Original Article

Comparative Evaluation of the Viability of L929 Murine Fibroblasts in the Presence of Different Concentrations of Propolis with and without Vitamin C as a Storage Medium for Avulsed Teeth

Zohreh Ahangari[1], Mandana Naseri[1], Zahra Yadegari[2], Mahdieh Nakhaee[3], Farhood Najafi[5], Mojgan Feli[1]

[1] Department of Endodontics, School Dentistry, Shahid Beheshti University of Medical Science, Tehran, Iran
[2] Departments of Dental Biomaterials, School of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3] Private Practice, Kerman, Iran
[4] Department of Resin and Additives, Institute for Color Science and Technology, Tehran, Iran

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Abstract

Introduction: This study aimed to assess the optimal concentration of propolis with and without vitamin C as a storage medium for avulsed teeth **Methods:** Following the preparation of L929 murine fibroblasts suspension, 5,000 cells were seeded to each well of a 96-well plate. After 24 h, the culture medium was replaced with 0.01, 0.005, 0.001, 0.0005, 0.0001, and 0.00005 concentrations of propolis(P) and propolis plus vitamin C(PC) using Dulbecco's Modified Eagle Medium. After 2, 24, and 72 h of incubation, the percentage of cell viability was determined by methyl thiazolyl tetrazolium assay, compared to the negative control group. Data were analyzed using the SPSS software (version 21). Two-way ANOVA was used to compare the means, while Tukey's test was applied for pairwise comparisons. **Results:** After 2 h, only the difference between the 0.001 concentration of P and PC was significant ($P<0.005$), such that cell viability was higher in the latter group. After 24 h, cell viability in 0.0005 and 0.00005 concentrations of P was significantly higher than that in the PC group. However, no significant difference was noted after 72 h. **Conclusion:** Cell viability was retained in all concentrations of propolis with or without vitamin C. On the other hand, with an increase in the concentration of propolis, cell viability decreased. Although PC was superior to propolis alone in cell viability; however, this effect decreased over time such that no significant difference was noted after 72 h.

Keywords: Avulsion, Cytotoxicity, Fibroblasts, Propolis, Vitamin C

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Introduction

Traumatic dental injuries are highly prevalent in primary and permanent teeth (1, 2). Avulsion is among such injuries. Duration of the extraoral time and the medium in which the avulsed tooth is stored during this period (until replantation) are among the most critical factors in replantation success (3). If an avulsed tooth is stored in an inappropriate and dry environment for over an hour, periodontal ligament (PDL) cells will not survive, and the fibers will be damaged. Under such circumstances, replacement resorption will probably pursue after replantation (4). Several studies have been conducted on different storage media to find a medium with minimal cytotoxicity (5-9).

Propolis is a biological material produced by honey bees (10). To date, over 180 constituents of propolis have been identified (11). Flavonoids are among its primary constituents with antioxidant, antibacterial, antifungal, antiviral, and anti-inflammatory properties (12). Some dental applications of propolis include its use for enhancing the healing of surgical wounds (10, 13), as a

pulp capping agent (14, 15), and for periodontal therapy (14). Evidence shows that propolis has properties comparable or superior to those of Hank's balanced salt solution (HBSS), and in appropriate concentrations, it can be used as a more affordable alternative to chemical storage media. However, its most appropriate concentration has not yet been identified (6, 7, 10).

Vitamin C is a water-soluble and non-toxic white powder that forms acid in aqueous solutions, thereby being referred to as ascorbic acid (16). It induces differentiation of mesenchymal cells, regulates the growth of tumor cells, has antioxidant properties, serves as a coenzyme for many enzymes, and causes hydroxylation, which is imperative for collagen synthesis (17, 18). Other properties of vitamin C include the induction of the proliferation of fibroblasts, as well as soft and hard tissue regeneration (19, 20). In the present study, vitamin C was used along with propolis to assess its effect on the viability of fibroblasts. Propylene glycol was employed as the solvent in this study, a transparent, colorless, and hydrophilic liquid used in the production of polymers. It is also used as a solvent in many non-water soluble pharmaceutical agents (21). This study aimed to assess the cytotoxicity of six different concentrations of propolis and propolis combined with vitamin C against L929 murine fibroblasts cell line after 2, 24, and 72 h of exposure to determine the optimal concentration of propolis and propolis plus vitamin C, as a storage medium for avulsed teeth.

Materials and Methods

This experimental study aimed to assess the cytotoxicity of different concentrations of propolis against the L929 murine fibroblasts cell line.

Preparation of propolis

Brown solid natural propolis was collected from the west of Iran. The 5% propolis solution was prepared in the Polymer Laboratory of Tehran University (Tehran, Iran) using propylene glycol solvent and water.

Culture of L929 murine fibroblasts

L929 murine fibroblasts cell line was obtained from the National Cell Bank of Iran (Pasteur Institute of Iran) and transferred to the Dental Material Biocompatibility Laboratory of the Dental Biomaterials Department, Shahid Beheshti School of Dentistry (Tehran, Iran). The cells were placed in a complete culture medium, including Dulbecco's Modified Eagle Medium (DMEM; Gibco, Spain) supplemented with 10% fetal bovine serum (Gibco, Spain) and antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin). They were incubated at 37°C, with 5% CO2 and 98% humidity. After proliferation and reaching 80% confluency, the cells were passaged using trypsin/EDTA. The cell suspension was prepared using the fourth passage cells. The cell suspension had a concentration of 25,000 cells/mL, and 5,000 cells were seeded in each well of a 96-well plate. The plates were incubated at 37°C, with 5% CO2 and 98% humidity.

Preparation of different concentrations of propolis, propolis plus vitamin C, and propylene glycol

The concentration of propolis stock solution was 5% (5 g propolis in 100 mL of propylene glycol and water), which was sterilized by a 0.22-µm membrane filter. This concentration was first diluted in complete culture medium in 0.01 ratio and then serially in 0.005, 0.001, 0.0005, 0.0001, and 0.00005 ratios.

The concentration of propolis with vitamin C stock solution included 5% propolis and 5% vitamin C in 100 mL propylene glycol and water. Similar to propolis concentration, this solution was also serially diluted in 0.01, 0.005, 0.001, 0.0005, 0.0001, and 0.00005 ratios using complete culture medium. Different concentrations (0.01, 0.005, 0.001, 0.0005, 0.0001, and 0.00005) of propylene glycol were also prepared as the vehicle control.

Exposure of the cells to different concentrations of propolis and propolis plus vitamin C

After 24 h incubation of cell culture plates, the culture medium of each well was removed, and then 200 µL of the prepared concentrations were replaced in each well. Six wells were allocated to each concentration (six repetitions) and six wells to each concentration of the vehicle control. Six wells were also specified for the negative control group and replaced with a complete cell culture medium. The positive control wells contained sterile distilled water, which is lethal for cells. Two plates containing the respective media were considered for assessment at 2, 24, and 72 h. All procedures were accomplished under sterile conditions in a laminar flow hood. All plates were incubated at 37°C, with 5% CO2 and 98% humidity for the respective periods.

Methyl thiazolyl tetrazolium assay for assessing the percentage of cell viability

For the Methyl thiazolyl tetrazolium (MTT) assay, yellow MTT salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in 5 mg/mL concentration was prepared using phosphate buffered saline and sterilized using a 0.22 µm membrane filter. After the

exposure time, the supernatant medium was completely removed, and all wells were rinsed with phosphate-buffered saline three times. The prepared MTT salt solution (5 mg/mL) was then diluted with DMEM media in a 1:10 ratio, and 200 µL of it was added to each well of a 96-well plate. The plates were incubated for 2 h and then removed, the supernatant medium was extracted, and 200 µL of dimethyl sulfoxide was added to each well to dissolve the formazan crystals. After mixing, the optical density of the solution (with a color change to dark blue) was measured at 570 nm wavelength with a reference filter of 620 nm using an ELISA Reader. The percentage of cell viability was calculated, compared to the negative control group at 2, 24, and 72 h using the following formula:

$$\text{Percentage of cell viability} = 100 \times \frac{\textit{Mean optical density in the test group}}{\textit{Mean optocal density in the negative control group}}$$

The MTT assay was conducted according to the ISO 2009-5-10993 standards.

The MTT data underwent statistical analysis using the SPSS software (version 21). The mean and standard deviation values were reported for each group. Two-way ANOVA was applied to compare the mean values, and Tukey's test was used for pairwise comparisons.

Two-way ANOVA was applied to compare cell viability after exposure to different concentrations of the tested media. The interaction effect was significant ($P<0001$). The mean and standard deviation of the ratio of viable cells to all cells were also reported. The mean values were compared by two-way ANOVA, and the independent t-test was applied to compare the percentage of cell viability at different time points within each group. Tukey's HSD test was used for pairwise comparisons.

Results

After 2 h, a significant difference was only noted between the 0.001 concentration of propolis and propolis plus vitamin C ($P<0.005$), such that the percentage of cell viability in the propolis plus vitamin C group was higher than that in the 0.001 propolis group. However, no significant difference was noted between other concentrations (Tables I and II). At this time, maximum viability was noted in the 0.0001 concentration of propolis, and the viability decreased in higher concentrations.

Table I. Pairwise comparisons of the media in six different concentrations at 2, 24, and 72 hours regarding the viability of fibroblasts

		P-value		
Media	Concentration	2 h	24 h	72 h
P, (P+C)		1.000	0.000*	0.011
P, PG	0.00005	1.000	0.002*	0.009
(P+C), PG		1.000	0.000*	1.000
P, (P+C)		1.000	0.057	0.160
P, PG	0.0001	1.000	0.000*	0.088
(P+C), PG		1.000	0.000*	1.000
P, (P+C)		1.000	0.002*	0.695
P, PG	0.0005	1.000	0.000*	0.090
(P+C), PG		1.000	0.000*	1.000
P, (P+C)		0.003*	0.596	1.000
P, PG	0.001	0.247	0.000*	0.000*
(P+C), PG		0.233	0.000*	0.000*
P, (P+C)		0.944	1.000	1.000
P, PG	0.005	0.382	0.000*	0.000*
(P+C), PG		1.000	0.000*	0.000*
P, (P+C)		0.904	1.000	1.000
P, PG	0.01	0.000*	1.000	1.000
(P+C), PG		0.000*	1.000	1.000

*PV<0.005, P=Propolis, (P+C)=Propolis+vitamin C, PG=Propylene glycol (vehicle control)

After 24 hours, the percentage of cell viability in 0.0005 and 0.00005 concentrations was 68.98% and 78.47% in the propolis and 57.13% and 63.34% in the propolis plus vitamin C groups, respectively. No significant difference was noted between the two groups in other concentrations (Tables I and II). At this time, maximum viability was observed in the presence of the 0.00005 propolis concentration, and the viability decreased in higher concentrations.

Table II. Mean percentage of viability of fibroblasts in the presence of different media in six different concentrations at 2, 24, and 72 hours

Media	Concentration	Percentage of cell viability±SD*		
		2h	24h	72h
P	0.00005	99.78±5.08	78.47±7.62	89.77±2.88
	0.0001	103.27±15.05	68.60±10.49	89.98±3.48
	0.0005	92.97±14.76	68.98±3.65	89.25±3.68
	0.001	92.30±10.71	53.08±5.90	79.78±11.97
	0.005	85.50±6.11	11.32±1.70	3.50±0.74
	0.01	11.98±1.48	3.92±0.18	2.84±0.48
P+C	0.00005	100.86±16.48	63.34±5.36	99.14±3.48
	0.0001	100.29±13.43	60.40±3.76	97.07±3.12
	0.0005	97.38±10.43	57.13±8.21	93.36±0.74
	0.001	113.19±7.27	48.34±6.75	73.93±5.56
	0.005	91.32±68	11.86±0.59	3.80±0.38
	0.01	18.52±4.90	6.00±0.28	3.78±0.162
PG	0.00005	98.88±13.39	90.40±6.07	99.25±7.43
	0.0001	102.15±6.63	93.93±6.54	980±12.2
	0.0005	96.85±11.66	83.46±7.41	96.46±5.40
	0.001	102.42±7.49	83.94±4.03	100.22±6.21
	0.005	94.35±7.06	61.12±6.33	۱۰۱.۱۳۳±۷,۵۸
	0.01	64.90±5.21	4.68±1.25	2.34±0.14

* Standard deviation, P=Propolis, (P+C)=Propolis+vitamin C, PG=propylene glycol (vehicle control)

After 72 h, no significant difference was noted between the propolis and propolis plus vitamin C groups in any concentration (P<0.005).

At this time, maximum viability was noticed in the presence of the 0.0001 propolis concentration, and the viability decreased in higher concentrations.

Discussion

The viability of PDL cells is highly crucial in the prognosis of replanted avulsed teeth (22, 23). Researchers have attempted to provide favorable conditions for higher viability of PDL cells. Evidence shows that drinking water, saliva, and saline are all ineffective in preserving the viability of PDL cells and are not recommended for long-term storage of avulsed

teeth since they cause early PDL cell death due to hypotonic property or bacterial contamination (4, 23). Although numerous storage media have been suggested for the storage of avulsed teeth, evidence reveals that products such as green tea, coconut juice, milk, and propolis (24) are more effective than synthetic products for this purpose (25). Attempts are ongoing to find media with ideal function to preserve the viability of PDL cells.

Propolis is a complex mixture of chemicals, and its composition depends on the geographical region and season of its collection. The propolis used in this study included 55% plant resin, 30% honey bee wax, 10% ether and aromatic oils, and 5% bee pollen (26).

Propylene glycol is an alcoholic solvent with no carcinogenicity or genotoxicity that has antimicrobial properties and FDA approval (27). In endodontic treatment, it is used as a vehicle for calcium hydroxide for easier handling and enhanced antimicrobial effects (27, 28). It also improves the mechanical and chemical properties of the mineral trioxide aggregate (29). In the present study, in addition to two experimental groups, a control solvent or vehicle group of propylene glycol was also considered to ensure that the observed cytotoxicity was not related to the solvent. The results showed that after 24 and 72 h, the viability of PDL cells in this group was higher in all concentrations, except for the 0.01 concentration.

Cytotoxicity assessment is the first step to evaluating the biocompatibility of materials (30). This test is easier and faster to be performed *in vitro* than *in vivo*. It can be more easily standardized *in vitro* because the confounding factors can be easily controlled and can serve as a type of primary screening. Therefore, the American National Standard Institute, the American Dental Association, the International Standard Organization, and the FDI World Dental Federation have all published guidelines for *in vitro* tests for assessing cytotoxicity (31). Different methods are employed for the *in vitro* assessment of cytotoxicity, such as the assessment of the changes in cell membrane permeability, replication assays, and functional assessments. The selection of an appropriate test depends on testing conditions and the chemical composition of materials (32).

Trypan blue staining is based on the assessment of changes in the permeability of cell membranes, which is used for staining dead cells. In this technique, cell viability is determined by counting the non-stained cells under a microscope or by other techniques (10, 33). Cells that can proliferate and form a colony are clonogenic, and losing this ability leads to their death. Accordingly, after exposure to different cytotoxic agents, the cells may still remain viable and produce protein and new DNA; however, if they lose their proliferation capacity, they are considered dead (34). Several problems exist with the conventional trypan blue staining which limit its application, such as the inability to detect dead cells if not counted within 3 to 5 min leading to an increase in the number of stained cells**.** Additionally, this staining cannot differentiate between viable functional cells and nonfunctional cells. Therefore, the MTT assay with L929 murine fibroblasts used in this study is a colorimetric assay for measuring cell metabolic activity (33, 35).

The reduction of yellow methyl tetrazolium salt by the mitochondrial dehydrogenase enzyme produces formazan crystal in viable cells, which causes a dark blue color change. The optical density of the colored solution is then measured by a spectrophotometer. The amount of produced formazan crystals has a direct correlation with the total number of viable cells. This technique has advantages such as simplicity, speed, and accuracy. In the MTT assay, direct and indirect methods may be used to expose the cells to materials. In the present study, the direct technique was used due to the nature of the media, and the filtration technique was used for sterilization. The sterilization of materials before exposure to cells is highly important, as any contamination with microorganisms can serve as a confounder (36, 37).

Some studies have shown that the PDL cells remain viable for a longer period in propolis, compared to HBSS and milk (4-7). Ahangari et al. (10) compared the percentage of cell viability at 1 and 3 h after storage in 10% and 50% propolis, milk, HBSS, and egg white. The percentage of cell viability was higher in the propolis group. Casaroto et al. (4) reported that the viability of PDL cells in propolis was better than that in milk, saline, and HBSS. However, Mahal et al. (38) indicated the equal efficacy of 15% propolis, egg albumin, and HBSS. Variations in the results can be due to the use of different concentrations and different constituents of propolis depending on the geographical location from which it was collected (39).

In the present study, after 2 h of exposure, cell viability in the propolis and propolis plus vitamin C groups was above 85% in almost all concentrations and decreased by ascending concentrations of propolis. Additionally, at this time, the results showed more cell viability in the presence of vitamin C rather than propolis alone in almost all concentrations. Several studies have shown that vitamin C causes the proliferation of fibroblasts (20).

A comparison of the results after 24 h indicated a reduction in cell viability in both groups of almost all concentrations, compared to 2 h groups, estimated at 48%, which may be due to neutralizing propolis and subsiding its active part. In contrast to the results

obtained at 2 h, cell viability was superior in the propolis group than that in the propolis plus vitamin C group, which may be due to the elimination of the vitamin C effect over time, making living conditions unsuitable for the cells. Saxena et al. (7) evaluated the viability of PDL cells by the MTT assay. The results showed that the combination of propolis and DMEM was superior to milk, saliva, and HBSS at 24 h, compared to 30 min. By contrast, the present study revealed that propolis with vitamin C was superior to propolis alone for short-term storage.

This study showed no significant difference between propolis and propolis plus vitamin C groups in any concentration at 72 h and descending of viability by an increase in concentration, which may be due to the toxicity of high concentrations of propolis. This was consistent with Saxena et al. (7) reporting that the combination of 10% propolis and DMEM had higher efficacy than 20% propolis and DMEM regarding cell viability (7).

External root resorption is a common complication following the replantation of avulsed teeth which occurs due to the death of PDL cells (40). Since propolis has anti-inflammatory and anti-microbial properties (24, 39), it can be used in low concentrations as a storage medium to preserve the viability of PDL cells and minimize the complications of replantation.

Conclusion

Propolis and propolis plus vitamin C at 2 h showed optimal biocompatibility and can be suggested as storage media for avulsed teeth in all concentrations, except for 0.01. Increasing the storage time to 24 and 72 h decreased the biocompatibility of the materials in 0.005 and 0.01 concentrations. However, the biocompatibility was still high in other concentrations. Natural substances, such as propolis, have shown optimal performance *in vitro*. *In vivo* studies are required to confirm these results.

Conflict of Interest

The authors deny any conflicts of interest related to this study.

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Corresponding Author
Mojgan Feli
Department of Endodontics, School Dentistry, Shahid Beheshti University of Medical Science, Tehran, Iran
Tel: +989183522394
Email: hfa.2000@yahoo.com