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Improved BALB/c mice granulosa cell functions using purified alginate scaffold

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Summary

Alginate, a non-toxic polysaccharide isolated from brown algae, is a widely used 3-dimensional (3D) porous scaffold for the granulosa cell and follicle encapsulation. However, impurities in commercial alginate can lead to alginate biocompatibility reduction. The aim of this study was to evaluate *in vitro* behavior of the granulosa cells seeded on the purified alginate in varying concentrations compared with matched non-purified ones. We produced a purified alginate using a simple and efficient method. Then, the granulosa cells from mice were isolated and seeded in various concentrations of (0.5%, 1% weight/volume) purified and non-purified alginate. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used on the 3rd, 5th and the 8th days of culture as an index of cell viability and proliferation. Furthermore, the secreted estradiol, progesterone and alkaline phosphatase enzyme (ALP) were measured in the granulosa cells culture media using radioimmunoassay kits. The cells cultured on purified and low concentration alginate showed a higher proliferation rate, sex hormone production and ALP activity. The results confirmed the impact of the alginate hydrogel properties on proliferative rate and function of granulosa cells in a 3D culture system.

Key words: Alginate, Granulosa cell, Purification, Scaffold

Introduction

The granulosa cells in the ovary play a crucial role in the process known as folliculogenesis. They are involved in steroidogenesis by producing estradiol and progesterone. The maintenance of the granulosa cell morphology and functional integrity depends on the spatial arrangement (Dzafic *et al*., 2013; Belani *et al*., 2014). In the traditional 2-dimensional (2D) culture system, the granulosa cells have limited interaction with the neighboring cells (Berkholtz *et al*., 2006). It was reported that cell-to-cell communication can enhance the proliferation and maturation of ovarian follicles (Joo *et al*., 2016). 2D culture system leads to granalosa cell apoptosis, while cell seeding within the 3D system maintains their cytoarchitecture, and cells can survive for many days (Berkholtz *et al*., 2006). In addition, researchers have reported that spheroid formation of bovine granulosa cells can imitate the stromal microenvironment and maintain the extracellular matrix (ECM) interactions (Hummitzsch *et al*., 2009).

Different types of 3D culture have been established and are suggested as useful systems for seeding the granulosa cells and follicle growth (Desai *et al*., 2010). Alginate, a non-toxic polysaccharide extracted from brown algae is a widespread applied system for ovarian follicle encapsulation. Alginate is composed of 1, 4-linked β-D-mannuronic and α-L-guluronic acid residues that polymerize and form 3D porous hydrogel (Pangas *et al*., 2003; Vigo *et al*., 2005). The pore structure of alginate hydrogel as a scaffold depends on some parameters, including the concentration of alginate and crosslinker type which support cell attachment and growth (Zhao *et al*., 2012; Singh *et al*., 2013; Lin *et al*., 2017). Alginate hydrogel has the ability to swell in an aqueous solution but will not dissolve in water (Pravdyuk *et al*., 2013; Mohanty *et al*., 2016). The affinity of sodium alginate to different bi-cations including Ca^{2+}, Ba^{2+} or Sr^{2+} can shape constant gel (Zhao *et al*., 2012). The alginate gel scaffold has some advantages including the ability to form hydrogel at room temperature and neutral pH (King *et al*., 2011; Andersen *et al*., 2015). In addition, the gel can dissolve rapidly using non-gelling ions and chelators, which allows for a better retrieval of the entrapped cells (King *et al*., 2011). However, commercial alginate has some disadvantages including the presence of a large amount of impurities such as proteins, endotoxins and polyphenols that can lead to a reduction in the alginate biocompatibility (Qi *et al*., 2009; Andersen *et al*., 2015). Jeong *et al*. (2011) showed the positive effect of purified alginate on the proliferation rate of retinal pigment epithelial cells (RPEs) in comparison with the non-purified alginate (Jeong *et al*., 2011). Also, various alginate concentrations, used for ovarian follicle 3D encapsulation, have an impact on follicle development (Pangas *et al*., 2003; Kreeger *et al*., 2005; Kreeger *et al*., 2006; Amorim *et al*., 2008). Some studies have reported that high concentration of alginate negatively affects the follicular somatic cell growth. West *et al*. (2007) indicated that low matrix stiffness increases the follicle growth, antrum formation and hormone production

(West *et al*., 2007). However, there is still debate on the alginate concentration ranging from 0.25% to 3% (w/v) rate (Dorati *et al*., 2016). In the present study, we produced a purified alginate as an efficient matrix and then investigated *in vitro* behavior of the granulosa cells seeded on the purified alginate in various concentrations and made a comparison with non-purified ones.

Materials and Methods

Alginate purification

Low-viscosity sodium-alginate (15-25 centipoise (cps) at Sigma, Cat number 180947) was purified using a protocol initially described by Qi *et al*. (2009). Briefly, alginate solution was prepared in distilled deionized water. Proteins were extracted by chloroform/butanol solution; then activated charcoal, equivalent to that of alginate-weight was added and stirred for 3 h. Charcoal/alginate solution was removed using filter paper, and then 0.22 µm filter. At the last step, alginate was precipitated with absolute ethanol. Alginate pellet was then prepared as 1% w/v solution in distilled water, filtered through a 0.22 µm syringe filter and then lyophilized for two days (Qi *et al*., 2009).

Primary cultures of the BALB/c mice granulosa cells

Female BALB/c mice at 8 weeks of age were superovulated with 20 IU of pregnant mares' serum gonadotrophin (PMSG); 48 h later, they were sacrificed via cervical dislocation. The granulosa cells were obtained from ovaries and cultured using the previously described methods with some modifications (Campbell, 1979; Sèdes *et al*., 2013). The ovaries of mature mice were collected and placed in phosphate buffered saline (PBS). Then, they were exposed to DMEM/F-12 containing (6.8 mM EGTA, and 0.2% BSA) followed by centrifugation at 1000 rpm for 15 min; and then they were washed twice and incubated in hypertonic sucrose solution (0.5 M sucrose, 1.8 mM EGTA, 0.2% BSA) in DMEM/F-12 for 5 min at 4°C (Belani *et al*., 2014). After centrifugation at 1500 rpm for 5 min, the ovaries were punctured with a 30-gauge needle to obtain cumulus-oocyte complexes and granulosa cells. The cells (mixed of the oocytes and granulosa cells) were pelleted by a 10 min centrifugation at 1500 rpm. Supernatant was discarded and the cells seeded into a 24-well culture plate (6×10^4 cells/well). The granulosa cells were separated from the oocytes by sequential washing with PBS and sub-cultured to eliminate the remaining oocytes. Every 2 to 3 days, half of the DMEM/F-12 media (200 µL) was replaced with fresh medium (Joo *et al*., 2016).

Granulosa cell encapsulation within the alginate hydrogel

The granulosa cells were suspended at a density of 3×10^5 cells/ml in 0.5% or 1% w/v purified and non-purified sodium-alginate. Gelation was performed by adding 200 µL alginate/cell suspension mixture to 50 mM $CaCl_2$ at 37°C for 30 min. The $CaCl_2$ was then removed, and alginate gel was washed thoroughly in the DMEM/F12. It should be noted that 4 groups were considered in 2 concentrations as follow:

Group I: Non-purified alginate 1% (control 1)
Group II: Non-purified alginate 0.5% (control 2)
Group III: Purified alginate 1%
Group IV: Purified alginate 0.5%

Cell proliferation and viability test

Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay on days 3, 5, 8 after cell seeding. On the due day, 50 µL of 5 mg/ml MTT solution in FBS-free DMEM (Sigma-Aldrich) was added into each well and incubated for 4 h at 37°C in dark. Then, MTT solution was aspirated and the formazan crystals were dissolved in 200 µL dimethylsulfoxide per well (Sigma-Aldrich) and optical densities of the stained solution were measured at 570 nm wavelength.

Measurement of the estradiol and progesterone concentrations

To evaluate the granulosa cells function in purified and non-purified alginate gel with different concentrations, estradiol and progesterone were measured in the granulosa cell culture media on the 3rd, 5th and 8th days using commercially available radioimmunoassay kits (IBL-Hamburg, Germany) according to the manufacturer's protocol. All samples were analyzed in triplicate and the data were expressed by mean±SD value at each point of time.

Alkaline phosphatase (ALP) assay

Photometric ALP kit (Pars Azmun, Iran, Tehran) was used to assess the ALP activity according to the manufacturer's instruction. Briefly, 20 µL of the culture medium was added to the 1000 µL freshly prepared solution containing 1 mol/L Diethanolamine (PH = 9.8), 0.5 mmol/L magnesium chloride and 10 mmol/L P-Nitrophenylphosphate for 1 min. Then, the optical density was evaluated at 405 nm. After the 1st, 2nd, and 3rd min, the average absorbance difference per min (ΔE/min) was calculated and multiplied in factor's number (2757) and the following formula:

$$\Delta E/min \times factor = ALP\ activity\ [U/L]$$

Statistical analysis

Statistical analysis was carried out using either two-way or one-way ANOVA followed by Tukey's post-hoc test. In this line, the observed value of the F-test statistic and its corresponding P-value was calculated from the Table of F percentages. A $P<0.05$ was considered to be statistically significant. In addition, the graphs were plotted by Prism v.5 software (Graph Pad Software, San Diego, CA, USA).

Results

Cell viability and proliferation test

The proliferation activity of the granulosa cells seeded in alginate scaffold was measured by MTT assay during the 3rd, 5th, and 8th days *in vitro*. A two-way repeated measures ANOVA was performed with the "culture days" as the within-subjects factor and "experimental groups" as the between-subjects factor. A significant difference was found for "culture days" [P<0.001, F(2,16)=12.82], "experimental groups" [P<0.001, F(3,8)=60.38], and the "culture days" by "experimental groups" interaction [P<0.01, F(6,16)= 10.60]. The data showed that the changes in cell growth rate during the culture days and these changes were different between the study groups (P<0.001). The granulosa cells could proliferate as the time progressed and the proliferation rate showed a significant increase in the cells cultured in 0.5% purified alginate in comparison with the cells cultured in control groups. The cell growth rate on the 3rd, 5th, and 8th days is shown in (Fig. 1; Tables 1-3).

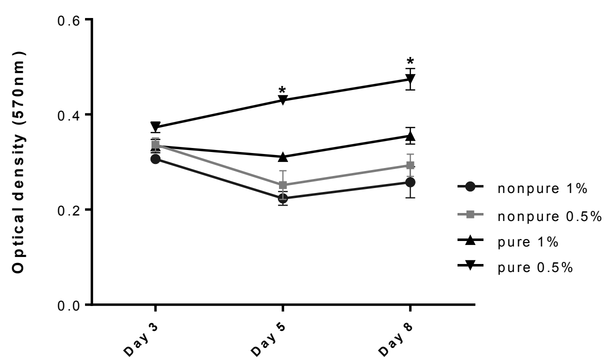

Fig. 1: Mean±SD of the percentage of granulosa cell proliferation in different alginate groups. On the days 5 and 8, a significant increase in cell proliferation was observed in the cells cultured in 0.5% purified alginate compared with the related control * P<0.01, purified alginate 0.5% vs all other groups

The one-way ANOVA was used to analyze the proliferation rate of granulosa cells during the culture days. The results showed a significant difference amongst the study groups in this respect on the 5th day [(P<0.001), F(3,8)=79.13] and on the 8th day [(P<0.001), F(3,8)=26.58]. On the days 5 and 8, a significant increase in cell proliferation was observed in the cells cultured in 0.5% purified alginate compared with the related control (P<0.01). This showed that purification of alginate and low concentration hydrogel affect the granulosa cell survival and proliferation.

Estradiol and progesterone secretion during the culture days

A two-way repeated measures ANOVA was performed to evaluate the estradiol and progesterone secretion with the "culture days" as the within-subjects factor and "experimental groups" as the between-subjects factor. A significant effect was observed for estradiol secretion [P<0.05, F(2,16)=3.89], "experimental groups" [P<0.001, F(3,8)=75.76], and the "culture days" by "experimental groups" interaction [P<0.01, F(6,16)=17.60]. The test also showed a significant effect on the progesterone secretion [P<0.001, F(1.07,8.60)=72.15], "experimental groups" [P<0.001, F(3,8)=29.21], and the "culture days" by "experimental groups" [P<0.01, F(3.22,8.60)=8.59]. The data showed that both estradiol and progesterone secretion changed during the culture days and these changes were different among the study groups (P<0.001). The present study also revealed significantly more steroid secretion in the 0.5% purified alginate on the 8th day in comparison with the control groups (Figs. 2A-B; Tables 1-3).

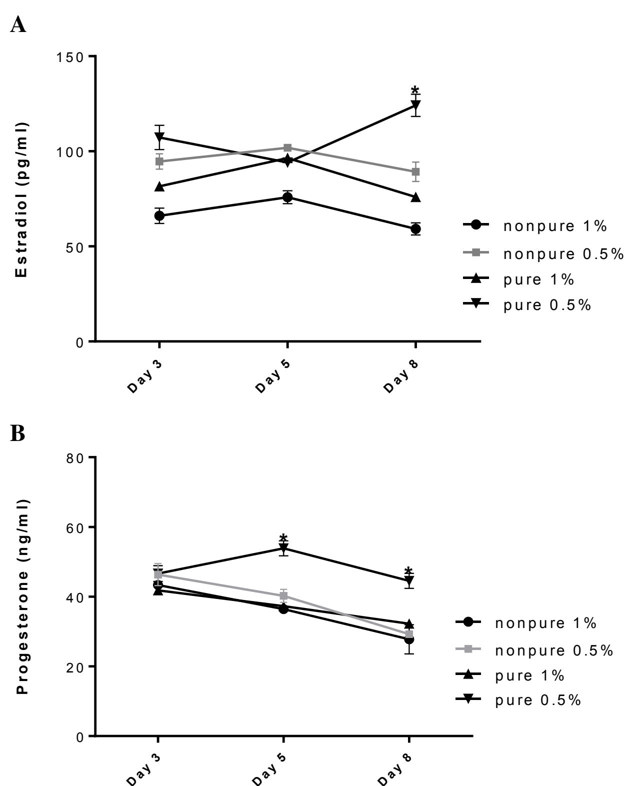

Fig. 2: Mean±SD of the percentage of steroid production by the granulosa cells in different alginate groups. (**A**) Granulosa cells produced significantly higher amounts of estradiol in the 0.5% purified alginate group on the 8th day of culture, and (**B**) progesterone was also produced significantly more by the cells cultured in 0.5% purified alginate after the 5th and 8th days *in vitro*. * P<0.01, purified alginate 0.5% vs all other groups

Also, one-way ANOVA was used to analyze the steroid production by the granulosa cells during the culture days. The results showed a significant difference amongst the study groups in this respect on the 8th day for estradiol [P<0.001, F(3,8)=60.55] and progesterone [P<0.001, F(3,8)=20.59]. In accordance with the growth and survival data, the steroid profiles showed estradiol levels increased significantly in 0.5% purified alginate on the 8th day of cultures (Fig. 2A). In contrast, progesterone (Fig. 2B) production was as early as on the 8th day although it increased significantly on the 5th day

in 0.5% purified alginate (P<0.01).

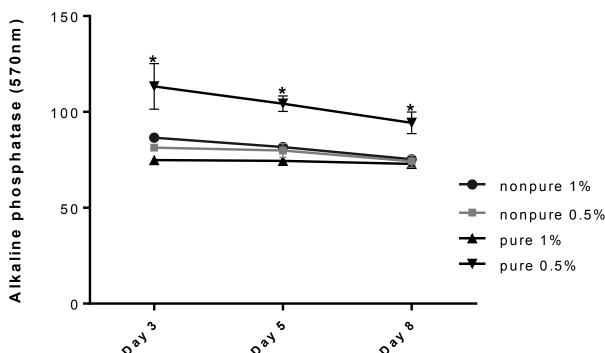

Fig. 3: Mean±SD of the percentage of alkaline phosphatase production in different alginate groups. The granulosa cells in the 0.5% purified alginate group showed high alkaline phosphatase production in comparison with the control group on the 3rd, 5th, and 8th days. * P<0.01, purified alginate 0.5% vs all other groups

Alkaline phosphatase

A significant effect was observed for "culture days" [P<0.05, F(2,16)=4.37], "experimental groups" [P<0.001, F(3,8)=76.93], and the "culture days" by "experimental groups" interaction [P<0.01, F(6,16)= 0.585]. This suggests that ALP production changed during the culture days and these changes were different between the study groups (P<0.001). The granulosa cells in the 0.5% purified alginate group showed high ALP production in comparison with the control group on the 3rd, 5th and 8th days (Fig. 3; Tables 1-3).

The one-way ANOVA was used to analyze the rate of ALP production during the culture days. The results showed a significant difference amongst the study groups in this respect on the 3rd day [P<0.01, F(3,8)=7.90], on the 5th day [P<0.001, F(3,8)=26.02], and on the 8th day [P<0.01, F(3,8)=11.57]. Assessment of ALP activity also revealed that the best condition for the granulosa cells activity was 0.5% purified alginate scaffolds (P<0.01).

Discussion

We hypothesized that matrix properties would influence the growth and steroidogenic capacity of granulosa cells seeded in alginate scaffold. In the present study, we purified the commercial alginate and compared it with non-purified ones. Furthermore, steroidogenesis was measured as an important functional feature of granulosa cells. Our results indicated a higher output of estradiol in 0.5% purified alginate. Also, progesterone level in the medium supernatant was significantly higher in 0.5% purified alginate and it could be detected as early as the 5th and 8th days of culture in comparison with the control groups. Also, the cells cultured on 0.5% purified alginate showed a higher proliferation rate and ALP activity.

Table 1: Mean±SD of the optical density, progesterone, estradiol and alkaline phosphatase in purified and non-purified alginate after 3th day *in vitro*

Groups	Optical density	Progesterone	Estradiol	Alkaline phosphatase
Nonpure 1%	0.30 ± 0.001	43.3 ± 0.2	66.06 ± 4.04	86.5 ± 1.6
Nonpure 0.5%	0.33 ± 0.01	46.3 ± 3.2	94.6 ± 4.05	81.3 ± 0.4
Pure 1%	0.33 ± 0.02	41.8 ± 1.5	81.5 ± 1.2	74.8 ± 1.3
Pure 0.5%	0.37 ± 0.01	46.6 ± 3.9	107.2 ± 11.1	113.2 ± 20.6*

* P<0.01, pure 0.5% vs. all other groups

Table 2: Mean±SD of the optical density, progesterone, estradiol and alkaline phosphatase in purified and non-purified alginate after 5th day *in vitro*

Groups	Optical density	Progesterone	Estradiol	Alkaline phosphatase
Nonpure 1%	0.22 ± 0.01	36.4 ± 0.2	75.8 ± 3.3	81.6 ± 1.7
Nonpure 0.5%	0.25 ± 0.03	40.2 ± 1.9	101.8 ± 1.8	79.9 ± 3.6
Pure 1%	0.31 ± 0.008	37.2 ± 2.3	96.4 ± 1.5	74.4 ± 3.6
Pure 0.5%	0.43 ± 0.008*	53.8 ± 3.7*	94.1 ± 3.02	104.3 ± 7.07*

* P<0.01, pure 0.5% vs. all other groups

Table 3: Mean±SD of the optical density, progesterone, estradiol and alkaline phosphatase in purified and non-purified alginate after 8th day *in vitro*

Groups	Optical density	Progesterone	Estradiol	Alkaline phosphatase
Nonpure 1%	0.25 ± 0.03	27.7 ± 4.1	59.2 ± 3.1	75.3 ± 0.9
Nonpure 0.5%	0.29 ± 0.02	29.2 ± 0.41	89.2 ± 5.1	74.2 ± 3.07
Pure 1%	0.35 ± 0.03	32.2 ± 1.5	75.9 ± 3.05	72.8 ± 1.05
Pure 0.5%	0.47 ± 0.03*	44.5 ± 3.7*	124.2 ± 10.2*	94.3 ± 9.7*

* P<0.01, pure 0.5% vs. all other groups

Several research groups had produced purified alginate with different procedures to remove the contaminants for culture of different cells (Langlois *et al*., 2009; Jeong *et al*., 2011; Selimoglu *et al*., 2012; Kim *et al*., 2013; Song *et al*., 2015; Sondermeijer *et al*., 2016). Song *et al*. (2015) reported that purified alginate provided a positive environment for chondrocytes growth (Song *et al*., 2015). Other researchers have also reported that the RPEs in purified alginate films had higher cell proliferation rate and maintained specific cell expression of genes (Jeong *et al*., 2011). In this study, we used a simple and efficient purification method to remove the alginate contaminants (Qi *et al*., 2009). Endotoxin, polyphenol and proteins are the most common contaminants in the alginate scaffold (Dusseault *et al*., 2006; Ménard *et al*., 2010). Langlois *et al*. (2009) reported that alginate contaminants had a deleterious effect on islets of Langerhans by triggering pro-apoptotic signaling pathways or the production of free radicals (Langlois *et al*., 2009). In the present study, we used some organic reagents such as chloroform/butanol that have been shown to be effective in removing protein contaminants (Qi *et al*., 2009). Kim *et al*. (2012) reported that reducing the protein content of alginate induces cell survival and proliferation (Kim *et al*., 2013). Also, in our study we used an activated charcoal, which is widely used in removing organic compounds from aqueous solution. It has been suggested that activated charcoal can reduce the amount of polyphenol in the alginate hydrogel (Qi *et al*., 2009). Other researchers have also reported that some chemical treatments, such as ethanol washing of alginate, are an effective step for decreasing the endotoxin levels. Endotoxins originate from the cell wall of Gram-negative bacteria that are important for their survival. Therefore, the last step of purification procedure in this study was precipitation of the alginate with ethanol (Dusseault *et al*., 2006).

The data from the current study also showed that alginate purification improved the viability and proliferation of granulosa cells. In comparison with the non-purified ones, there was a significant increase in survival and proliferation rates after 8 days of seeding in the purified alginate. These results are in line with those of Qi *et al*. (2009) who reported a higher proliferation of osteoblast on the purified alginate film in comparison with the non-purified ones. Findings of other studies suggest that the ECM affects the secretion of the steroid hormone (Woodruff and Shea, 2007). Some researchers have shown that 3D culture within collagen hydrogels had an effect on the ovarian follicle survival and supported hormone production. An increased 17β-estradiol level was reported in various concentrations of collagen hydrogels by Joo *et al*. (2016), however, the progesterone production was not influenced significantly by the collagen concentrations.

Different alginate concentrations such as 3% (West *et al*., 2007), 1.5% (Kreeger *et al*., 2005; Xu *et al*., 2006a, b), 1% (Heise *et al*., 2005; Amorim *et al*., 2008), 0.5% (Xu *et al*., 2009) and 0.25% (Mainigi *et al*., 2011) have been used in follicular growth, but there is still debate about optimum alginate concentration for higher survival and growth rate of follicles in different species. Our study showed that, alginate concentration had an effect on the murine granulosa cell proliferation and function. After the 8th day, the viability of the granulosa cells, estradiol and progesterone production in the 0.5% purified alginate gel was higher than those cultured in 1% alginate. In line with our study, West *et al*. (2007) revealed that low matrix stiffness accelerates the follicle growth and hormone production. It is assumed that the higher production of estradiol and progesterone in the 0.5% purified alginate group could be attributed to the enhanced granulosa cell numbers. On the other hand, decreasing the percentage of alginate may enhance the diffusion of macromolecules through hydrogel and further granulosa cell potential. Some researchers have also reported that non-human primate follicles cultured in high alginate stiffness had a higher cell survival and morphology (Xu *et al*., 2009; Hornick *et al*., 2012). It has been suggested that different species require special physical environments to support the cell survival and growth.

Phosphatase enzymes were engaged in both growth and atresia of the follicles. Alkaline phosphatase enzyme is a lysosomal enzyme that is involved in the DNA turnover and protein transport (Ganguly, 2013). In the current study, a higher ALP was produced by the cells cultured within 0.5% purified alginate scaffolds after the 8th day. On the other hand, our results also showed that progesterone secretion was significantly higher in the granulosa cells seeded within 0.5% purified alginate after 8 days. This finding is in line with those of the previous studies reporting that phosphatase activity was conducted by higher concentration of progesterone and androgen (Ganguly, 2013; Deka *et al*., 2014). Therefore, in the present study the higher ALP activity might be due to a progesterone dominant environment after the 8th day of seeding.

In conclusion, our results indicated that the cells cultured on purified and low concentration alginate had a higher proliferation rate, sex hormone production and ALP activity.

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Conflict of interest

The authors declare no conflict of interest.

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