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ORIGINAL ARTICLE

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Comparative Evaluation of the Cytotoxic Effect of Different Intracanal Medicaments on Stem Cells of the Apical Papilla: A Cell Culture Study

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Abstract

Background: Regenerative endodontics involves biologically driven techniques aimed at restoring compromised tissues, such as dentin, root structures, and the cellular components of the pulp–dentin complex. The effectiveness of these procedures largely relies on the viability of stem cells. Antibiotic pastes used for microbial elimination are often limited in concentration due to their potential toxicity to stem cells, while lower concentrations raise concerns regarding efficacy. Phytomedicines have been used extensively due to their efficacy and fewer adverse effects. Carnosic acid is one such phytomedicine which has proven to have a good effect against degenerative diseases because of their good regenerative potential. Moreover, studies have shown carnosic acid having better disinfection capacity than triple antibiotic pastes (TAP) as intracanal medicament. However, its regenerative potential in endodontics is yet to be known. Therefore, this study aims to investigate the cytotoxicity of carnosic acid on SCAPs from permanent human teeth in comparison with triple antibiotic paste. **Objective**: To evaluate and compare the cytotoxic effect of carnosic acid on stem cells from the apical papilla (SCAPs) of permanent human teeth Methodology: Stem cells removed from immature teeth were cultivated. After cultivation and third cell passage, modified TAP (metronidazole, ciprofloxacin, and clindamycin) and carnosic acid were placed in cell culture medium. After 1 and 3 days, cell viability in the culture medium was assessed using MTT method ([4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and ELISA (Enzyme-linked immunosorbent assay). **Results**: Carnosic acid has shown more stem cells viability and hence is less cytotoxic than mTAP. **Conclusion**: Within the limitations of the current study, it can be concluded that Carnosic acid has a lower cytotoxic effect on the SCAPs than mTAP. Hence, its use as ICM in regenerative endodontics looks promising. However, further clinical studies are required.

KEYWORDS
Carnosic acid, stem cells, regeneration, intracanal medicaments, triple antibiotic paste

1 | INTRODUCTION

Regenerative endodontics is a biologically driven approach designed to restore damaged tissues such as dentin, root structures, and the cellular components of the pulp–dentin complex. This concept was initially introduced by Dr. Nygaard Ostby in 1961.[1] Regenerative endodontics relies on three fundamental elements: stem cells, scaffolds, and growth factors.[2] The objectives of this treatment include alleviating symptoms, promoting healing, enhancing root length and thickness, and achieving a favorable response to vitality testing.[2] Since microbial reduction in regenerative procedures is primarily accomplished through appropriate irrigation and intracanal medicaments, the choice of material becomes crucial to ensure effective disinfection while maintaining a balance between antimicrobial efficacy and biocompatibility with stem cells.[3] Calcium hydroxide has been routinely used as intracanal dressing in the regenerative procedure.[4] Due to the polymicrobial nature of the root canal niche, antibiotic paste combinations were tried.[4] Triple antibiotic paste consisted of metronidazole, minocycline and ciprofloxacin.[1] But the presence of minocycline poses the risk of staining and discoloration of the tooth.[5] Cefaclor, clarithromycin, clindamycin, amoxicillin

and other antibiotics have been tried to overcome the problem of discoloration.[5] But, allergic potential, host immune resistance and concentration dependent survival of stem cells continued to be some of the challenges. Reports have highlighted the cytotoxic impact of triple antibiotic paste (TAP) on primary pulp stem cells in deciduous teeth. In addition, concerns have been raised regarding the damaging influence of modified TAP (mTAP) on stem cells derived from the apical papilla (SCAPs) of immature permanent teeth.[4] Hence, other phytochemicals have been explored, such as carnosic acid, obtained from the leaves of the rosemary plant (Salvia rosmarinus), which possesses both antimicrobial and antioxidant properties, as suggested by Nieto et al.[6] This material has shown good regenerative potential and low tissue toxicity as suggested by Mirza et al.[7] However, there are not many studies comparing the cytotoxicity of carnosic acid and TAP on long term usage as ICM in regenerative procedures. Therefore, the aim of present study was to assess and compare the cytotoxicity of modified Triple antibiotic paste (mTAP) and Carnosic acid (CA) on Stem cells of apical papilla (SCAPs) of extracted intact human mandibular third molars.

2 | MATERIALS AND METHODOLOGY

SAMPLE SELECTION: After obtaining informed consent from the patients, four healthy immature third molars with open apex (more than 1.5 mm diameter) extracted for orthodontic purpose in the age group of 17-21 years with no history of systemic diseases were extracted to obtain SCAPs. Teeth with caries, previous restoration, endodontic treatment, periapical pathosis and fully developed roots were excluded. Two to five days before tooth extraction, the patients underwent dental prophylaxis, and on the day of extraction, the patients received tooth prophylaxis as well. HARVESTING OF SCAPs AND CULTURE: Teeth were extracted with sterile instruments and were immediately placed in sterile phosphate buffered saline (PBS) (TM Media). SCAPs were then isolated from the apical papilla tissue of incompletely developed tooth using sterile tweezers and placed in a digestive solution containing trypsin. The cell culture medium was changed every two days. Cell passage was performed after cell density in cell colonies reached about 80-70%. Third passage cells were used to assess the cytotoxicity of the drugs. Flow cytometric analysis was performed in the third passage to evaluate the nature of SCAPs and the expression of surface markers.

PREPARATION OF ANTIBIOTICS: Carnosic Acid (CA) (>91% purity; Alpspure Lifesciences Private Ltd, India) was used as one of the test agents. A stock solution was prepared and diluted to obtain a final working concentration of 50 µg/mL. Antibiotic combination consisted of metronidazole (400 mg), ciprofloxacin (500 mg) and clindamycin (500 mg). The enteric coating of these medicines was removed and then crushed with the help of a clean mortar and pestle. Each antibiotic was weighed by a digital scale with an accuracy of 0.1mg. Then 50 µg/ml of each medication was prepared and equal proportions of each medication in the prepared concentration was mixed for mTAP (modified Triple Antibiotic Paste). Solvent used for antibiotics was the cell culture medium. The prepared medications were added to cell culture plates. 24 and 72 hours later, cell viability in the culture medium was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. Group I : SCAPs exposed to a combination of mTAP over a period of 24 and 72 hours. Group II : SCAPs exposed to CA over a period of 24 and 72 hours. Group III: Negative control group.

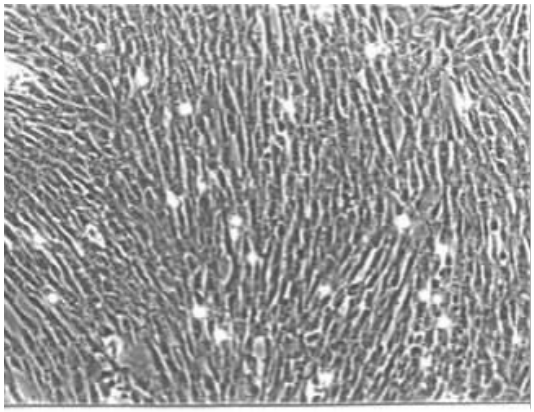

FIGURE 1 Cell density

3 | DATA ANALYSIS

Sample size of 60 was established pertaining to wells in the microtiter plate. Absorbance value of each well for both the groups was measured through ELISA reader (Rayto, RT-2100C). Statistical analysis was done using SPSS statistical software (version 26.0 IBM Corp.). Statistical tests such as Levene's test for equality of variances and independent t test for equality of means was calculated. The significance level in all tests was kept <0.05.

4 | RESULTS

Cell viability was calculated by normalizing the absorbance values of experimental groups to the negative control group (considered as 100% viability). At 24 hours, the mean absorbance value for the CA group was 0.05249 ± 0.005124, whereas for the mTAP group it was 0.04187 ± 0.003851. When normalized to the negative control, CA demonstrated a significantly higher percentage of cell viability compared to mTAP ($p < 0.000001$).

At 48 hours, the mean absorbance value for the CA group was 0.05532 ± 0.005116, while the mTAP group showed a mean value of 0.04286 ± 0.003671. Upon normalization to the control group, CA again exhibited significantly greater cell viability than mTAP ($p < 0.000001$). Thus, relative to untreated control cells, SCAPs exposed to CA maintained higher viability compared to those exposed to mTAP at both time intervals.

TABLE 1 Cytotoxicity evaluation at 24 hours

Group	N	Max (24 hrs)	Min (24 hrs)	Mean	Std. Deviation	p value
CA	60	0.05761	0.04736	0.05249	0.005124	<0.000001
TAP	60	0.04572	0.03802	0.04187	0.003851	

TABLE 2 Cytotoxicity evaluation at 48 hours

Group	N	Max (48 hrs)	Min (48hrs)	Mean	Std. Deviation	p value
CA	60	0.06043	0.05020	0.05532	0.005116	<0.000001
TAP	60	0.04653	0.03918	0.04286	0.003671	

FIGURE 2 Triple Antibiotic Paste preparation

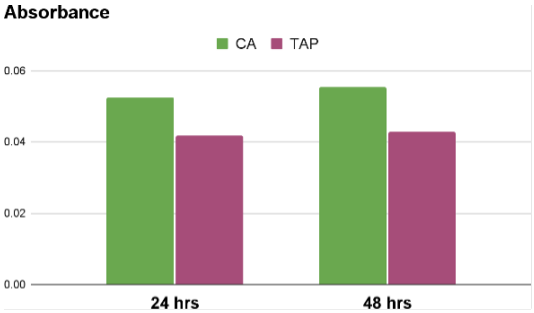

FIGURE 4 Graphical representation of results

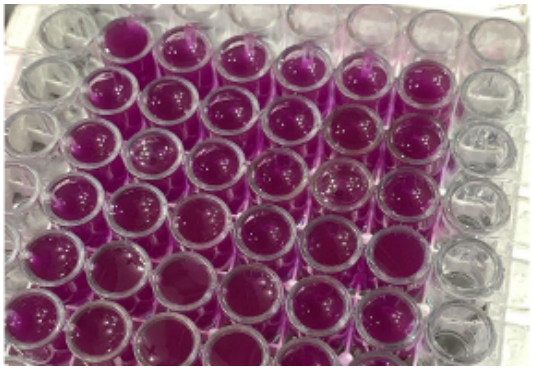

FIGURE 3 Microtiter plate with samples

5 | DISCUSSION

Regeneration refers to the restoration of tissue continuity by replacement with identical tissues, preserving the original architecture and function.[8] In dentistry, regenerative endodontics focuses on the reformation of dentin and pulp-like tissues.[2] This procedure is indicated for teeth with necrotic pulp and incompletely developed apices, provided that post or core restorations are not required.[1] Following a proper irrigation protocol and disinfection strategy is crucial; incomplete eradication of the bacterial load results in a change in stem cell phenotypic expression from dentinogenic to osteogenic[9]. Calcium hydroxide intracanal medication upregulates phosphorylated extracellular signal-related kinases only at low concentrations. At higher concentrations, it has been shown to affect SCAP attachment. Different antibiotic combinations have also been tried and tested and have given quite promising results. But the development of resistant bacterial strains, allergic reactions and damage to DNA of SCs is quite concerning and points to look forward towards an alternative.[4] Carnosic acid appears to be a promising alternative in this domain. According to Loussouarn et al., its antioxidant activity is attributed to the presence of polyphenols.[10] The four phenolic hydroxyl groups (-OH) suppress lipid peroxidation, promote cell proliferation, mitosis, and differentiation, thereby enhancing signaling pathway activity. Its antimicrobial effect arises from interactions with the cell membrane, leading to leakage of cellular contents. Carnosic acid interacts with the cell membrane, leading to alterations in genetic material and nutrient balance, disruption of electron transport, leakage of intracellular components, and modifications in fatty acids. Furthermore, it engages with membrane proteins, resulting in structural changes and loss of membrane functionality.[10] Crozier et al. also reported that carnosic acid exhibits anti-allergic properties by suppressing allergen-induced responses, including calcium (Ca^{2+}) mobilization, ROS production and subsequent degranulation and late responses by modulation of tyrosine kinase Syk and downstream effectors TAK1 (Ser412) and Akt (Ser473) as well as NFκB signaling.[11] A study by Mirza et al. highlighted the property of carnosic acid to transcribe cytoprotective genes.[7] According to Zampini et al. it has a lower minimum inhibitory concentration (MIC) and is effective against multidrug resistant bacteria by acting as an effective pump modulator.[12] The pluripotency of CA is through the upregulation of the KEGG pathway as described by Ferdousi et al. PDGFRB and ROCK1 upregulation is responsible for angiogenic growth and MAPK & IKBKB pathway being responsible for its ability for neo-neuronal growth.[13] Dessai et al reported carnosic acid as an intracanal medicament performs better than triple antibiotic paste

and calcium hydroxide to eradicate Enterococcus faecalis from root canal pointing towards its better antimicrobial efficacy.[14] The concentration responsible for antimicrobial action of CA is as low as 6.25-12.5 µM as reported by Othman et al.[15] Mirza et al stated that Carnosic acid exerts a neuroprotective role that may serve to strategize novel therapeutic approaches for debilitating neurodegenerative disorders by regeneration.[7] Also, Lou et al reported rosemary to have the potential to stimulate hepatocyte proliferation leading to liver regeneration.[16] The results obtained in this study shows a higher absorbance value for carnosic acid both at 24 hours and at 48 hours interval which is 0.05249 and 0.05532 respectively. Whereas, mTAP shows absorbance value of 0.04187 and 0.04286 at 24 and 48 hours respectively. This suggests the lower cytotoxic levels in the CA group and higher percentage of cell survival and is statistically significant. The reasons associated are the good antimicrobial efficacy at a lower concentration. This low concentration helped in stem cell survival, attachment, differentiation and proliferation. It is multipotent in nature. Antibiotics have shown detrimental effects on stem cells, whereas carnosic acid has helped in the survival of those SCs.

6 | CONCLUSION

Within the limitations of the current study, it can be concluded that Carnosic acid has a lower cytotoxic effect on the SCAPs than mTAP. Hence, its use as ICM in regenerative endodontics looks promising. However, further clinical studies are required.

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