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Potential of the propolis as storage medium to preserve the viability of cultured human periodontal ligament cells: an *in vitro* study

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Abstract – *Aim: In vitro* experiments were carried out to evaluate the potential of propolis, a natural resin known for its wide therapeutic window, as storage medium to preserve the viability of cultured human periodontal ligament (PDL) cells. *Materials and Methods:* Primary cultures of human PDL cells were subjected to either independent exposure of propolis (2.5%, 5.0%, 10.0%, and 20.0%), Hank's balanced salt solution (HBSS), milk (0.5%), artificial saliva, Dulbecco's modified Eagle's medium (DMEM) or combination of propolis 10% + DMEM, propolis 20% + DMEM for 30 min to 24 h at 37°C. Cell viability was assessed using standard endpoints i.e., tetrazolium bromide salt (MTT), neutral red uptake, and trypan blue dye exclusion assay. *Results:* In general, combinations of propolis 10% + DMEM, propolis 20% + DMEM, and DMEM alone were found to be better than other media used in this study. The difference in the potentials of these media to maintain the cell viability reached to the statistically significant levels by 24 h, when compared with other media used viz., propolis 2.5% ($P < 0.01$), propolis 5.0% ($P < 0.05$), propolis 10.0% ($P < 0.05$), propolis 20.0% ($P < 0.001$), HBSS ($P < 0.001$), and milk ($P < 0.01$). Trypan blue dye exclusion assay could be recorded the most sensitive among all the assays selected to study the cell viability of PDL cells. *Conclusions:* Study indicates that combinations of propolis 10% + DMEM, propolis 20% + DMEM, and DMEM alone are equally good as storage media of choice to keep PDL cells viable during extra-alveolar period up to 24 h. Other more readily available medium such as milk may serve as appropriate alternative storage medium for shorter time periods i.e., up to 12 h.

The damage to the attachment apparatus during an avulsion injury is unavoidable, so maintaining the viability of the periodontal ligaments (PDL) attached to the avulsed tooth is critical. Thus, extra-alveolar period has been suggested as one among the important factors for the success of transplantation/replantation of avulsed teeth (1, 2). Ideally, the tooth should be replanted immediately after the injury to preserve the viability of the PDL cells and eventually to optimize healing and minimize root resorption (3). Although immediate replantation has been shown to have the best prognosis, (3–5) unfortunately, this occurs very rarely (5, 6). If in any case, the immediate replantation of an exarticulated tooth is not possible, the storage conditions should be recommended to maximize the preservation of the PDL cells during extra-alveolar period (2, 7–12). Similarly, during transplantation procedures, when the tooth is surgically transferred from its initial position into a new socket, an appropriate storage medium is required to maintain the vitality of the cells during the extra-alveolar period of tooth during which new socket is being prepared. Likewise, in an intentional replant, a tooth is extracted and then replaced in the same socket after treatment, and a suitable medium is needed for storage of teeth during this procedure (13, 14).

Cvek et al. (15) demonstrated that 13% of teeth kept in a dry state for 15 min, 40% of those kept in a dry state for 20–40 min, and 100% of those stored dry for >60 min showed signs of ankylosis. Therefore, it is the most important to prevent the PDL cells from drying. It has been suggested to keep the avulsed tooth under the patient's tongue until it is brought to the dentist (16, 17). Thus, human saliva has also been suggested as a potential storage medium (16). However, milk has been proven to be a more suitable storage medium for avulsed teeth, mainly because of its physiological osmolality, neutral pH, presence of essential nutrients, and lack of active toxic components (8, 9, 12, 18). Many other media have also been tried to evaluate their possible potential as storage media to preserve the viability of PDL cells, such as Hank's balanced salt solution (HBSS) (10, 11, 19–21), Gatorade® (Gatorade, PepsiCo Inc., Anderson

Hill Road, NY, USA) (9, 22), contact lens solutions (9, 23), conditioned medium (13), Viaspan and Custodiol (organ transplant media) (24, 25), and egg albumen (26, 27). However, none of these storage media could be adopted as ideal because of their mixed efficacy and other limitations. So, the search for a more suitable transportation medium is still on.

Recently, a natural resin made from the buds of conifers and poplar trees, bee wax, and other bee secretions has caught the attention to be used as storage medium to protect the PDL cells (10, 11, 20, 21) owing to its antimicrobial (28), antifungal (28, 29), antioxidant (28), and anti-inflammatory (30) activities. Thus, the present investigations were designed to evaluate the potential of propolis alone as well as in combination with Dulbecco's modified Eagle's medium (DMEM) as storage/transport medium to preserve the viability of cultured PDL cells. The experiments were carried out using primary cultures of human PDL cells under *in vitro* conditions for the exposure periods of 30 min to 24 h.

Materials and methods

Samples collection

The teeth were collected from healthy individuals having no caries beyond the cemento-enamel junction and no periodontal disease. The sex and race of the patients were not considered for this study and hence not recorded. Extracted teeth were immediately placed in minimum essential medium (MEM) with low glucose (Gibco BRL, Grand Island, NY, USA) + penicillin 10 000 IU/100 ml, streptomycin 10 000 IU/100 ml, and fungizone 250 μg/100 ml medium (PSF) to maintain periodontal cell viability during transport and also help to reduce sample contamination by initiating antibacterial/antifungal activity.

Cell culture

The primary cultures of human PDL fibroblasts were obtained following the methods of Pant et al. (31). Briefly, the teeth were washed in MEM containing antibiotic–antimycotic, then adherent soft tissues were removed from the crown as well as the coronal one-third of the root and discarded. The crown and coronal one-third of the root were then placed in 5.25% NaClO for 2 min to reduce bacterial contamination, as well as to kill any remaining gingival epithelial cells. The middle third of the root was scraped to obtain PDL tissue specimens. The tissue specimens were placed in a sterile petri dish containing a thin layer of MEM with 10% fetal bovine serum. The PDL tissue was disaggregated using 0.2% collagenase and 0.125% trypsin for 30 min at 37°C, and the cells were collected by centrifugation at 100 ***g*** for 5 min. The pellet of packed cells was then resuspended in the six-well culture plates in complete MEM and incubated at 37°C with an atmosphere of 95% air and 5% CO_2 for the attachment. Growth was permitted until the cells attained a confluence. These cells were trypsinized (trypsin 0.05%–EDTA 0.53 mM) and passaged into T-25 culture flasks to expand the cell population (first cell passage). The cells of third and fourth passages were trypsinized and pooled for experimentation (to control the cell variability). Cell number for experimentation was determined using an Electronic Coulter Counter (Model Zf; Coulter Electronics, Hialeah, FL, USA). The number of viable cells in each batch was measured by the trypan blue dye exclusion test before each experiment, and batches showing more than 95% viability were used for the experiment.

Preparation of propolis and other media

Dry tablets of propolis were commercially procured in the product name of Forever Bee Propolis® from M/s Forever Living Products, India. Following grinding, stock solution of propolis was prepared by dissolving it in dimethyl sulfoxide (DMSO). Working dilutions of propolis i.e., 2.5%, 5%, 10%, and 20% were prepared by diluting the stock solutions using minimal essential medium (Sigma Chemical Co., St Louis, MO, USA). Pasteurized low fat content milk was used having the composition −3.5 g protein, 0.5 g lipids, 5.0 g carbohydrates, 124 mg calcium, and 0.19 mg riboflavin per 100 ml. HBSS and DMEM were procured from Sigma Chemicals Co. Mixtures of propolis and DMEM were prepared in sterilized double distilled water. Artificial saliva was prepared according to the composition given by Leung et al. (32).

Exposure of PDL cells to storage media

The trypsinized cells (1×10^4 cells well^{-1} in 100 μl culture medium) were plated in 96-well plates and allowed to adhere for 24 h. Then, the medium was replaced with the low serum medium (MEM + 2% fetal bovine serum + PSF) to make the cells more receptive, and the plates were returned to the incubator for 18 h. Subsequently, the medium in the wells was replaced with the experimental solutions. The low serum containing MEM served as the positive control. Six replicates were used to examine each test solution and control sets. The exposure periods were for 30, 60, 180 min (short-term exposure) and 6, 12, and 24 h (long-term exposure) at 37°C.

Cell viability assays

Trypan blue dye exclusion test

In principle, the cells with damaged membrane allow the trypan blue dye to pass through membrane into cytoplasm, whereas undamaged cells exclude dye. The test was conducted to study the cell viability by assessing the loss of membrane integrity following the method of Pant et al., (33) with desired modifications. In brief, immediately after the completion of respective incubations, cells were aspirated and subjected to stain with trypan blue dye (0.4% solution) at a ratio of 1:5 (dye: cell suspension) and placed in hemocytometer. The counting for live (unstained transparent) and dead (blue stained) cells was made at 100× magnification in phase-contrast inverted

microscope (Leica DMIL, Wetzlar, Germany). The untreated sets were also run parallel under the identical conditions and served as control.

MTT assay

The assay shows the ability of the cells to convert the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide tetrazolium salt into a purple MTT formazon, by mitochondrial dehydrogenase activity of live cells, which is measured spectrophotometrically at 550 nm. MTT assay provides an indication of mitochondrial integrity and activity, which is interpreted as a measure of percent cell viability. The MTT assay was performed following the method of Siddiqui et al., (34) with desired modifications. In brief, cells (1×10^4 per well) were seeded in 96-well tissue culture plates and then exposed to different storage media for different time intervals as described earlier. Tetrazolium bromide salt (5 mg ml^{-1} of stock in PBS) was added (10 μl $well^{-1}$) in 100 μl of cell suspension for 4 h. At the end of incubation, the reaction mixture was carefully taken out and 200 μl of DMSO was added to each well by pipetting up and down several times until the content was homogenized. The plates were kept on rocker shaker for 10 min at room temperature and then readings were taken at 550 nm using Multiwell Microplate Reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT, USA). Untreated sets run under the identical conditions were served as basal control, whereas cells treated with manganese (10^{-3} and 10^{-4} M) were used as positive control.

Neutral red uptake (NRU) assay

The assay shows the lysosome-mediated catabolic activity of viable cells by engulfing and accumulating the neutral red vital dye. The accumulation of neutral red dye is measured spectrophotometrically at 540 nm. The assay provides an indication of catabolic impairment of the cells caused by any physical and/or physiological stress, which is interpreted as measurement of cell viability. NRU assay was performed following the protocol of Siddiqui et al., 2008 (34) with desired modifications. Briefly, cells were exposed to various storage media for different time periods described earlier. On the completion of respective incubation periods, the test solution was aspirated and cells were washed twice with PBS. Cells were then incubated for 3 h in a medium supplemented with neutral red dye (50 μg ml^{-1}). The medium was washed off rapidly with a solution containing 0.5% formaldehyde and 1% calcium chloride. Cells were then subjected to incubation further for 20 min at 37°C in a mixture of acetic acid (1%) and ethanol (50%) to extract the dye. The plates were read at 540 nm using Multiwell Microplate Reader (Synergy HT). The obtained values were compared with the values of control sets, which were run under identical conditions in MEM only.

Statistical analysis

The results are expressed as mean and standard error of means (SEM) for at least six wells in three independent experiments, as indicated in the Figs 1–3 and Table 1. ANOVA was employed to detect differences between the groups of treated and control. $P < 0.05$ was taken to indicate significant differences.

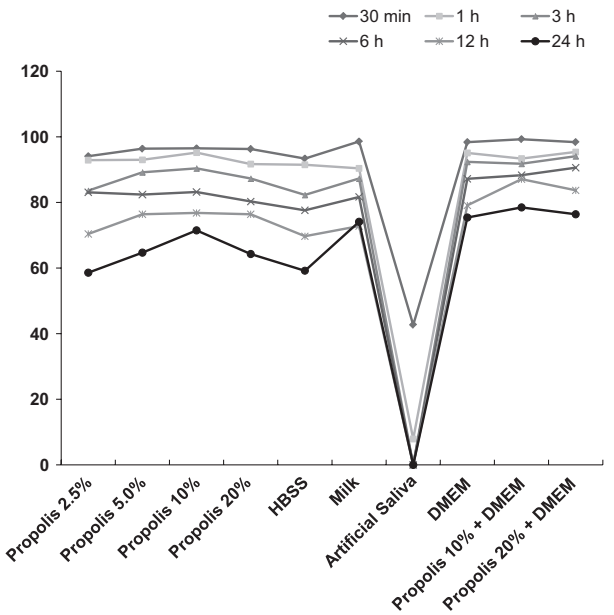

Fig. 1. Effect of propolis and its combination with Dulbecco's modified Eagle's medium on the cell viability of cultured human periodontal ligament cells kept for various time periods (trypan blue dye exclusion assay: loss of membrane integrity). The values are mean of three independent experiments. SE values were < 7.0% of the values.

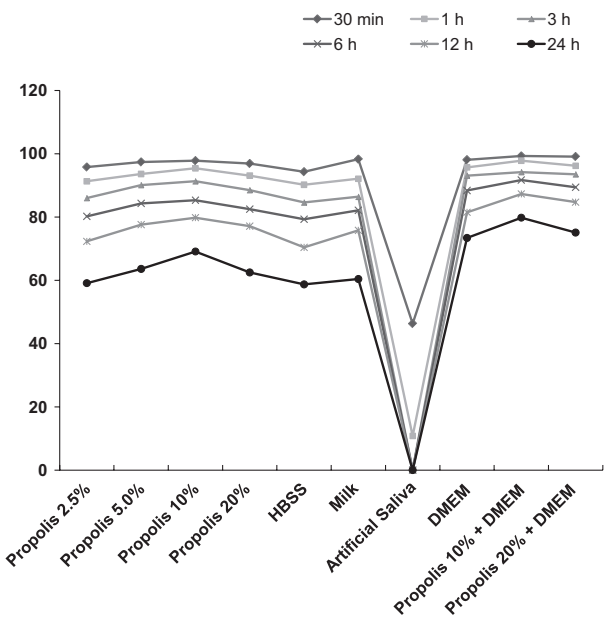

Fig. 2. Effect of propolis and its combination with Dulbecco's modified Eagle's medium on the cell viability of cultured human periodontal ligament cells kept for various time periods (MTT assay: mitochondrial enzymatic activity). The values are mean of three independent experiments. SE values were < 7.0% of the values.

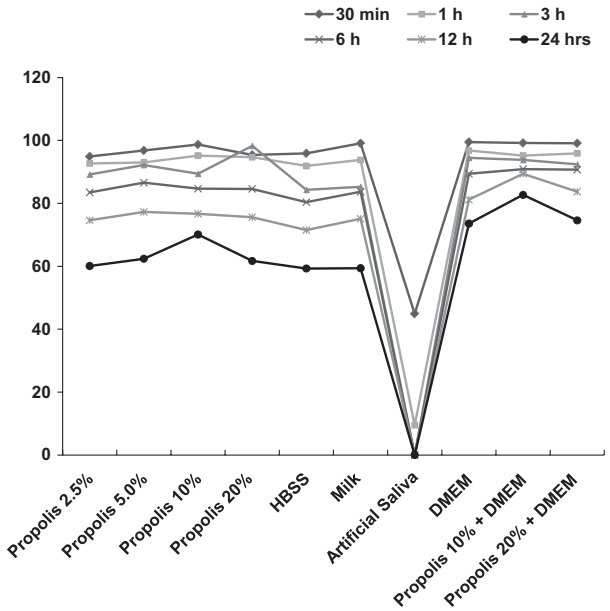

Fig. 3. Effect of propolis and its combination with Dulbecco's modified Eagle's medium on the cell viability of cultured human periodontal ligament cells kept for various time periods (NRU assay: lysosomal activity). The values are mean of three independent experiments. SE values were < 7.0% of the values.

Results

Data highlights for loss of percent cell viability are summarized in Tables 1 and 2 and Figs 1–3. Statistically insignificant decrease in cell viability was observed in time-dependent manner. No significant changes were observed in the viability of cells until 3 h of exposure except for artificial saliva. Cell survival was significantly ($P < 0.001$) affected in artificial saliva within 30 min and this loss of percent cell viability gradually increased in subsequent incubations and brought down toward absolute lethality by 12 h. Until 3 h, differences were insignificant among the propolis group; however, the results were comparable between individual concentrations of propolis and propolis combination with DMEM.

Trends of time-dependent loss of percent cell viability became prominent with the extended periods of incubations i.e., 6, 12, and 24 h. By 24 h, propolis 10% was found to be the most efficacious to preserve the viability of PDL cells among all the concentrations of propolis used in the study. However, in trypan blue dye exclusion assay, milk has shown better performance than propolis 10% on independent exposure group. Among the co-exposure group, propolis 10% + DMEM has shown the best performance and was able to retain the cell viability up to 79.8 ± 3.4%, 82.7 ± 3.4%, and 78.5 ± 5.6% at 24 h in MTT, NRU, and trypan blue assays, respectively. Overall performance of coexposure of propolis + DMEM was found to be better than any of individual exposure all through the exposure tenures i.e., 30 min to 24 h. In general, around 80% and 90% cells were viable and physiologically functional until 6 h in independent storage media except artificial saliva as well as in coexposure of propolis + DMEM, respectively. The cell survival was found to significantly better in propolis 10% + DMEM at 12 and 24 h, when compared with propolis (2.5%, 5.0% and 20%) and HBSS. However, this difference could not reach to significant level, when compared with DMEM and propolis 20% + DMEM. Trypan blue dye exclusion assay was found to be most sensitive among the endpoints used in the study.

To identify the suitability of propolis, if any, significance analysis was carried out comparing the data of propolis 10% + DMEM with the rest of the media used in the study (Table 2). It has appeared that propolis 10% + DMEM was statistically ($P < 0.05$) better than propolis 2.5%, 20%, and HBSS even at 6 h of incubation. By the end of 12 h, difference level of significance ($P < 0.01$, $P < 0.001$) was increased from most of the media used, except milk, DMEM, and propolis 20% + DMEM. The magnitudes of statistical differences were further increased by 24 h. But the differences in percent cell viability were insignificant all through the

Table 1. Percent cell viability of cultured periodontal ligament fibroblasts following different storage periods in various storage media according to trypan blue exclusion assay (cell viability % + SE value)

	Incubation period (min h^{-1})					
	30 min	1 h	3 h	6 h	12 h	24 h
Storage media	Trypan blue exclusion assay (cell viability % + SE)					
Propolis 2.5%	94.1 ± 2.4	92.9 ± 2.4	83.6 ± 2.5	83.1 ± 3.4	70.4 ± 2.5	58.6 ± 3.1
Propolis 5%	96.4 ± 3.2	93.0 ± 3.1	89.2 ± 3.4	82.4 ± 4.2	76.4 ± 2.1	64.7 ± 3.5
Propolis 10%	96.5 ± 3.4	95.2 ± 3.2	90.4 ± 3.5	83.2 ± 3.5	76.8 ± 2.2	71.5 ± 2.4
Propolis 20%	96.3 ± 2.5	91.7 ± 2.4	87.3 ± 1.9	80.3 ± 1.9	76.4 ± 3.1	64.3 ± 3.4
HBSS	93.4 ± 3.4	91.5 ± 2.3	82.3 ± 1.8	77.6 ± 1.6	69.7 ± 4.1	59.2 ± 3.6
Milk	98.6 ± 4.2	90.4 ± 5.2	87.3 ± 2.6	81.7 ± 2.5	72.8 ± 2.5	74.1 ± 1.5
Artificial saliva	42.7 ± 5.3	7.9 ± 4.2	0 ± 0	0 ± 0	0 ± 0	0 ± 0
DMEM	98.4 ± 2.3	95.1 ± 3.4	92.4 ± 2.3	87.2 ± 6.1	79.1 ± 4.3	75.4 ± 6.7
Propolis10% + DMEM	99.3 ± 3.5	93.4 ± 1.3	91.8 ± 3.4	88.3 ± 3.1	87.1 ± 3.5	78.5 ± 5.6
Propolis20% + DMEM	98.4 ± 3.1	95.4 ± 1.5	94.1 ± 3.5	90.6 ± 3.2	83.7 ± 3.6	76.4 ± 4.3

DMEM, Dulbecco's modified Eagle's medium; HBSS, Hank's balanced salt solution.

Table 2. A cross-sectional statistical analysis to identify the significance levels between propolis 10% + DMEM and rest of the media used in the study. One-way ANOVA followed by Dunnett test was employed to calculate the significance values

	Incubation period (min h^{-1})					
	30 min	1 h	3 h	6 h	12 h	24 h
Storage media	(Propolis 10% + DMEM) significance level					
Propolis 2.5%	NS	NS	NS	<0.05	<0.001	<0.01
Propolis 5%	NS	NS	NS	NS	<0.01	<0.05
Propolis 10%	NS	NS	NS	NS	<0.01	<0.05
Propolis 20%	NS	NS	NS	<0.05	<0.01	<0.001
HBSS	NS	NS	NS	<0.05	<0.001	<0.001
Milk	NS	NS	NS	NS	NS	<0.01
Artificial saliva	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
DMEM	NS	NS	NS	NS	NS	NS
Propolis20% + DMEM	NS	NS	NS	NS	NS	NS

NS = non-significant.
$P < 0.05$ = significant.
$P < 0.01$ = greater significant.
$P < 0.001$ = highly significant.
DMEM, Dulbecco's modified Eagle's medium; HBSS, Hank's balanced salt solution.

incubations, i.e., up to 24 h, when compared between propolis 10% + DMEM vs DMEM and propolis 20% + DMEM. The values for artificial saliva were highly significant for 30 min of incubation (Table 2).

Discussion

In the present investigations, propolis preparation was found to be better than other storage media used. This might be attributed to its antibacterial (28), antimycotic (28, 29), antioxidant (28), and anti-inflammatory (30) properties. As inflammatory responses (14, 34), ankylosis (14, 33) along with loss of cell viability in PDL cells attached to avulsed tooth because of bacterial contaminations and oxidative stress–mediated events (1) are well reported during extra-alveolar period. A thick literature is available showing better prognosis of replantation of avulsed tooth by minimizing these post-traumatic events during extra-alveolar period (2, 9, 10, 20, 21, 35). Our results indicate that the percentage of viable cells increases by using 10% of propolis concentration followed by a decline at 20% of propolis concentration (Tables 1 and 2). Our findings are in well coordination with earlier study carried out by Özan et al. (21). The antagonistic effects of propolis at 20% concentration might be because of its own cytostatic/cytotoxic responses.

In general, a synergism in the cell viability and preservative potential was observed in propolis and DMEM combination. DMEM is a complex mixture of nutrients and supplements required to culture the variety of cells and tissues of human and animal origin, thus reported as a potential medium for both short (36) and long (37) storage of avulsed tooth. We hypothesized that propolis provides antibacterial, antimycotic, antioxidant, and anti-inflammatory properties, whereas DMEM acts as extended source of energy and other required nutritional supplements with osmotic stability (38, 39). In combination, propolis 10% + DMEM was found better than propolis 20% + DMEM. This finding also confirms that 20% concentration of propolis might have some cytotoxic/cytostatic response on the cell physiology (21). Although propolis 10% + DMEM shows better viability than DMEM alone as well as propolis 20% + DMEM groups, the difference could not reach statistically significant level. Thus, our study suggests the equal suitability for all three storage media viz., DMEM, propolis 10% + DMEM, and propolis 20% + DMEM to keep the cells viable and better prognosis even after longer extra-alveolar period during accidental avulsion or elective replantation of tooth.

In short-term exposures, i.e., up to 3 h, all the tested storage media were found to be equally effective in maintaining PDL cells' viability except in the case of artificial saliva. This was quite anticipated because storage media used such as propolis, milk, DMEM are isotonic solutions with nutrients required to prevent the energy failure and suggested as good storage media (2, 10, 11, 21) for quite long period, while HBSS is a buffer to keep salt balancing with the maintenance of isotonic conditions (40). So, until 3 h, energy failure was not an issue for loss of cell viability in these storage media. At the same time, cells' own homeostasis system also is triggered and tries to maintain the energy supply and normal physiology using stored cytosolic materials. However, in case of artificial saliva, a significant ($P < 0.001$) decrease in cell viability was seen even at 30 min, further decrease with the increase in incubation, and reached toward 100% cell death by 3 h. The microscopic examinations had also been shown cell swelling and membrane rupture at large scale, which was as a result of hypotonic environmental conditions because of very low osmolality (66 mOsm kg^{-1}) coupled with energy failure constraints (21, 35). Our findings for artificial saliva have been closely associated with the findings of earlier studies carried out under similar setups (16, 38).

By the end of long-term exposure, i.e., 24 h, it was clearly apparent that DMEM was the best among the individual exposures. We believe that this might be

attributed to its composition as complete nutrition for cultured cell growth along with osmotic balancing, which kept the PDL cells physiologically more active, viable, and healthy for longer period of time outside the oral cavity. Such kinds of promising results with DMEM have also been reported in the past (38, 39). The combination of propolis 10% + DMEM stood at first position in overall ranking till the end of exposure period, i.e., 24 h. This may be suggested because of energy supply from DMEM (38, 39) and restriction on inflammation, oxidative stress, bacterial and fungal infections through propolis (28–30). Our result also shows the linearity with earlier findings showing milk as one among the good media for the storage of PDL cells (20, 26). Such promising results with milk may be suggested because of physiological osmolality as well as high nutritious values (10, 23).

Trypan blue dye exclusion assay was found to be the most sensitive assay among all the assays carried out in this study. This may be attributed because of direct visualization of loss of membrane integrity, which leads to the cell death. Whereas MTT and NRU assays are indirect assays and give the status of impaired activity of mitochondria and lysosomes respectively. Owing to organelles specificity, these tests have been reported to give false positive/negative/masking of the results (40). Quite often, cell dies because of loss of membrane integrity as it is the first site to interact with any xenobiotic, but even within the dead cells, some enzymatic activity in mitochondria and lysosomes is reported. Under such circumstances, MTT and NRU assays are known to show some higher values than the exact (34).

Conclusion

All the tested storage media except artificial saliva were found to be equally effective in maintaining PDL cell viability up to 3 h. Present investigations indicate the equal suitability of all three storage media viz., DMEM, propolis 10% + DMEM, and propolis 20% + DMEM to keep PDL cells viable up to 24 h of extra-alveolar period during accidental avulsion or elective replantation of tooth. Other more readily available medium such as milk may also serve as appropriate alternative storage medium for shorter time periods, i.e., up to 12 h. Trypan blue dye exclusion assay was found to be most sensitive among the assays selected in the present investigations.

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