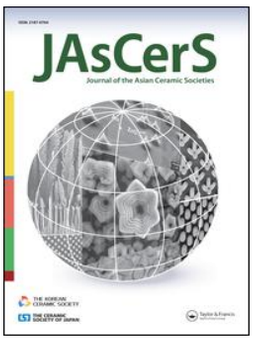

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In vitro evaluation of tooth-colored yttria stabilized zirconia ceramics

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ABSTRACT

Effects of $MoCl_3$ and $NiCl_2$, originally incorporated as coloring agent, on the cellular response of 3 mol% yttria stabilized zirconia (3Y-TZP) ceramics was investigated. $MoCl_3$ and $NiCl_2$-$MoCl_3$ incorporated, tooth-colored 3Y-TZP ceramics were produced through cold isostatic pressing at 100 MPa followed by pressureless sintering at 1450°C for 2 h. Aging was performed on the sintered ceramics using distilled water in a reactor at 134°C at 2.3 bar pressure for 2 h. The phases developed during different stages of processing were identified by X-ray diffraction (XRD) analysis. In vitro cell culture studies were carried out using L929 fibroblast cell line. The cell viability and proliferation studies revealed that none of the specimens showed cytotoxicity with respect to coloring. Confocal laser scanning microscope (CLSM) analyses suggested that all of the specimens exhibited good in vitro cytocompatibility. Enhancement in cell attachment, adhesion, and proliferation was observed in all specimens via scanning electron microscope (SEM) analysis. Although the coloring process did not improve the proliferation performance of the aged specimens, the incorporation of transition metals enhanced the in vitro performance of 3Y-TZP ceramics.

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Y_2O_3; ZrO_2; cell culture; tooth; biomedical applications

1. Introduction

In the last two decades, zirconia (ZrO_2) ceramics produced from tetragonal ZrO_2 polycrystals stabilized with 3 mol % yttria (Y_2O_3; 3Y-TZP ceramics) have received great attention as biomaterials due to their exceptional physical, mechanical, and biological properties as well as superior esthetics [1–4]. As a result of the advancements in CAD-CAM technology, 3Y-TZP ceramics are confidently used in applied dentistry [5–8]. Nontoxicity to the surrounding tissues makes ZrO_2 ceramics promising biomaterial candidates for prosthetic dentistry and dental implantology applications [9,10]. In addition, 3Y-TZP ceramics can meet the specifications of ISO standards for surgical applications to be used for dental restorations [11]. In spite of its exceptional biological properties, the white color of ZrO_2 is a serious esthetic disadvantage [12]. The appearance of a natural tooth is difficult to obtain even after the veneering process [13]. Therefore, 3Y-TZP ceramics are frequently shaded to mimic the tone of natural teeth. The hue of the dental zirconia block can be tailored by adding a suitable coloring agent into zirconia powder [14] or by immersing the block in a color solution [15]. The quality of color families was distinguished from each other by the hue [16]. Therefore, different metal salts have been tried for esthetic coloration of ceramic crowns. For instance, Shah *et al.* [6] investigated the effect of $BiCl_3$ and $CeCl_3$ on 3Y-TZP. Chen *et al.* [17] evaluated the coloration of zirconia ceramics by Fe_2O_3, Bi_2O_3, and CeO_2.

Recently, Kaplan *et al.* [15] have published the features of the tooth-colored 3Y-TZP ceramics with improved LTD properties along with mechanically and esthetically acceptable properties, for dental applications. The esthetic appearances of ceramics are improved by infiltration of color solutions. However, the incorporation of coloring agents might cause adverse effects on its biological properties. Although transition metal dopants provide required mechanical properties and biocompatibility to gingival fibroblast [18], several studies [19–26] on mechanical properties have achieved limited success in the extension of the service life of 3Y-TZP ceramics. Hence, in vitro studies are vital for biomedical applications to predict the performance of tooth-colored 3Y-TZP ceramics in service.

Biocompatibility of materials is a prerequisite for the purpose of tissue integration in the living organism, assessment of that may be carried out by the cellular reaction via cell culture studies and various cellular

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assays. A number of studies have been performed to improve cell adhesion, proliferation, and differentiation for tissue regeneration [27–29]. Cell attachment is the preliminary step for cell proliferation, differentiation, and maturation [30]. The zirconia implants performed well in vitro cytocompatibility in terms of osteoblast attachment, proliferation, and differentiation on the surfaces [4,9,27–30]. A detailed in vitro cytocompatibility study of the 3Y-TZP ceramics infiltrated with nickel chloride ($NiCl_2$), molybdenum chloride ($MoCl_3$), and $NiCl_2$+ $MoCl_3$ solutions, and subsequently aged at isothermal conditions is necessary to understand the effects of $NiCl_2$ and $MoCl_3$ as coloring agents on the tissue integration of 3Y-TZP ceramics.

This study was carried out to understand the effects of $NiCl_2$ and $MoCl_3$, used as coloring agents, on the cellular response of 3Y-TZP ceramics. The objective was to enhance the ability of cell attachment and proliferation of the natural tooth-colored 3Y-TZP ceramics through the infiltration of coloring solutions.

2. Materials and methods

This work is a continuation of the investigation in which the effects of $MoCl_3$ and $NiCl_2$ solutions on coloring and aging of 3Y-TZP ceramics has been studied. The reader is referred to reference 15 for the details on the preparation of the specimens.

2.1. Specimen preparation

The 3Y-TZP powders (Tosoh Co., Code: TZ-3YB-E) were dry pressed uniaxially at a pressure of 25 MPa for the compaction. Nominal dimensions of the disc-shaped compacts were 20 mm in diameter and 4 mm in thickness. Then, the disc was compressed by applying an isostatic pressure of 100 MPa. After that, the isostatically pressed discs were pre-sintered in air at 950°C for 1 h. The heating and cooling rates were 3°C/min and 5°C/min, respectively.

$MoCl_3$ (99.95% purity) and $NiCl_2$ (98% purity) powders, supplied from Sigma-Aldrich, were used for coloring agents. Predetermined amounts of $MoCl_2$ and $NiCl_2$ -$MoCl_3$ powders were dissolved in distilled water (100 mL) to prepare the coloring solutions. Shade on the pre-sintered compacts was accomplished by immersing the specimens into 0.1 wt % $MoCl_3$ and 0.25 wt % $NiCl_2$-$MoCl_3$ solutions for 5 s. After shading, the infiltrated compacts were kept in an oven at 100°C overnight for drying. The dried compacts were finally sintered in air at 1450°C for 2 h. The heating and cooling rates applied during pressureless sintering were 5°C/min.

Table 1. Specimen codes.

Specimen Code	Color Solution Concentration (wt %)	Aging Time (h)
Control	None	-
0.1Mo	0.1% $MoCl_3$	-
0.25NiMo	0.25% [$NiCl_2$ + $MoCl_3$]	-
Control-A	None	2
0.1Mo-A	0.1% $MoCl_3$	2
0.25NiMo-A	0.25% [$NiCl_2$ + $MoCl_3$]	2

2.2. Aging

A test for aging was performed in accord with ISO 13,356 standard [31]. Aging was carried out in distilled water in a reactor (ITO Instruments® I max). A 2.3 bar of water vapor pressure was applied to the specimens at 134°C for 2 h for accelerated aging. In addition to five colored specimens, an uncolored specimen was also aged at the same conditions for comparison purpose. Specimens were coded regarding the type of coloring agent concentration used and application of aging as indicated in Table 1. Henceforth, the specimens will be referred to as the codes given in Table 1.

2.3. Characterization

The sensitivity of the specimens to aging was characterized by X-ray diffraction (XRD) analysis employing an XRD diffractometer (AXS D8 Advance A25, Bruker). The phases present in the samples were detected in 2θ range of 20°-70° at the scanning rate of 2 °/min with CuKα radiation at 40 kV and 40 mA. The XRD patterns were taken at 2θ range of 23°-35° at a scanning rate of 0.5 °/min for the calculation of the amount of monoclinic phase in the specimens. The percentages of phase alteration from tetragonal to monoclinic, which took place inside specimens as a result of aging, were calculated using the method reported by Garvie and Nicholson [32]. The monoclinic and tetragonal peaks diffracted from m(−111), m(111), and t(101) planes, respectively were taken into account for the calculations.

Attachment, growth, and cellular morphology were examined by scanning electron microscope (SEM, Zeiss Ultra SEM) after the 3rd and 7th days of the cultivation of cells. SEM images were taken at 1000X. A confocal laser scanning microscope (CLSM, Zeiss LSM 510, Carl Zeiss, USA) was employed to take the CSLM images.

2.3.1. Cell culture studies

The cellular proliferation and attachment were examined by culturing L929 ATCC CCL-1 mouse fibroblast cells. The disc-shaped specimens were placed in 24-well plate petri dishes and exposed to ultraviolet (UV) radiation for 1 h to ensure sterilization. The cell viability and proliferation studies were performed for a period of 7 days with a cell seeding concentration of 5×10^5 cell/ml in a culture medium that consisted of DMEM/

F12 + 10% (v/v) FBS + 1% (v/v) L-glutamine + 1% (v/v) penicillin/streptomycin (100 units/ml penicillin, 100 µg/ml streptomycin). The petri dishes containing the cultured cells on the specimens were kept in an incubator at 37°C, 5% CO_2. Bovine serum albumin (BSA, Amresco, USA), phosphate buffer saline (PBS, Amresco, USA), Dulbecco's modified Eagle medium (DMEM/F12, Sigma Aldrich, USA), Triton X-100 (Sigma Aldrich, USA), DRAQ5 (Sigma Aldrich, USA), ethanol (96%, v/v), penicillin/streptomycin (Sigma Aldrich, USA), fetal bovine serum (FBS, Sigma Aldrich, USA), glutaraldehyde (Sigma Aldrich, USA), L-glutamine (Sigma Aldrich, USA) and Alexa Fluor 488 phalloidin (AF-488, Life Technologies, USA) were chemicals used for the cell culture studies.

2.3.2. Cell attachment studies

The cellular attachment was accomplished in TCPS (Tissue Culture Polystyrene) to investigate the adhesion behavior of cells to specimens. The adhesion performance was evaluated by using hemocytometry counting technique for 3 h at selected intervals of 30, 60, 90, 120, 150, and 180 min. Unattached cells were removed by discarding the medium from each well. The attached cells were harvested after incubation for 15 min in a Trypsin/EDTA solution at 0.1% (w/v). The remaining cells were counted for each interval by trypan blue dye exclusion technique.

2.3.3. MTT assay

Cellular viability was examined by using tetrazolium salt3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the in vitro cytocompatibility of specimens was tested during 7 days with defined time points. For MTT assay, the specimens were placed in 24-well plate and cells were seeded at the top of the materials. Before the analysis at each time points, the medium was discarded, and the specimens were rinsed 3 times with PBS. Then, 600 µl medium with MTT solution was added and incubated for 3 h. 1 mL dimethylsulfoxide (DMSO) was added to the medium-MTT solution to dissolve the formazan and incubated for 1 h. 200 µL solution from each well was placed in a 96-well plate. Quantification of the cellular viability was conducted by measuring the absorbance at 540 nm using a microplate reader (Dynamical LEDETECT96). Each measurement was repeated three times.

2.3.4. Confocal laser scanning microscope (CLSM) analysis

In order to examine cell adhesion and proliferation on the Control, 0.1Mo, and 0.25NiMo, fibroblasts were stained and analyzed by using CLSM at day 3 and day 7. Before each staining, medium was removed, rinsed 3 times with PBS, and fixed with 2.5% (v/v) glutaraldehyde for 30 min. Then, cells were permeabilized with 0.1% Trion X-100 for 5 min and blocked with BSA for 30 min. Before imaging, staining was performed with AF-488 for 20 min and DRAQ5 for 10 min. The excitation and the emission wavelengths used for CLSM images were λ_{ex}: 488 nm, λ_{em}: 500–550 nm, and λ_{ex}: 635–640 nm, λ_{em}: 655–700 nm for AF-488, and DRAQ5, respectively.

2.4. Statistical analysis

All in vitro studies were carried out in triplicate data sets (n = 3) and analyzed by one-way analysis of variance (ANOVA) followed by Tuckey's test using IBM SPSS version 23.0 statistics software. The significance level was taken $p < 0.05$ and data were labeled with (*) for $p < 0.05$ and (**) for $p < 0.01$.

3. Results

The appearances of the uncolored and colored sintered 3Y-TZP ceramics are given in Figure 1. The results of the color measurements for the specimens were presented in the previous publication [15]. XRD patterns of the specimens, shown in Figure 2, present the phases developed in the 3Y-TZP ceramics after each treatment. The XRD peaks were located at 2θ of 30.7°, 34.3°, 35.5°, 50.0°, 50.5°, 59.2°, 60.5°, and 63.2°

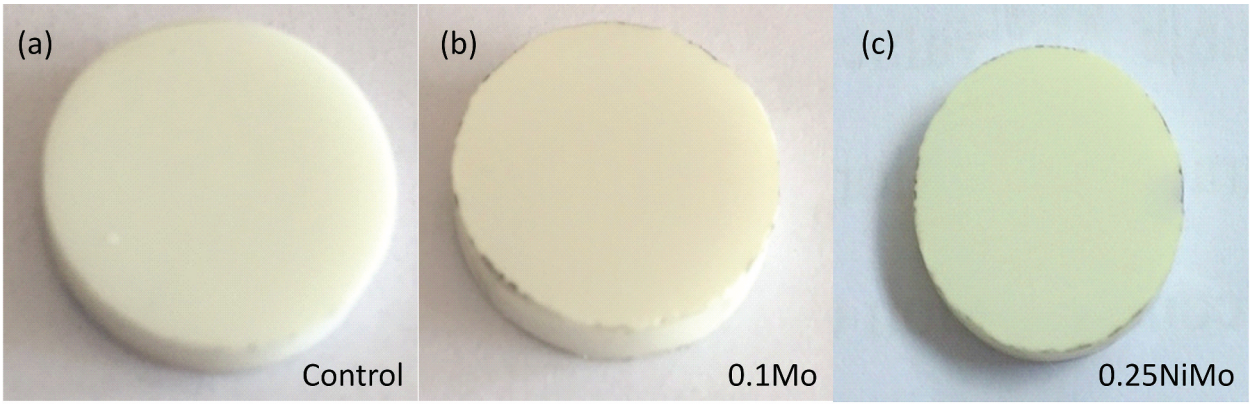

Figure 1. Appearances of the control and the colored yttria stabilized zirconia ceramics.

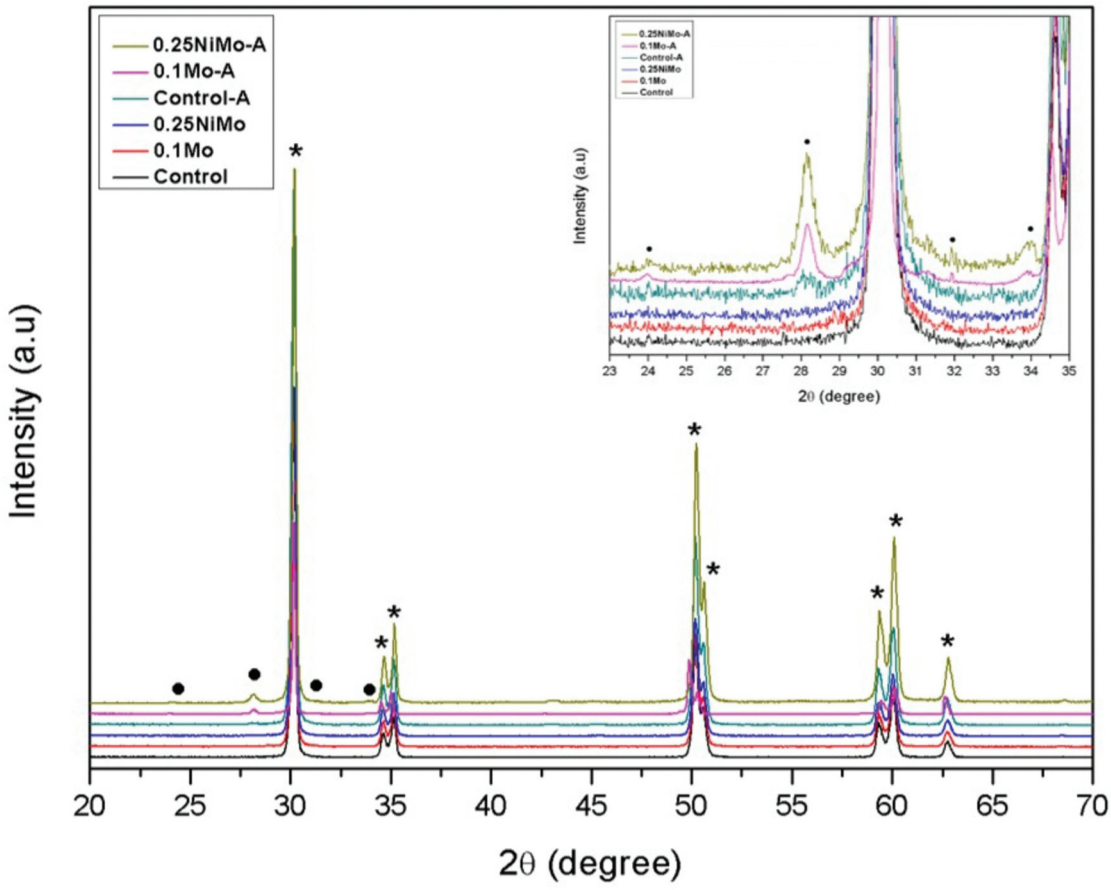

Figure 2. XRD patterns of the specimens. *Tetragonal, •Monoclinic.

belonging to the t(101), t(002), t(110), t(112), t(200), t(103), t(211), and t(202) planes, respectively. The finding suggests that tetragonal ZrO_2 (JCPDS 01–072-2743) existed in all of the specimens. The XRD peaks belonging to monoclinic ZrO_2 at 2θ of 24.2° and 28.3° corresponding to the m(100) and m(−111) were also detected in the specimens as shown in the inset in Figure 2.

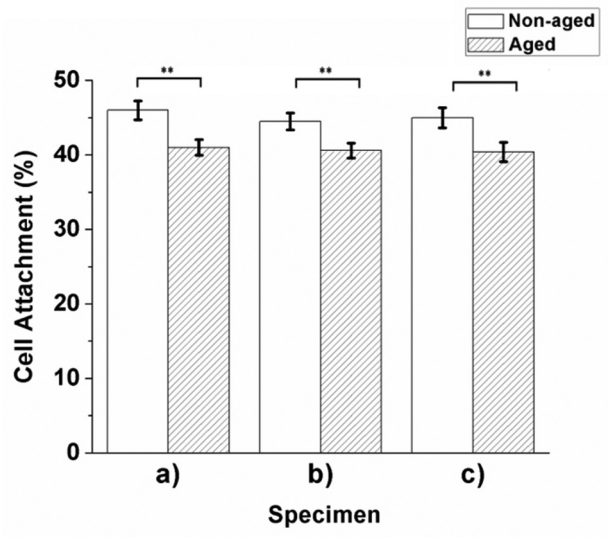

Figure 3. Percentage of cell attachment for the specimens (a) Control, (b) 0.1Mo, and (c) 0.25NiMo. The symbol "**" indicate the significant differences (**p < 0.01).

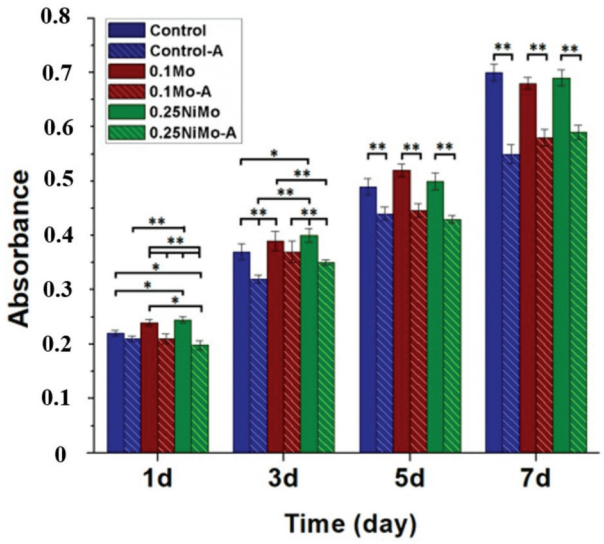

Figure 4. MTT assay of the specimens. * p < 0.05 and ** p < 0.01.

The percentages of the L929 fibroblast cells attached to the specimens after 3 h were shown in Figure 3. The cell viability and cytotoxicity on the specimens were measured by the MTT assay for 7 days. As shown in Figure 4, cell viability on all of the specimens

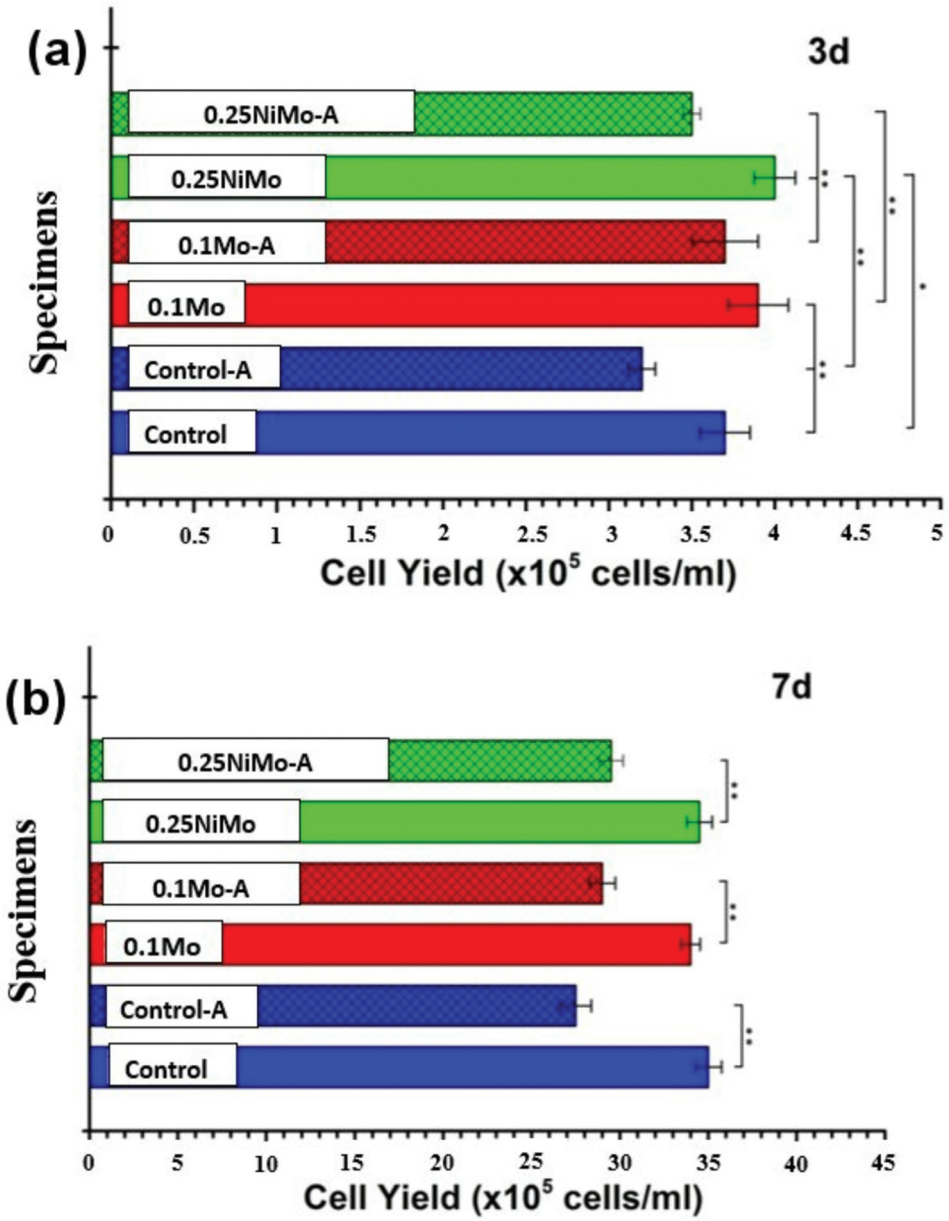

Figure 5. Cell yield of the specimens at the end of (a) 3rd day, and (b) 7th day of the culture. * $p < 0.05$ and ** $p < 0.01$.

exhibited a steady increase showing no evidence of cytotoxicity.

The cell yield on the specimens was measured at the 3rd and 7th days of the culture by MTT assay. As shown in Figure 5, the highest cell yield was obtained for unaged specimens. The cell yield was not affected by the infiltration solution. Figure 6 shows the SEM images of Control, 0.1Mo, and 0.25NiMo after cell culture durations. The CLSM images were taken after staining by AF-488/DRAQ5 on the 3rd and 7th days of the cultivation durations as shown in Figure 7.

4. Discussion

Monoclinic phase content of all of the specimens was calculated less than 2% before aging. In the XRD pattern of 0.1Mo-A and 0.25NiMo-A, additional peaks belonging to the monoclinic phase were detected at 2θ of 31.9° and 34.1° for m(111) and m (002), respectively due to the aging process. After the aging, the monoclinic phase content of the Control-A specimen remained almost the same, but increased to 3.38% and 4.46% for 0.1Mo-A and 0.25NiMo-A specimens, respectively. As a result of aging by the hydrothermal treatment, surface morphology has been affected as seen by rougher morphology and phase transformation. Similar findings were reported in a previous publication [33]. Non-aged specimens exhibited better performance than the aged specimens in terms of proliferation of the cells. However, among the aged specimens, a more monoclinic phase content of 0.25NiMo-A specimen resulted in better cell attachment than either Control-A or 0.1Mo-A specimens.

L929 fibroblast cells have attached to all of the specimens after a duration of 3 h. The lowest cell

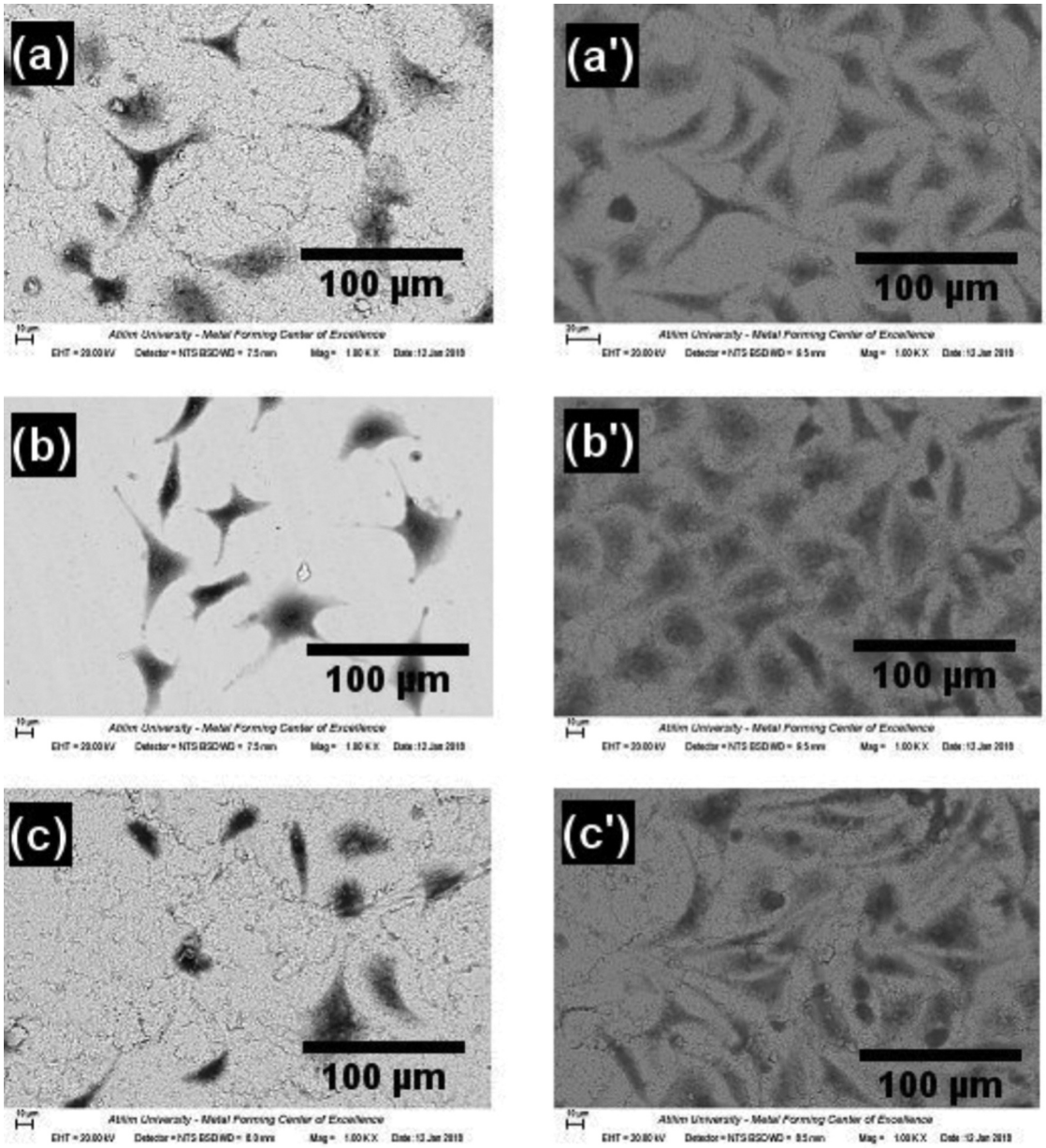

Figure 6. SEM images taken after cell culture durations of 3rd day (left) and 7th day (right) for (a)-(a') Control, (b)-(b') 0.1Mo, and (c)-(c') 0.25NiMo.

attachment with ~ 45% was observed for aged specimens. As shown in Figure 3, the infiltration of color solutions did not affect adhesion and proliferation the fibroblasts. Similar result was also reported previously by Ito *et al*. [34] who concluded that the commencement of cell adherence on micro and nano-topographies upon the specimen surface was identical.

The MTT assay was performed to calculate the cell viability and proliferation. On the basis of the capacity of mitochondrial dehydrogenase enzymes existing in living cells, viability and proliferation were evaluated [35]. At the end of the 7-day culture, Control, 0.1Mo, and 0.25NiMo specimens showed the highest cell viability. The findings are consistent with the cell attachment results. Although the incorporation of coloring agents did not influence the cellular adhesion and proliferation, the aging process decreased the proliferation of cells as also reported previously by Kilic *et al*. [36].

The effects of the aging process and the infiltration of shading solutions, as discussed above, show parallel relation to each other. None of the specimens indicated any cytotoxicity but, showed good in vitro cytocompatibility. It is evident that all of the specimens

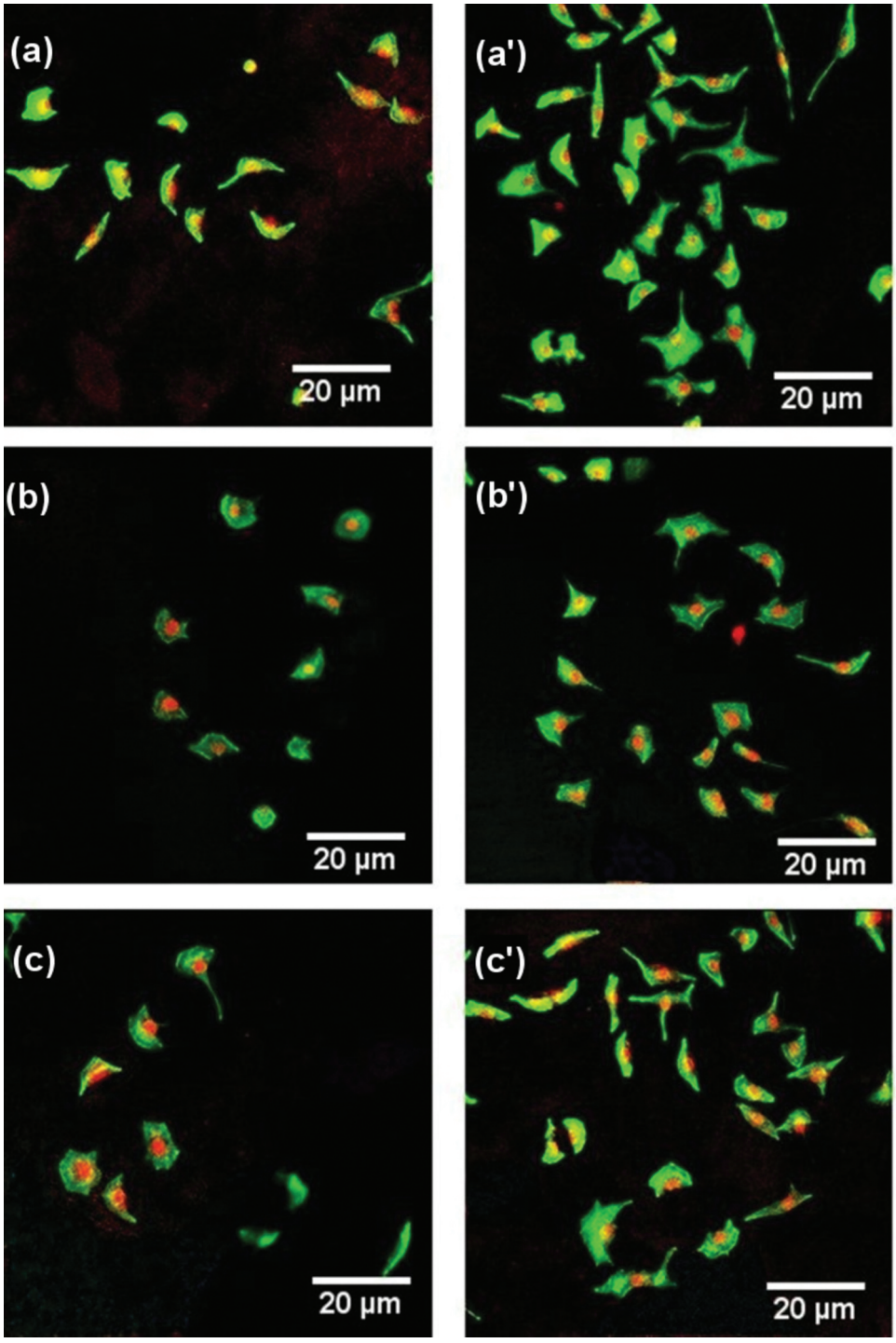

Figure 7. CLSM images taken after the culture test at 3rd day (left) and 7th day (right) for (a)-(a′) Control, (b)-(b′) 0.1Mo, and (c)-(c′) 0.25NiMo at 1000 X.

supported cell adhesion and growth at the end of 3rd and 7th days of the culture. Furthermore, cells tended to preserve their i-like structure on the specimens and to stretch their axons along with the specimens as seen in Figures. 6(a′), 6(b′) 6(c′).

Control, 0.1Mo, and 0.25NiMo specimens showed improvement in cell attachment, adhesion, proliferation and spreading efficiency on 7th day of the cultivation compared to the 3rd day as shown in Figure 7. In addition, all of the specimens exhibited good in vitro cytocompatibility in CLSM analysis.

5. Conclusions

For the long-term esthetic success of the dental restoration, the color stability and aging process is an essential controlling aspect. One of the necessary requirements for restorative materials is that it

maintains color stability over time in the oral environment. The incorporation of coloring agents has a promising effect on the cellular response of sintered and hydrothermally aged 3Y-TZP ceramics. Cytotoxic effect was not detected in the colored 3Y-TZP ceramics. However, aging causes a decrease in the proliferation of cells. This study confirms that shading by transition metal solutions does not influence the adhesion and proliferation of gingival fibroblasts and enhances the in vitro performance of 3Y-TZP ceramics. Therefore, tooth colored 3Y-TZP ceramics could be utilized as a promising dental restorative material.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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