AL-AZHAR
Dental Journal
For Girls

The Official Publication
of The Faculty of Dental
Medicine For Girls,
Al-Azhar University
Cairo, Egypt.

Print ISSN 2537-0308 • Online ISSN 2537-0316

ADJ-for Girls, Vol. 9, No. 2, April (2022) — PP. 331:339

Effect of Propolis and Castor Oil Cement as Pulpotomy Materials on the Properties and Osteogenic Differentiation of Stem Cells Isolated from Human Exfoliated Deciduous Teeth (SHED)

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Codex : 16/22.04

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http://adjg.journals.ekb.eg

DOI: 10.21608/adjg.2022.29419.1257

Pediatric Dentistry & Orthodontics
(Pediatric Dentistry, Orthodontics)

ABSTRACT

Purpose: The aim of this research was to assess the effect of propolis and castor oil cement as pulpotomy materials on the stemness properties and osteogenic differentiation potential of stem cells isolated from human exfoliated deciduous teeth (SHED). **Materials and methods:** SHEDs were isolated from the pulp of five deciduous anterior teeth indicated for extraction. SHEDs were cultured with propolis and castor oil cement extracts. SHEDs cultured in MTA extracts and basal media were used as positive and negative controls respectively. Stemness properties of SHEDs were assessed in the form of proliferation, migration and the ability to enhance wound healing. Moreover, osteogenic differentiation potential of the isolated SHEDs was evaluated. **Results:** No significant statistical difference between propolis and castor oil cement regarding cell proliferation was detected. SHEDs cultured in Castor oil cement showed higher migration ability than those cultured in propolis. The ability to enhance the healing of the wound was more in castor oil cement group rather than in the propolis group. Finally, castor oil cement induced osteogenic differentiation while no formation of calcified nodules was noticed in the presence of propolis. **Conclusions:** Because of the limitation of the current research it was came to conclusion that Castor oil cement may have greater potentials as a pulpotomy material than propolis.

KEYWORDS

Propolis, Castor Oil Cement, pulpotomy, Stem Cells.

- Paper extracted from Doctor thesis titled "Effect of Propolis and Castor Oil Cement as Pulpotomy Materials on the Properties and Osteogenic Differentiation of Stem Cells Isolated from Human Exfoliated Deciduous Teeth (SHED)"

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INTRODUCTION

Repair of dental pulp is still a massive clinical obstacle as human teeth have a little ability to regenerate[1]. Dental pulp has an enormously proliferative stem/progenitor cells possessing self-regeneration and differentiation aspects that commonly remain inactive when they are in the dental pulp and quickly react after insult[2].

Stem cells are defined as undifferentiated cells that have the ability to synthesize new more stem cells (auto-renewal). These cells can differentiate to multiple special cell lineages that have further particular properties which ensures repair and regeneration of tissue throughout life[3].

It was known that stem cells extracted from human exfoliated deciduous teeth (SHED) constitute a portion of postnatal stem cells. SHED is a good source of mesenchymal stem cells that can be potentially capable of proliferating and differentiating extensively[4].

Pulpotomy is almost the popular way for protection of pulp vitality of primary teeth. It is the most favourable method if only the coronal pulp is inflamed as a result of invasion of the bacteria that occurs after carious, traumatic or due to iatrogenic reasons. So, the choice of pulpotomy materials mostly influences the abiding results[5].

Lately, a continual study has been done to discover additional biocompatible and naturally made substances for use in dental practice[5]. Propolis was instructed to be used as; a mouth rinse, endodontic irrigant, in treatment of dentinal hypersensitivity, and recurrent aphthous stomatitis. Moreover, it is used as a storage medium for avulsed teeth[6]. Castor oil cement also exhibited a good potential as a pulp capping material. The chemical component of this substance has a series of fatty acids whose molecular components are also found in the human body fatty tissue. Accordingly, the cells do not consider castor oil cement as a foreign body[7].

It is believed that the pulp reaction changes when exposed to different available products which could cause serious damage to it[8]. Cell culture methods are beneficial in assessing the biocompatibility of substances used in dental procedures. Infact, in vitro assays with cell cultures are frequently used to explain the methods involved in various biological reactions and to evaluate cell behavior in certain circumstances[9].

Therefore, this work was performed to assess the action of propolis and castor oil cement as pulpotomy materials on the stemness properties and osteogenic differentiation potentials of stem cells isolated from human exfoliated deciduous teeth (SHED).

MATERIALS AND METHODS

The specimens were divided into five groups:

- Untreated cells as the negative control group
- Propolis group
- Castor oil cement group
- Dimethyl sulfoxide (DMSO) (vehicle) group
- Mineral trioxide aggregate as a positive control group

The armamentarium used in this study and their manufactures are illustrated in (Table 1).

1- Isolation and culture of SHEDs:

This study was approved by the Research Ethics Committee (REC) of the Faculty of Dental Medicine for Girls, Al-Azhar University (**REC17-93**). Teeth were obtained from children according to ethical guidelines of the Faculty of Dental Medicine for Girls, Al-Azhar University. Signed informed consent was obtained from the parents/guardians (n= 5 teeth from 7 to 9 years).

Extirpated pulp was minced into fragments then digested using 0.2% collagenase type two for 1 hour at 37°C. Isolated cells were cultured in DMEM supplemented with penicillin/streptomycin and 15% FBS and then incubated at 37ºC and 5% CO2. When the adherent cells became 70%-80% confluent they were passaged. The cells at the fifth passage were used in the subsequent experiments.

Table (1): *The armamentarium used in this study*

Item category	Manufacture	Location
Dulbecco's Modified Eagle's Medium (DMEM)	Lonza Yerviers SPRL	Belgium
Penicillin/streptomycin	Invitrogen Co.	USA
Fetal bovine serum (Fbs)	FBS, Life Science	United Kingdom
Collagenase type two	Serva Electrophores	Germany
Propolis	Beehives	Egypt
Castor oil	DE TUINEN	Netherland
Methylene diphenyl diisocyanate (MDI)	Sigma	Germany
Calcium carbonate	Oxford	India
Mineral trioxide aggregate (MTA)	ANGELUS	Brazil
Cell counting kit 8 (CCK-8)	Sigma	Germany
Giemsa stain	Biodiagmostic	Egypt
Osteogenic differentiation kit	Gibco®, StemPro®	USA
Alizarin red stain	Loba chemic	India
Migration kit	Greiner bio-one	Switzerland
0.22 µm filter	Thermo Fisher Scientific, Nalgene™	USA
Inverted phase-contrast light microscope	DMi1-Leica	Germany
Water bath shaker	MaxturdyTM- DAIHAN Scientific	South Korea
Microplate reader	BMG Lab-Tech	Germany

2- Preparation of materials condition media:

The ethanolic extract of propolis was prepared by dissolving 5 grams of propolis in 100ml of 70% ethanol at 37°C. The solution was incubated in a water bath at 60°C at 250 rpm for another 24 hours in dark. The dissolved propolis was centrifuged at 4000 rpm for 10 minutes and then was filtered using a 0.22 µm filter. The solution was left to evaporate in a water bath at 50°C. The resultant extract was dissolved in dimethyl sulfoxide and diluted with MDEM to a final concentration of 0.1%[10,11].

The castor oil cement was prepared by mixing castor oil, prepolymer (MDI) and calcium carbonate (CaCo3) (in a ratio 1:1:1) until homogenization was obtained[12]. MTA was prepared according to manufacturer instruction. The materials were sterilized by ultraviolet light for 24 hours. DMEM was added to the prepared castor oil cement and MTA and incubated for 24 hours at 37°C.

3- Cell proliferation assay:

Cell proliferation was measured using CCK-8, SHEDs ($1x10^4$/well) were cultured in 96-well plate in DMEM supplemented with 15% FBS for 24 h. Then the media were replaced by the prepared conditioned media of; propolis, castor oil cement, MTA and DEMSO for 24 hours and 48 hours. At each time interval, 10µl/well of CCK-8 was added and incubated for 4h. Absorbance was measured using a microplate reader at 450 nm. The experiments were performed in triplicate[13-15].

4- Cell migration assay:

A. Transwell migration assay: Cell migration was assessed using a two-chamber Transwell system (8mm pore size and 6.5 mm diameter). The material extract supplemented with 15% FBS was added to the lower wells of a 24-well plate. Migration Chambers were put in place and $0.5x10^5$ cells were suspended in 100 ul serum-free

DMEM and seeded on the migration chambers. The upper chambers were transferred to the lower wells and incubated at 37°C for 24 hours. The cells were then fixed in 4 % formaldehyde for 2 minutes and stained with Giemsa stain for 15 minutes. Cells failed to migrate were gently wiped off with a cotton swab from inside the chamber. The wells were then observed under an inverted microscope and the numbers of migrating cells in each well were counted in random fields. The experiments were performed in triplicate independently[16-18].

B. Wound healing assay: SHEDs were cultured in a 6 well plate and incubated for 24 hours until reaching 70-80 % confluency. A scratch was made using a sterile 1-ml pipette tip. Material extracts supplemented with 15% FBS were added and pictures were taken under the microscope to calculate the width of the wound using image J software. The cells were incubated for 24 hours. Cells were then stained using Gemisa stain and Pictures were taken to analyze migration and wound healing of the cells using Image J software. The experiments were performed in triplicate independently[19-21].

5- Osteogenic differentiation assay:

In osteogenic differentiation medium supplemented with 15% FBS were used as a positive control. SHEDs were also cultured with various material extracts supplemented with 15% FBS. All cells were incubated for 21 days and the media was changed every 3 days. After 21 days, the cells were fixed in 10% formaldehyde solution and then stained with 2% alizarin red. The experiments were performed in triplicate[22-24].

Statistical analysis:

Analysis of data was performed using SPSS 23 (Statistical Package for Scientific Studies) for Windows. The description of variables was presented in the form of mean, and standard deviation (SD). Data were explored for normality using Shapiro-Wilk test. Comparisons between groups for quantitative variables were carried out with the one-way analysis of variance (ANOVA) test followed by Tukey's Post hoc test. Results were expressed in the form of P-values. The significance level was set at $P \leq 0.05$.

RESULTS

1- Cell proliferation:

Statistically, the difference among the proliferation rates of SHEDs in the castor oil cement group, propolis group and DMSO group after 24h and 48h when compared to those in the -ve Control group and +ve Control group was not significant ($p>0.05$). Moreover, DMSO as a vehicle for propolis, showed little influence on the proliferation rate of the cultured cells (Table 2 and Fig. 1).

Table (2): *The mean absorbance rate of all groups after 24h & 48h*

Time	Control	MTA	Castor oil cement	Propolis	DMSO	P-value
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
24h	2.68±0.08	2.51±0.29	2.83±0.03	2.83±0.09	2.87±0.11	0.068
48h	2.91±0.07	2.88±0.10	2.86±0.04	2.96±0.18	2.83±0.17	0.750

SD: Standard deviation

2- Cell migration:

Image J analysis showed there was no detectable variations in the count of migrated cells in castor oil cement, DEMSO and negative control groups ($p>0.05$) while the count of migrated cells in castor oil cement group was significantly more than that of MTA and propolis groups($p<0.0001$). Moreover, the count of migrated cells in the propolis group was significantly lower than all other groups ($p<0.0001$) (Table 3 and Fig. 1).When the mean percentage of wound healing was analyzed using image J, the difference among all groups on day one was not significant ($p>0.05$). However, on day two a statistically significant increase in wound area was detected in the propolis group ($p<0.0001$) (Table 4 and Fig. 1).

3- Osteogenic differentiation assay:

On day thirty, all groups were stained with Alizarin red stain. Castor oil cement group and MTA group gave positive results indicating a successful osteogenic differentiation when compared with the osteogenic differentiation group. The lowest calcium accumulation was detected in the –ve control group. In the propolis group, on day four, the proliferation rate of cells started to decline. No formation of calcified nodules was noticed, on the contrary, cells started to degenerate starting six till complete degeneration on day thirteen. Staining with Alizarin red was not possible at this stage.

Table (3): *The mean numbers of migrated cells in all groups*

Control	MTA	Castor oil cement	Propolis	DMSO	P-value
Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
266.67±19.22[a]	222.56±19.65[b]	273.22±21.64[a]	19.33±5.81[c]	264±18.27[a]	**<0.0001***

SD: Standard deviation, *statistically significant p-value < 0.05
[a,b, and c:] Different small letters indicate significant differences between the two groups

Table (4): *The mean percentage of wound healing*

	Control	MTA	Castor oil cement	Propolis	DMSO	P-value
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Day 1	23±14.23	21±10.13	20±15.64	9±12.89	20±9.03	0.130
Day 2	[a] 91±2.20	[a] 83±3.27	[a] 86±6.05	[b] -13±17.23	[a] 90±2.99	***0.0001>**

SD: Standard deviation, *statistically significant p-value < 0.05
[a,b, and c:] Different small letters indicate significant differences between the two groups

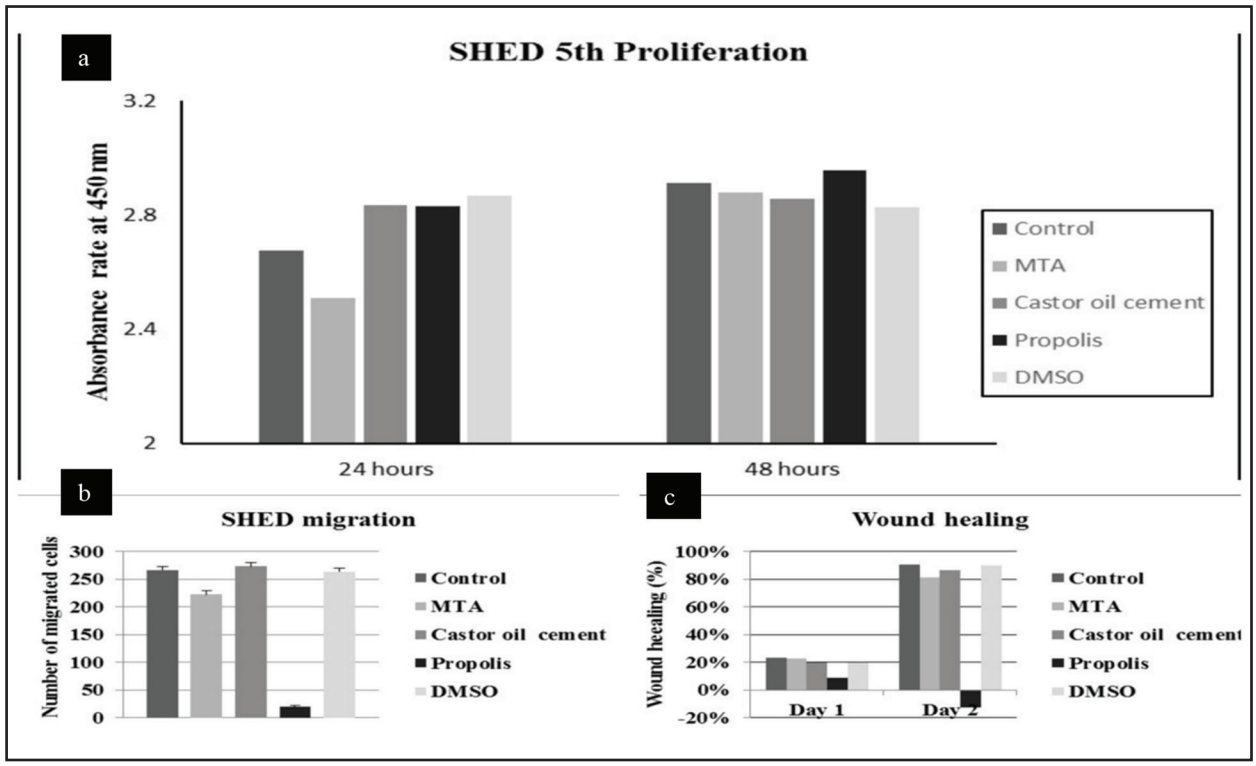

Figure (1) Bar graph revealing the mean absorbance rate of all groups after 24h & 48h (a), Bar chart showing the mean numbers of migrated cells within all groups (b) and Bar chart showing the percentage of wound healing on day one and two in all groups (c).

DISCUSSION

SHEDs in the tooth pulp tissue reveal the ability of auto-regeneration and multilineage differentiation and are a cause of pulp cure. When pulp exposure occurred and capped by biocompatible substances, SHEDs proliferate, migrate to the affected area, and differentiate to odontoblast-like cells, that successively accumulate reparative dentine. An ideal pulpotomy material should evaluate the capability of stimulating and modulating the healing technique generated by SHEDs[25].

In this study, Propolis induced SHEDs proliferation which agrees with a previous study which showed that SHEDs proliferated only when treated with the lowest concentration of propolis[14]. Also, the current study is relatable to previous studies which showed that lower concentrations of Propolis increased the proliferation of HDPSCs and human fibroblasts[10,26]. The outcomes of the current work were in accordance with a study on the influence of MTA and Brazilian propolis on properties of DPSCs that showed that both propolis and MTA induced the proliferation of DPSCs[5]. The mechanism for the increased proliferation in this research were linked into the active ingredients present in Propolis; flavonoids are believed as the most pharmacologically active constituent and potent antioxidant[27]. Although, a contrast action was showed by other researchers who noted that propolis has an anti-proliferative influence on cutaneous fibroblast[28].

Castor oil cement was able to induce SHEDs proliferation. This agrees with a previous study that revealed that castor oil cement and MTA extracts had induced cell proliferation of transfected human pulp cells (tHPCs)[29].

A study came in agreement with the outcomes of this work stated that caffeic acid in addition to caffeic acid phenethyl ester, the active ingredients in propolis, disturbed the migration of malignant breast cells[30]. In contrast to the outcomes of the

present research, a study showed that the migratory ability of BMMSCs was accelerated when propolis was present[31].

Castor oil cement in this study had the ability to induce SHEDs migration significantly more than propolis and MTA. This can be considered the first results concerning the effect of Castor oil on SHEDs migration ability since no previous study has evaluated its effect.

Moroever, the difference among all groups on day one considering the mean area of the induced wound was not significant. However, on day two statistically significant increase in wound area was found in the propolis group. These findings correlate with a study that reported notification on the action of propolis on glioblastoma and normal fibroblast cell lines. It was observed that propolis had a potent antagonistic action on wound healing of the two cell lines[32]. In contrast to the present study, some authors have claimed that propolis promotes visually faster wound closures on connective tissue fibroblasts[33].

Also, the difference among castor oil cement and MTA regarding the area of wound healing was not significant, however, the castor oil cement showed slightly better wound healing than MTA on day two. This result came in agreement with a study that confirmed that the combination of calcium carbonate and castor oil polymer resulted in a polymer mix that revealed greater wound healing in rabbit tibia[34]. In contrast to the current study, delaying in the healing of the wound was found in a previous study when castor oil cement was existed in the cervical third of rodent alveolus[35].

Propolis failed to induce differentiation of SHEDs, this agrees with a previous study which showed that BMMSCs revealed deficient osteogenic differentiation in the existence of propolis, assuming that propolis extract can have an inhibitory action on osteogenic differentiation[31]. In contrast to the current results, a previous study showed that propolis exhibited significantly higher mineralization promotion effects on DPSCs than MTA[5]. Also, castor oil cement had successfully induced osteogenic differentiation of SHEDs. Although no other studies have assessed the influence of castor oil cement on the osteogenic differentiation of human stem cells.

Zoology researches have revealed that castor oil cement induced fibroblastic neoformation of bone after implantation into rat alveolus, granules were implanted into rodent lung tissue and surrounded by a fibrous capsule which turned less in thickness than normal and by the 6th week after operation, the examined spot was nearly fully covered with mature osseous tissue[34].

The limitations of this study were due to the lack of investigations of the antimicrobial effects of propolis and the inability to regard its various methods of extraction. Studies on castor oil cement were not abundantly available.

CONCLUSIONS

Under the limitations of that research, it was came to conclusion that:

Both propolis and castor oil cement can induce the proliferation of stem cells isolated from human deciduous teeth. Regarding propolis, it was found that it has the lowest effect on migration of SHEDs, and it either couldn't stinulate wound healing or induce osteogenic differentiation. As regards castor oil cement, it has a superior effect on the migration of SHEDs, a good effect on wound healing and can successfully induce osteogenic differentiation of SHEDs.

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