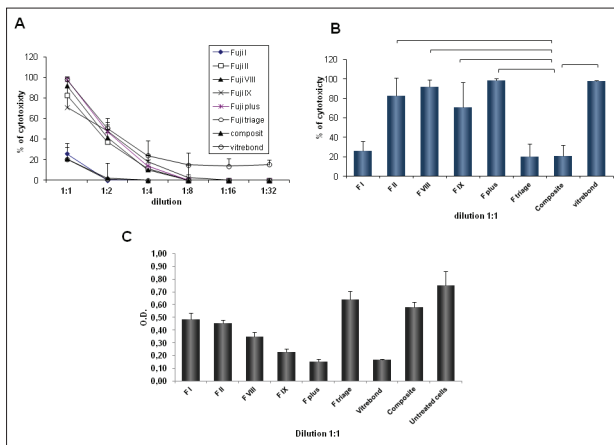
The main function of dental pulp is the formation of dentin. In this process, dental pulp mesenchymal stem cells differentiate into odontoblasts, the cells that deposit collagen matrix for further mineralization. Pulp also continues to produce dentin in order to repair damage (Briso et al., 2006). For these reasons, it is important to protect the pulp during restorative dentistry, applying several layers of specific materials between the restorative material and the dental tissue (Briso et al., 2006; Modena et al., 2009). Calcium hydroxide-based products have been largely used to protect the pulp, due to their ability to stimulate sclerotic and reparative dentin formation and protect the pulp against antibacterial action (Foreman and Barnes, 1990; Stanley et al., 1997). Glass ionomer cements (GICs) were introduced by Wilson and Kent in 1971 as a mixture of calcium or strontium aluminofluorosilicate glass powder (base) and a water-soluble polymer (acid) as a convenient material for prevention of pulp tissue damage and the penetration of bacteria (Wilson and Kent, 1971). Conventional GICs have valuable advantages over other materials used in dentistry: low solubility, ability to release fluoride, good adhesion to dentin and coefficients of thermal expansion and elasticity modes similar to dentin (Maijer and Smith, 1998). GICs also uptake fluoride from topical fluoride applications, allowing them to act as long-term fluoride-releasing agents (de Souza Costa et al., 2003). Several variations of GICs have been developed. The addition of resinous compounds, 2-hydroxyethyl methacrylate (HEMA), to conventional GICs improved their mechanical properties and enhanced flexural and diametral tensile strength, elastic modulus and resistance to wear (Xie et al., 2000). However, modified GICs have a very important disadvantage: reduction in biocompatibility due to the release of uncured

monomers causing pulp irritation and higher cytotoxicity in comparison with conventional GICs (Stanley et al., 1967).

Dental pulp tissues of human exfoliated deciduous teeth contain a mesenchymal stem cell (MSC) population, called stem cells from human exfoliated deciduous teeth (SHED) (Miura et al., 2003). These cells have typical stem cell properties: clonogenicity, self renewal and multipotency to differentiate into osteoblasts, adipose tissue and neural cell-like cells (Miura et al., 2003); they also have immunomodulatory properties. MSCs isolated from different fetal and adult tissues are considered the most appropriate source for cell-based therapy (Porada and Almeida-Porada, 2010). It was shown *in vivo* that SHEDs govern bone repair in bone defects in mouse calvarias (Seo et al., 2008) and swine mandible (Yamaza et al., 2010). When applied systemically, they attenuate autoantibody levels and IL-17 production (Yamaza et al., 2010). SHEDs also express the capacity to form a dentin/pulp complex when subcutaneously transplanted into immunocompromised mice (Miura et al., 2003). More importantly, SHED can be reprogrammed into iPS (inducible pluripotent stem) cells at a higher rate than fibroblasts (Yan et al., 2010). The recent finding has shown that SHED obtained from the cryopreserved dental pulp tissue of human deciduous teeth retained the main MSC properties: self-renewal, multipotency, *in vivo* dentin/bone-regeneration and *in vitro* immunomodulatory function (Ma et al., 2012). Furthermore, SHEDs that have been isolated from recovered dental pulp tissue showed therapeutic efficiency *in vivo* on immune disorders in MRL/*lpr* mice and also in skeletal and bone defects in the calvarias of immunocompromised mice (Ma et al., 2012).

We have previously reported the cytotoxic effects of GICs on dental pulp stem cells (DPSC)

isolated from postnatal pulp (Kanjevac et al., 2012). Exfoliated deciduous teeth are easily accessible tissues and cryopreserved dental pulp tissues of human exfoliated deciduous teeth could be an important source for SHEDs, which could be used for syngeneic cell-based tissue engineering and immune therapy. In this study, we examined the cytotoxic and genotoxic effects of commonly used GICs, Fuji I, Fuji II, Fuji VIII, Fuji IX, Fuji Plus, Fuji Triage and Vitrebond, on SHEDs and compared them with effects of composite. We found significant cytotoxic effect of Fuji II, Fuji plus, Fuji VIII, Fuji IX and Vitrebond on SHEDs, while Fuji I, Fuji VIII, Fuji Triage and Vitrebond inhibited further mitosis of SHED. In addition, we found no genotoxic effects of the examined GICs on SHED.



**Fig. 1.** The cytotoxicity of GIC eluates on SHEDs evaluated through the MTT assay. The percentages of dead SHED after 24-h exposure to GIC eluates are expressed as the means + standard error (SE) from 9 independent experiments (each dilution was performed in triplicate per experiment). (A) Dose-dependent curves show that Fuji I, Fuji triage and Composite had no cytotoxic effect if they were used in dilution 1:2, while Fuji II, Fuji VIII, Fuji IX, and Fuji plus were not cytotoxic in dilution 1:8. Vitrebond retained a cytotoxic effect even in dilution 1:32. (B) at 1:1 Fuji II, Fuji VIII, Fuji IV dilution, the Fuji triage and Vitrebond eluates exerted significantly higher cytotoxic effects on SHED compared with the composite eluates (\*  $p < 0.001$ ), as evaluated by the MTT assay. (C) Mean values + standard error (SE) of optical density from 9 independent experiments. Optical density of the cells exposed to tested eluates is shown, and the cells cultured only in growth medium.

## MATERIALS AND METHODS

### Tested materials

Eight biomaterials, Fuji I, Fuji II, Fuji VIII, Fuji IX, Fuji plus, Fuji Triage (GC Corporation, Tokyo, Japan), Vitrebond (3M ESPE, London, UK) and Te-Econom composite (Ivoclar Vivadent AG, Liechtenstein) were used in this study. Separate GIC samples were prepared according to the manufacturers' directions at room temperature and then placed into open plastic rings 5 mm in diameter and 2 mm deep. After consolidation, the samples were removed from the rings and dry heat sterilized for 1 h at 170°C. The samples were then incubated in complete culture medium (150  $\mu$ L per sample) for 72 h at the 37°C and in a 5% CO<sub>2</sub> atmosphere. The sample dimensions and immersion conditions were chosen to approximate the GICs mass and the dentin-exposed surface area typically used in restorative dentistry patient procedures. Sterilization of samples in 70% ethanol was also done. Since there was no difference in the concentration of released ions and pH value of medium between sterilized samples, the medium exposed to each heat sterilized GICs was used for testing in a cell-culture system.

### Culture of human SHED

Human SHED purchased from AllCells, LLC (Emeryville, California, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100 I.U./mL penicillin G and 100  $\mu$ g/mL streptomycin (Sigma-Aldrich chemical, Munich, Germany). SHEDs in 4 passages were used throughout these experiments.

### MTT assay for cytotoxicity

The effects of GICs on SHED viability were determined using the MTT colorimetric technique

(Mosmann, 1983). SHEDs were diluted with DMEM medium to  $1 \times 10^5$  cells/mL and aliquots ( $1 \times 10^4$  cells/100  $\mu$ L) were placed in individual wells in 96-multiplates. The next day, medium was exchanged with 100  $\mu$ L of each GIC eluate, differently diluted in growth medium (dilutions from 1:1 to 1:32). Each eluate was tested in triplicate. Cells were incubated at 37°C in a 5% CO<sub>2</sub> incubator for 24 h. Cells cultured in growth medium only were used as control.

After incubation, the GIC eluate, or medium for control, was removed and 15% MTT solution (5 mg/mL in PBS) in pure DMEM was added to each well. After a additional 4 h of incubation at 37°C in a 5% CO<sub>2</sub> incubator, the MTT solution was removed and DMSO (150  $\mu$ L) with glycine buffer (20  $\mu$ L) was added to each well to dissolve the crystals. The plates were shaken for 10 min. The optical density of each well was determined at 595 nm using an Anthos Zenyth 3100 Microplate Multimode Detector. The percentage of cytotoxicity was calculated using the formula: percentage of cytotoxicity =  $100 - ((E-B)/(C-B) \times 100)$ , where B is optical density of medium alone, C is optical density of cells cultured in growth medium, and E is optical density of cells exposed to GIC eluates, so that the percentage of cytotoxicity of each GIC eluate is calculated in relation to the cytotoxicity of the medium alone which was 0%.

### Apoptosis assay

For detection of apoptosis, the Annexin V binding capacity of treated cells was examined by flow cytometry using an Annexin V FITC Detection Kit (BD Pharmingen, San Jose, CA, USA) according to the manufacturer's protocol. After SHED reached subconfluency, the medium was replaced with GIC eluate diluted 1:1 in complete DMEM

(volume, 4 mL). SHEDs exposed to GIC eluate were placed at 37°C in a 5% CO<sub>2</sub> incubator for 24 hours. Cultured cells were washed twice with cold phosphate-buffered saline (PBS, Sigma Aldrich) and resuspended in 1 x binding buffer (10 x binding buffer: 0.1 M HEPES/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl<sub>2</sub>) at concentration  $1 \times 10^6$  per mL. Annexin FITC and propidium iodide (PI) were added to the 100  $\mu$ L of cell suspension and incubated for 15 min at room temperature (25°C) in the dark. After incubation, 400  $\mu$ L of 1 x binding buffer was added to each tube and stained cells were analyzed within 1 h using FACS Calibur (BD, San Jose, USA) and Flow Jo software (Tri Star). Since Annexin V FITC staining precedes the loss of membrane integrity that accompanies the later stage identified as Annexin V FITC and PI positive; viable cells are Annexin V FITC and PI negative. Cells that are in late apoptosis or already dead are both Annexin V FITC and PI positive.

### Cytogenetic Analysis

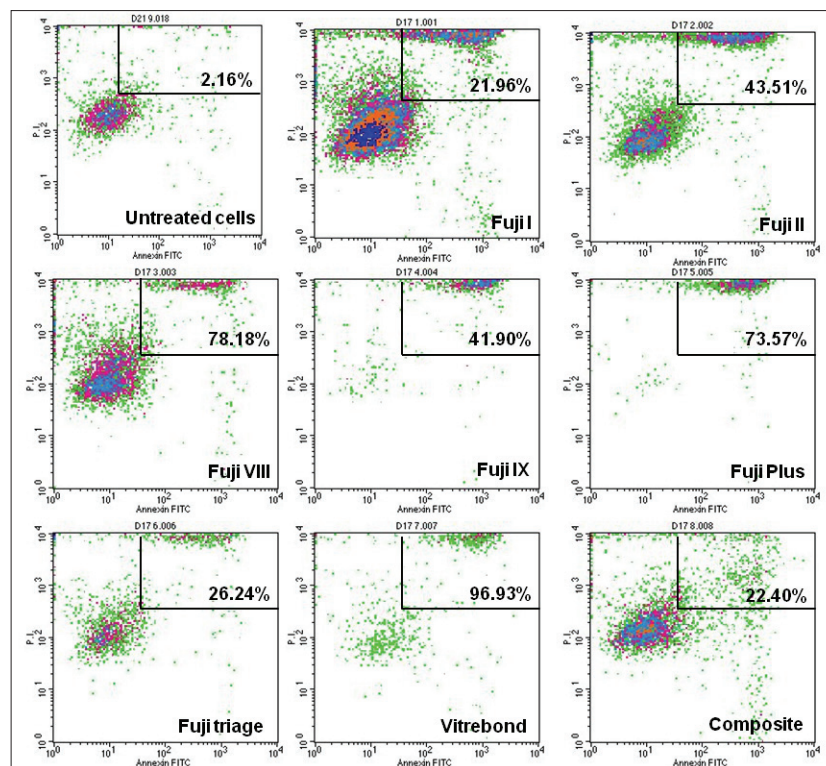
To test eventual chromosome abnormalities induced by GICs, cytogenetic analysis was done (Foudah et al., 2009). After exposure to GIC eluates (dilution 1:4) for 24 h, SHEDs were incubated in growth medium without GIC eluate for 48 h (5% CO<sub>2</sub>, 37°C) to recover proliferation. Then vinblastine (1  $\mu$ g/mL) was added to the culture flask to arrest the cell cycle in mitosis and incubated for another 48 h. After exposure to mitotic arrestant, the cells were dislodged from the flask using trypsin-EDTA  $1 \times$  in PBS (Gibco, Invitrogen) for 3 min at 37°C, collected into 15-mL centrifuge tubes, and pelleted by centrifugation at 125 G for 10 min. The pellet was suspended in 9 mL of 0.56% KCl for 15 min at 37°C. Following incubation, 1 mL of freshly prepared fixative (methyl alcohol-glacial acetic acid, 3:1) was added,



and the suspension was centrifuged at 125xg for 10 min. The pelleted cells were resuspended in 5 ml of fresh fixative for 30 min at room temperature, then centrifuged at 125xg for 10 min and again resuspended in fresh fixative. This procedure was repeated three times. Finally, the pellet was resuspended in 70% acetic acid. About three drops of cell suspension were dropped onto cold, dry slides tilted at an angle of 30° and air-dried for 2-3 days. This procedure allowed metaphase spreads in which the two chromatids of each chromosome lay close together and were relatively long. The slides were placed in a cuvette with 5N HCl for 10 min, washed in water and stained with 10% Giemsa. One hundred mitoses were analyzed per sample.

### Quantification of fluoride, aluminum and strontium ions in medium exposed to GICs

The fluoride (F) concentrations of each eluate were assayed by high performance liquid chromatography using a ChromeleonR Chromatography Workstation (Dionex, Wien, Austria) equipped with a GP50 gradient pump, conductivity detector, ASRS ultra 4 mm. An Ionpac AS15 column and an AG15 guard column were used. Potassium hydroxide was used as the eluent. The flow rate was 1.0 mL/min. All results were analyzed on Chromeleon 6.7 Chromatography Management Software. Aluminum (Al) and strontium (Sr) concentrations were assayed



**Fig. 2.** The representative dot plots of the apoptosis assay with percentages of late apoptotic cells. The highest percentage of late apoptotic cells (propidium iodide (+) AnnexinV (+)) was attained after treatment with Vitrebond (96.93%), Fuji VIII (88.48%) and Fuji Plus (79.57%). A moderate percentage of late apoptotic cells was attained after treatment with Fuji IX (41.90%) or Fuji II (43.51%). The lowest percentage of late apoptosis was attained after treatment with Fuji I (21.96%), Fuji triage (26.24%) and composite (22.40%). The results of a representative experiment are presented.

by inductively coupled plasma-optical emission spectroscopy (ICP-OES) using a Thermo Scientific iCAP 6500 Duo ICP (Thermo Fisher Scientific, Cambridge, UK) according to manufacturer's instruction.

### Statistical analysis

One-way ANOVA tests and Student's *t* test were used to analyze the data. Bivariate correlation was used for correlation between the concentration of a single ion and cytotoxicity, while correlation between combined ions and cytotoxicity was analyzed with partial correlation:  $p < 0.05$  was considered statistically significant. Correlation was expressed by Pearson's coefficient (*r*). All statistics were carried out using SPSS 13.0 and SPSS 19.0 for Windows software.

## RESULTS

### Glass ionomer cements had significant cytotoxic effect on SHED

The cytotoxic effects of GIC eluates on stem cells from human exfoliated deciduous teeth (SHED) were evaluated by MTT assay. The results clearly show the cytotoxic activity of all tested GICs against SHED. There is a significant difference in cytotoxicity among the tested GICs. The cytotoxicity of GICs decreases with its increasing dilution (Fig. 1A). There is no cytotoxicity of GIC eluates diluted 8, 16 and 32 times. Concentrated eluates had low pH and induced maximal cytotoxic effect, almost 100% (data not shown), and for further analyses the dilution 1:1 was used. Fuji I and Fuji triage (dilution 1:1) had some cytotoxic effect on SHED but was significantly lower than the other tested GICs (Fig. 1B). Comparison of the cytotoxicity of GICs and

composite (Te-Econom) on SHED showed significantly stronger ( $p < 0.001$ ) cytotoxicity of Fuji Plus (98.5% cytotoxicity), Vitrebond (97.92%), Fuji VIII (92.06%), Fuji IX (82.71%) and Fuji II (71.07%) than of composite (Te-Econom). There was no significant difference in cytotoxicity on SHED among Fuji I (25.88%), Fuji Triage (20.38%) and composite (20.76%) (Fig. 1B).

The comparison of the cytotoxic effects of GICs with results obtained previously (Kanjevac et al., 2011) showed significantly more cytotoxic effects of GICs on SHED than on DPSC. Fuji II ( $p < 0.05$ ), Fuji VIII ( $p < 0.005$ ), Fuji plus ( $p < 0.005$ ) and Fuji Triage ( $p < 0.05$ ) exerted significantly stronger cytotoxicity on SHED when compared with their effect on DPSC.

### SHED exposed to glass ionomer cements were in late apoptosis

Another assay was used to analyze the cytotoxic effects of GICs on SHED cells. Similar results were obtained using an apoptosis assay (Fig. 2). Most of the SHED cells were in late apoptosis (Annexin V+ PI-). The highest percentage of cells in late apoptosis was found after treatment with Fuji VIII (88.18%), Vitrebond (96.93%), and Fuji Plus (79.57%). The lowest toxic effect was observed with Fuji I, Fuji triage was similar to Composite, as evaluated by the apoptosis assay. These results were in good correlation with cytotoxicity obtained using the MTT assay ( $r = 0.808$ ).

### GICs eluates do not induce aberrations in chromosomes of SHED in vitro

In order to test the eventual mutagen potential of GICs, classical cytogenetic analysis was done with chromosomes obtained from SHED treated with GIC eluates (dilution 1:4) for 24 h and

subsequently incubated in growth medium to eventually recover proliferative potential.

It was impossible to obtain chromosomes in mitosis from cells treated with Fuji II, Fuji VIII, Fuji Triage and Vitrebond. SHED treated with Fuji I, Fuji IX, Fuji plus and Te-Econom recovered proliferative capacity (evaluated by phase contrast microscopy) and chromosome aberrations were not found (Fig. 3).

GICs cytotoxicity is in correlation with concentration of coupled fluoride, aluminum and strontium ions

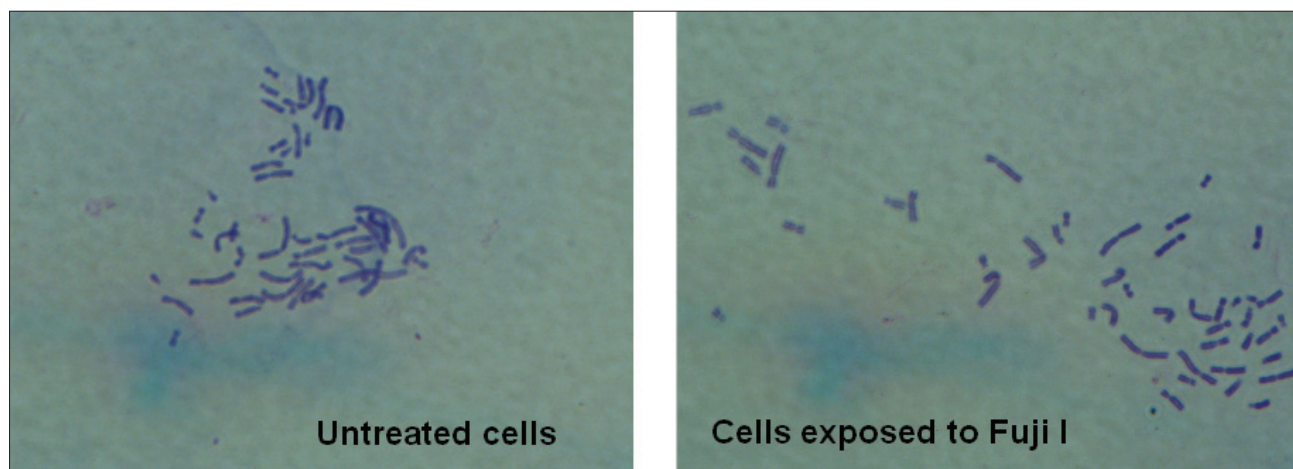
There was a strong correlation between the concentration of fluoride and the cytotoxic activity of GIC eluates on SHED evaluated by MTT assay (Pearson's correlation coefficient ( $r = 0.815$ ,  $p < 0.05$ ). The GIC eluates with the most toxic effects (Fuji Plus, Vitrebond and Fuji VIII)) released the highest amount of fluoride while opposite results were found for composite and Fuji I (Fig. 3). A correlation between the aluminum and strontium ion concentration in eluates and cytotoxicity on SHED was not found.

Interestingly, a stronger correlation was found between the combination of fluoride and strontium ions in eluates and their cytotoxic activity, while the strongest correlation with cytotoxicity on SHED was found for the combination of all three tested ions ( $r = 0.900$ ).

A similar result was found for the correlation between the cytotoxic effect on DPSC and the combined concentration of fluoride and strontium (Kanjevac et al., 2012).

## DISCUSSION

SHED is a population of highly proliferative, clonogenic cells, differentiating into a diversity of cell types including dentin/bone-forming cells (Miura et al., 2003; Sakai et al., 2010), endothelial cells (Sakai et al., 2010), neural cells (Nourbakhsh et al., 2011) and myocytes (Kerkis et al., 2008) *in vitro* and *in vivo*. SHED express STRO-1 and CD146, two MSC markers also present in DPSC, but SHED exhibited higher proliferation rates than DPSC. Another difference between



**Fig. 3.** Chromosome analysis after exposure to GIC eluates. Representative images of chromosomes from untreated cells and cells exposed to Fuji I eluates with no observed chromosome aberrations.



SHED and DPSC is the expression of markers of embryonic stem cells (Oct4, Nanog, SSEA-3 and SSEA-4) on SHED (Kerkis et al., 2006). SHED cells have been also applied for tissue engineering in large animals, including bone defects, muscular dystrophy and dentin defect (Zheng et al., 2009; Zheng et al., 2012), as well as in small animal models for bone defect and spinal cord injury (Seo et al., 2008, de Mendonça Costa et al., 2009, Sakai et al., 2012).

Glass ionomer cements are widely used for crown cementation, intracoronary restoration, in the orthodonty, cavity lining, etc. However, there is much evidence of their toxic effects. GICs contain organic monomers and different ions that can diffuse through the dentin tubules and reach the pulp tissue. These molecules can affect the vitality of the odontoblasts and interfere with pulp homeostasis and healing (Bouillaguet, 2006, Teti et al., 2009). Resin-modified glass ionomer can cause the inhibition of cell growth and differentiation *in vitro* (Imazato et al., 2009., dos Santos et al., 2012), while *in vivo* studies have indicated their low biocompatibility, Vitrebond induced necrosis and intense inflammatory infiltrate (do Nascimento et al., 2000).

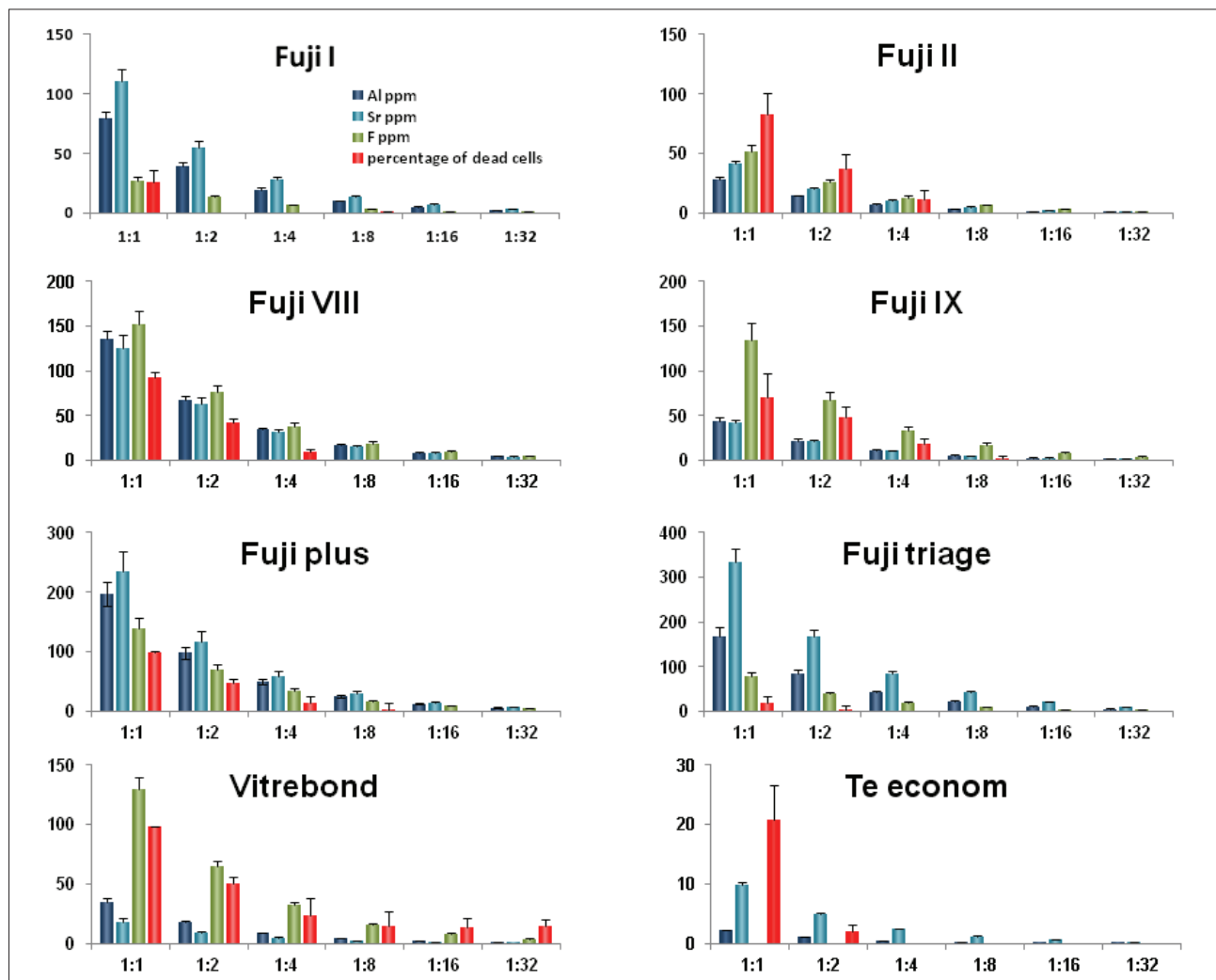
Our MTT assay results clearly showed the dose-dependent cytotoxic effect of GIC eluates on SHED cells. All tested GIC eluates in dilutions from 1:1 to 1:4 had toxic effects on SHED, while dilutions 1:8, 1:16 and 1:32 had no effect. Statistical analysis of the cytotoxic effects of eluates (dilution 1:1) showed the significantly stronger effect of Fuji Plus (98.5% dead cells), Vitrebond (97.92%), Fuji VIII (92.06%), Fuji IX (82.71%) and Fuji II (71.07%) on SHED than the control (Te-Econom composite, 20.76%), but also Fuji Triage (20.38%) and Fuji I (25.88%). Comparison of the effects of GIC eluates on

SHED and our previous results (Kanjevac et al., 2012) indicates that SHED is significantly more sensitive than DPSC. A statistically significant difference between the effects of GICs on SHED and DPSC was found for the eluates of Fuji II ( $p < 0.05$ ), Fuji VIII ( $p < 0.005$ ), Fuji plus ( $p < 0.005$ ) and Fuji Triage ( $p < 0.05$ ). These results are in line with previous reports about the *in vitro* cytotoxic effects of GICs (especially RMGIC) on odontoblasts (Aranha et al., 2006, de Souza Costa et al., 2003), fibroblasts (Souza et al., 2006., Thonemann et al., 2002), macrophages (Meryon et al., 1983), mouse lymphoma cell lines (Ribeiro et al., 2006a), dental pulp cells (Stanislawski et al., 1999; Kong et al., 2009) and mouse embryonic stem cells (Nguyen Ngoc et al., 2012) evaluated by MTT assay. The results of apoptosis assay are, in general, very similar to those obtained with MTT assay. Slight differences between the percentages of dead cells measured by MTT assay and late apoptotic cells measured by flow cytometry are results of the different assay principles. Enzymatic activity is measured by MTT assay, while flow cytometry of cells stained with Annexin V and propidium iodide detects changes in the cell membrane.

Eluates of Fuji II, Fuji VIII, Fuji Triage and Vitrebond inhibited mitosis in the remaining live cells. Two days after exposure to GICs, no mitosis was detected. In the remaining cells exposed to eluates of Fuji I, Fuji IX, Fuji plus and Te-Econom composite there was normal mitotic activity with no detected chromosome aberrations. There are no literature data about the effects of GICs on chromosome aberrations in SHED cells, but the genotoxic effects of some GICs were found in Chinese hamster ovary (CHO) cells (Ribeiro et al., 2006b), normal cultured human lymphocytes (Bakopoulou et al., 2009) and mouse fibroblasts (Angelieri et al., 2012).

There is a strong correlation between the fluoride ion concentration in eluates and the cytotoxic effect on SHED (measured by MTT assay) of these eluates (Pearson's correlation coefficient ( $r = 0.815$ ,  $p < 0.05$ )). This is in line with our previous report that GIC-induced SHED necrosis correlates with fluoride concentration (Kanjevack et al., 2011). Aluminum concentra-

tion in the eluates was highest for Fuji plus, Fuji triage and Fuji VIII, while the lowest aluminum concentrations were found for composite, Fuji II, Vitrebond, Fuji IX and Fuji I. These results are in correlation with previous results (Vieira et al., 1999; Nicholson and Czarnecka, 2009; Billington et al., 2006; Kovarik et al., 2005). There is no correlation between the concentration



**Fig. 4.** Cytotoxic effect of GIC eluates on SHEDs correlates with the coupled concentrations of fluoride, aluminum and strontium. Bars represent the percentages of cytotoxicity (MTT assay) of GIC eluates in different dilutions and concentrations of F (measured by HPLC), Al and Sr (measured by ICP-OES) for each GIC in appropriate dilution. Concentrations of F, Al and Sr in medium only were below detectable levels. There is a strong correlation between the concentration of released F ions and the cytotoxic effect of GIC eluates on SHEDs (Pearson's correlation coefficient ( $r = 0.815$ ,  $p < 0.05$ )) measured by the MTT assay. However, a stronger correlation existed between SHED cytotoxicity and the coupled concentrations of released aluminum, strontium and fluoride ions ( $r = 0.900$ ).

of Al ions and the cytotoxicity of SHED. Similar results were found for strontium concentrations, i.e. there is no correlation with cytotoxicity. The lowest concentrations were found in composite, Vitrebond, Fuji II and Fuji IX eluates while highest concentrations were found in Fuji triage, Fuji plus, Fuji VIII and Fuji I eluates. Concentrations of Sr ions released in the given time and volume are in line with previous reports (Kudalkar and Damle, 1997). However, there is significant correlation between coupled concentrations of fluoride, aluminum and strontium and the percentage of dead SHED, and the strongest correlation was found for the coupled concentration of fluoride and strontium ( $r = 0.900$ ).

The concentrations of released fluoride in GIC eluates were several times higher than concentrations proven to be cytotoxic. Although the released fluoride concentration declines over time (Markovic et al., 2008), the fluoride concentrations in saliva after GIC restoration are several times higher in comparison to salivary concentrations in a control group (Seo et al., 2008; Koch et al., 1990; Chatzistavrou et al., 2010).

## CONCLUSION

The results of our study indicate the potential harmful effects of GICs on SHED, the cells that could be isolated from stored pulp and could be used in cell-based therapy in a syngeneic host. These results indicate caution in the usage of GICs in restorative dentistry, especially if used near pulp tissue.

**Acknowledgments:** This study was supported by Ministry of Science and Technology, Republic of Serbia (grants: ON175069, ON175071 and ON175103) and by Faculty of Medical Sciences, University of Kragujevac (grant: JP26/10).

**Authors' contributions:** Conceived and designed the experiments: TK MM AL. Performed the experiments: TK, MM, ZT, OMDJ, MJ. Analyzed the data: MM TK. Contributed reagents/materials/analysis tools: OMDJ ZT. Wrote the paper: TK MM.

**Conflict of interest disclosure:** The authors have declared that no competing interests exist.

## REFERENCES

- Angelieri, F., Joias, R.P., Bresciani, E., Noguti, J. and D.A. Ribeiro (2012). Orthodontic cements induce genotoxicity and cytotoxicity in mammalian cells in vitro. *Dent. Res. J. (Isfahan)*. **9**(4), 393-398.
- Aranha, A.M., Giro, E.M., Souza, P.P., Hebling, J. and C.A. de Souza Costa (2006). Effect of curing regime on the cytotoxicity of resin-modified glass-ionomer lining cements applied to an odontoblast-cell line. *Dent. Mater.* **22**(9), 864-869.
- Bakopoulou, A., Mourelatos, D., Tsiftoglou, A.S., Giassin, N.P., Mioglou, E. and P. Garefis (2009). Genotoxic and cytotoxic effects of different types of dental cement on normal cultured human lymphocytes. *Mutat. Res.* **672**(2), 103-112.
- Billington, R.W., Williams, J.A. and G.J. Pearson (2006). Ion processes in glass ionomer cements. *J Dent.* **34**(8), 544-555.
- Bouillaguet, S. (2004). Biological risks of resin-based materials to the dentin-pulp complex. *Crit. Rev. Oral. Biol. Med.* **15**, 47-60.
- Briso, A.L.F., Rahal, V., Mestreneur, S.R. and E. Jr. Dezan (2006). Biological response of pulps submitted to different capping materials. *Braz. Oral. Res.* **20**(3), 219-225.
- Chatzistavrou, E., Eliades, T., Zinelis, S., Athanasiou, A.E. and G. Eliades (2010). Fluoride release from an orthodontic glass ionomer adhesive in vitro and enamel fluoride uptake in vivo. *Am. J. Orthod. Dentofacial. Orthop.* **137**(4), 458.e1-8.
- de Mendonça Costa, A., Bueno, D.F., Martins, M.T., Kerkis, I., Kerkis, A., Fanganiello, R.D., Cerruti, H., Alonso, N. and M.R. Passos-Bueno (2009). Reconstruction of large cranial defects in nonimmunosuppressed experimental design with human dental pulp stem cells. *J. Craniofac. Surg.* **19**, 204-210.
- de Souza Costa, C.A., Hebling, J., Garcia-Godoy, F. and C.T. Hanks (2003). In vitro cytotoxicity of five glass-ionomer cements. *Biomaterials.* **24**(21), 3853-3858.
- Diaz-Arnold, A.M., Holmes, D.C., Wistrom, D.W. and E.J. Jr Swift (1995). Short-term fluoride release/uptake of glass ionomer restoratives. *Dent. Mater.* **11**, 96-101.
- do Nascimento, A.B., Fontana, U.F., Teixeira, H.M. and C.A. Costa (2000). Biocompatibility of a resin-modified glass-ionomer cement applied as pulp capping in human teeth. *Am. J. Dent.* **13**, 28-34.

- dos Santos, R.L., Pithon, M.M., Martins, F.O., Romanos, M.T. and A.C. Ruellas (2012). Evaluation of cytotoxicity and degree of conversion of glass ionomer cements reinforced with resin. *Eur J Orthod.* **34**(3), 362-366.
- Foreman, P.C. and Barnes, I.E. (1993). A review of calcium hydroxide. *Int. Endod. J.* **23**(6), 283-297.
- Forss, H. (1993). Release of fluoride and other elements from light-cured glass ionomers in neutral and acidic conditions. *J. Dent. Res.* **72**(8), 1257-1262.
- Foudah, D., Redaelli, S., Donzelli, E., Bentivegna, A., Miloso, M., Dalprà, L. and G. Tredici (2009). Monitoring the genomic stability of in vitro cultured rat bone-marrow-derived mesenchymal stem cells. *Chromosome. Research.* **17**, 1025-1039.
- Imazato, S., Horikawa, D., Nishida, M. and S. Ebisu (2009). Effects of monomers eluted from dental resin restoratives on osteoblast-like cells. *J. Biomed. Mater. Res. B. Appl. Biomater.* **88**, 378-386.
- Kanjevac, T., Milovanović, M., Volarević, V. and N. Arsenijević (2011). Fluoride release from glass ionomer cements correlates with necrotic death of human dental pulp stem cells. *Ser. J. Exp. Clin. Res.* **12**(2), 67-70.
- Kanjevac, T., Milovanovic, M., Volarevic, V., Lukic, M.L., Arsenijevic, N., Markovic, D., Zdravkovic, N., Tesic, Z. and A. Lukic (2012). Cytotoxic effects of glass ionomer cements on human dental pulp stem cells correlate with fluoride release. *Med. Chem.* **8**(1), 40-45.
- Kerkis, I., Ambrosio, C.E., Kerkis, A., Martins, D.S., Zucconi, E., Fonseca, S.A., Cabral, R.M., Maranduba, C.M., Gaiad, T.P., Morini, A.C., Vieira, N.M., Brolio, M.P., Sant'Anna, O.A., Miglino, M.A. and M. Zatz (2008). Early transplantation of human immature dental pulp stem cells from baby teeth to golden retriever muscular dystrophy (GRMD) dogs: Local or systemic? *J. Transl. Med.* **6**, 35.
- Kerkis, I., Kerkis, A., Dozortsev, D., Stukart-Parsons, G.C., Gomes Massironi, S.M., Pereira, L.V., Caplan, A.I. and H.F. Ceruti (2006). Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers. *Cells. Tissues. Organs.* **184**, 105-116.
- Koch, G. and S. Hatibović-Kofman (1990). Glass ionomer cements as a fluoride release system in vivo. *Swed. Dent. J.* **14**(6), 267-273.
- Kong, N., Jiang, T., Zhou, Z. and J. Fu (2009). Cytotoxicity of polymerized resin cements on human dental pulp cells in vitro. *Dent. Mater.* **25**(11), 1371-1375.
- Kovarik, R.E., Haubenreich, J.E. and D. Gore (2005). Glass ionomer cements: a review of composition, chemistry, and biocompatibility as a dental and medical implant material. *J. Long. Term. Eff. Med. Implants.* **15**(6), 655-671.
- Kudalkar, S.A. and S.G. Damle (1997). The comparative evaluation of release profiles of aluminum, fluoride, sodium and strontium by resin modified and conventional glass polyalkenoate cements in neutral and acidic medium--an in vitro study. *J. Indian. Soc. Pedod. Prev.* **15**(1), 1-9.
- Ma, L., Makino, Y., Yamaza, H., Akiyama, K., Hoshino, Y., Song, G., Kukita, T., Nonaka, K., Shi, S. and T. Yamaza (2012). Cryopreserved dental pulp tissues of exfoliated deciduous teeth is a feasible stem cell resource for regenerative medicine. *PLoS. One.* **7**(12), e51777. <http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0051777>
- Maijer, R. and D.C. Smith (1998). A comparison between zinc phosphate and glass ionomer cement in orthodontics. *Am. J. Orthod. Dentofacial. Orthop.* **93**, 273-279.
- Markovic, D.Lj., Petrovic, B.B. and T.O. Peric (2008). Fluoride content and recharge ability of five glassionomer dental materials. *BMC. Oral. Health.* **8**, 21.
- Meryon, S.D., Stephens, P.G. and R.M. Browne (1983). A comparison of the in vitro cytotoxicity of two glass-ionomer cements. *J. Dent. Res.* **62**(6), 769-73.
- Miura, M., Gronthos, S., Zhao, M., Lu, B., Fisher, L.W., Robey, P.G. and S. Shi (2003). SHED: Stem cells from human exfoliated deciduous teeth. *Proc. Natl. Acad. Sci. USA.* **100**, 5807-5812 (2003).
- Modena, K.C., Ckasas-Apayco, L.C., Atta, M.T., Costa, C.A., Hebling, J., Sipert, C.R., Navarro, M.F. and C.F. Santos (2009). Cytotoxicity and biocompatibility of direct and indirect pulp capping materials. *Journal. of Applied. Oral. Science.* **17**, 544-554.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* **65**(1-2), 55-63.
- Nguyen Ngoc, T.D., Son, Y.O., Lim, S.S., Shi, X., Kim, J.G., Heo, J.S., Choe, Y., Jeon, Y.M. and J.C. Lee (2012). Sodium fluoride induces apoptosis in mouse embryonic stem cells through ROS-dependent and caspase- and JNK-mediated pathways. *Toxicol. Appl. Pharmacol.* **259**(3), 329-337.
- Nicholson, J.W. and B. Czarnecka (2009). Review paper: Role of aluminum in glass-ionomer dental cements and its biological effects. *J. Biomater. Appl.* **24**(4), 293-308.
- Nourbakhsh, N., Soleimani, M., Taghipour, Z., Karbalaie, K., Mousavi, S.B., Talebi, A., Nadali, F., Tanhaei, S., Kiyani, G.A., Nematollahi, M., Rabiei, F., Mardani, M., Bahramiyan, H., Torabinejad, M., Nasr-Esfahani, M.H. and H. Baharvand (2011). Induced in vitro differentiation of neural-like cells from human exfoliated deciduous teeth-derived stem cells. *Int. J. Dev. Biol.* **55**, 189-195.
- Porada, C.D. and Almeida-Porada (2010). Mesenchymal stem cells as therapeutics and vehicles for gene and drug delivery. *Adv. Drug. Deliv. Rev.* **62**, 1156-1166.
- Ribeiro, D.A., Marques, M.E. and D.M. Salvadori (2006 a). Biocompatibility of glass-ionomer cements using mouse lymphoma cells in vitro. *J. Oral. Rehabil.* **33**(12), 912-917.
- Ribeiro, D.A., Marques, M.E. and D.M. Salvadori (2006 b). Genotoxicity and cytotoxicity of glass ionomer cements on Chinese hamster ovary (CHO) cells. *J. Mater. Sci. Mater. Med.* **17**(6), 495-500.
- Sakai, K., Yamamoto, A., Matsubara, K., Nakamura, S., Naruse, M., Yamagata, M., Sakamoto, K., Tauchi, R., Wakao, N., Imagama, S., Hibi, H., Kadomatsu, K., Ishiguro, N. and M.



- Ueda (2012). Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. *J. Clin. Invest.* **122**, 80-90.
- Sakai, V.T., Zhang, Z., Dong, Z., Neiva, K.G., Machado, M.A., Shi, S., Santos, C.F. and J.E. Nör (2010). SHED differentiate into functional odontoblasts and endothelium. *J. Dent. Res.* **89**, 791-796.
- Seo, B.M., Sonoyama, W., Yamaza, T., Coppe, C., Kikuri, T., Akiyama, K., Lee, J.S. and S. Shi (2008). SHED repair critical-size calvarial defects in immunocompromised mice. *Oral. Dis.* **14**, 428-434.
- Shen, C. and L. Autio-Gold (2002). Assessing fluoride concentration uniformity and fluoride release from three varnishes. *J. Am. Dent. Assoc.* **133**(2), 176-182.
- Souza, P.P., Aranha, A.M., Hebling, J., Giro, E.M. and C.A. Costa (2006). In vitro cytotoxicity and in vivo biocompatibility of contemporary resin-modified glass-ionomer cements. *Dent. Mater.* **22**(9), 838-844.
- Stanislawski, L., Daniau, X., Lauti, A. and M. Goldberg (1999). Factors responsible for pulp cell cytotoxicity induced by resin-modified glass ionomer cements. *J. Biomed. Mater. Res.* **48**(3), 277-288.
- Stanley, H.R. and C.H. Pameijer (1997). Dentistry's friend: calcium hydroxide. *Oper. Dent.* **22**(1), 1-3.
- Stanley, H.R., Swerdlow, H. and M.G. Buonocore (1967). Pulp reactions to anterior restorative materials. *J. Am. Dent. Assoc.* **75**, 132-141.
- Teti, G., Mazzotti, G., Zago, M., Ortolani, M., Breschi, L., Pelotti, S., Ruggeri, A. and M. Falconi (2009). HEMA down-regulates procollagen alpha1 type I in human gingival fibroblasts. *J. Biomed. Mater. Res.* **90**(1), 256-62.
- Thonemann, B., Schmalz, G., Hiller, K.A. and H. Schweikl (2002). Responses of L929 mouse fibroblasts, primary and immortalized bovine dental papilla-derived cell lines to dental resin components. *Dent. Mater.* **18**(4), 318-323.
- Vieira, A.R., de Souza, I.P. and A. Modesto (1999). Fluoride uptake and release by Composites and glass ionomers in a high caries challenge situation. *Am. J. Dent.* **12**(1), 14-18.
- Wilson, A.D. and B.E. Kent (1971). The glass ionomer cement, a new translucent dental filling material. *Journal of applied chemistry & biotechnology.* **21**, 313.
- Xie, D., Brantley, W.A., Culbertson, B.M. and G. Wang (2000). Mechanical properties and microstructures of glass-ionomer cements. *Dent. Mater.* **16**, 129-138.
- Yamaza, T., Kentaro, A., Chen, C., Liu, Y., Shi, Y., Gronthos, S., Wang, S. and S. Shi (2010). Immunomodulatory properties of stem cells from human exfoliated deciduous teeth. *Stem. Cell. Res. Ther.* **1**(1), 5.
- Yan, X., Qin, H., Qu, C., Tuan, R.S., Shi, S. and G.T. Huang (2010). iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. *Stem. Cells. Dev.* **19**(4), 469-480.
- Zheng, Y., Liu, Y., Zhang, C.M., Zhang, H.Y., Li, W.H., Shi, S., Le, A.D. and S.L. Wang (2009). Stem cells from deciduous tooth repair mandibular defect in swine. *J. Dent. Res.* **88**, 249-254.
- Zheng, Y., Wang, X.Y., Wang, Y.M., Liu, X.Y., Zhang, C.M., Hou, B.X. and S.L. Wang (2012). Dentin regeneration using deciduous pulp stem/progenitor cells. *J. Dent. Res.* **91**, 676-682.