Original Article

Chemical preconditioning escalates chondrogenic activity in explant cultured human dental pulp stem cell study model for future temporomandibular joint regeneration

ABSTRACT

Context: Human dental pulp stem cells (hDPSC) derived from dental pulp in conducive environment activated by chemicals can enhance chondrogenic cells for future animal model temporomandibular joint model.

Aim: The study aims at evaluating the chemicals preconditioning (curcumin and rapamycin) efficacy toward chondrogenic proliferation of human dental pulp stem cells.

Settings and Design: The *in vitro* study model with 10 premolar teeth extirpated pulp was processed under sterile chemical conditions. The cells viability was checked with calorimetric assay for adipogenic and chondrogenic, osteogenic lineages. The viability of the cells and the concentration of curcumin (CU) and rapamycin (RP) required for cell differentiation toward chondrogenic lineage were assessed.

Material and Methods: The hDPSC was evaluated after explant long-term cultivation with characterization and chemical conditioning with dimethyl sulfoxide (DMSO) as control. MTT assay was used for cytotoxicity evaluation, cell viability, and proliferation. The dose optimization was observed with RP and CU. Chondrogenic proliferation was assessed with standard staining method of 0.1% Safranin O and 0.1% Alcian blue.

Statistical Design: The flow cytometry analysis revealed good results for CD 90 compared to others. The intergroup analysis was done by ANOVA, and intragroup analysis was done by *Post hoc* Tukey's test. The intragroup analysis showed *P* value < 0.05 for RP in comparison between the various preconditioning agents CU and RP. The dosage of 10 μg/ml RP was considered statistically significant.

Results: The flow cytometer analysis revealed good results for CD 90 compared to other surface markers. The dosage of 10 μg/ml RP was having good chondrogenic cell proliferation. The intragroup analysis showed *P* value < 0.05 for RP in comparison between the various preconditioning agents CU and RP. The calorimetric assay (MTT) quantitative analysis of the chondrogenic cells with Safranin O stain the standard deviation (SD = 0.017 for rapamycin), Alcian blue (SD = 0.49 for RP) in comparison to DMSO (control) and CU.

Conclusion: RP activates mTOR pathway and hence stabilizes the stem cell maintenance of human dental pulp stem cell and the dose quantified can be used for future animal temporomandibular joint animal model.

Keywords: Cartilage regeneration, curcumin, human dental pulp stem cells, rapamycin

Lakshmi Shetty, Pushkar P. Waknis, Avinash Kharat[1], Ramesh Bhonde[1], Uday Londhe, B. M. Rudagi, Supriya M. Kheur[1], Kalyani Bhate

Department of Oral and Maxillofacial Surgery, Dr. D. Y. Patil Dental College and Hospital, Dr. D. Y. Patil Vidyapeeth, Pune, Maharashtra, [1]Regenerative Medicine Laboratory, Dr. D. Y. Patil Dental College and Hospital, Dr. D. Y. Patil Vidyapeeth, Pune, Maharashtra, India

Address for correspondence: Dr. Lakshmi Shetty, Department of Oral and Maxillofacial Surgery, Dr. D. Y. Patil Dental College and Hospital, Dr. D. Y. Patil Vidyapeeth, Pimpri, Pune - 411 018, Maharashtra, India.
E-mail: drlakshmishetty21@gmail.com

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INTRODUCTION

Temporomandibular joint disorders (TMD) are indicative of damage to the temporomandibular joint (TMJ) disc, synovial tissues, and mandibular condyle. The cartilage undergoes degenerative changes and clinically presents itself with

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TMD. Degeneration of cartilage layer with disorganized cellular units of surface fibrocartilage.[1] Mesenchymal stem cells (MSC) display a potential for osteogenic, chondrogenic, adipogenic lineages with appropriate media for propagation in an *in vitro* model.[2-4]

Human dental pulp stem cells (hDPSCs) have demonstrated better proliferation and cartilage-regeneration capacity. Pharmacological preconditioning hDPSC with different chemical and biological culture models needs to be studied for TMJ regeneration.[5-7] CU has been the gold standard ancient Indian drug with several enhancing qualities of regeneration, boosts growth of stem cells, and good action against oxidative stress. The easy availability the drug has always been the best natural source for promoting the differentiation of human source of MSC into lineages such as chondrogenic and osteogenic.[8] RP drug which works on the m-Tor pathway has been time tested for various disease models[6] including vascular anomalies. There has been no research on this drug being used for cartilage animal model studies for future TMJ animal models. Thus this study aims to understand the effect of both these chemical preconditioning drugs and their effect on chondrogenic differentiation of hDPSCs *in vitro* for future TMJ animal model benchmark study.

MATERIALS AND METHODS

Study sample: 10 samples of asymptomatic orthodontic extracted premolar teeth were taken after written informed. The sample size was obtained with sample size estimation using G power v. 3.1.9.2 by using calculated effect size from previous literature by Patil VR[Effect size 1.26, alpha error-0.05, Power 95%]

The human dental pulp was extirpated from the above samples under sterile conditions [Figure 1].

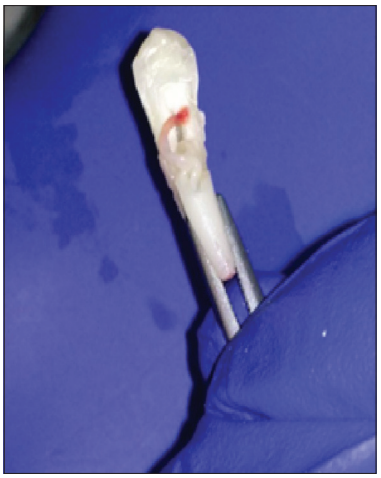


Figure 1: Human dental pulp extracted from premolar tooth

Phase 1: Basic research

1. Extraction of teeth and removal of pulp
2. *In vitro* cultivation of hDPSCs
3. Characterization of the cultivated hDPSCs for stem cell surface markers.
4. Chondrogenic differentiation of the cultivated hDPSCs under chemical preconditioning agents (CU and RP) [Suppl 1] Methodology flow design.

a. Sample Collection

Teeth (pre-molar) were collected from healthy adults aged 14–28 with appropriate oral hygiene ($n = 10$). The patients received chlorhexidine mouth rinse to reduce oral microbes prior to extraction. The pulp extraction was carried out in sterilized conditions using bur chuck type airotor hand piece, then the pulp was gently removed using sterile forceps; immersed in tubes containing PBS with double-strength antibiotic-antimycotic solution and transferred to laboratory immediately.

b. Human dental pulp cell (DPSCs) isolation and culture

Pulp tissue was minced into ~ 1 mm fragments (E0), which were placed in 60 mm culture dishes employing two different patterns (checker board and random). Drops of fetal bovine serum (FBS) were introduced on the tissues sufficient to cover them completely. The dishes were incubated for 24 hours at 37°C with 5% CO_2. After incubation, explants were maintained in DMEM supplemented with 20% FBS and antibiotic-antimycotic solution (AA) at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was changed every 2–3 days and the cell outgrowth was monitored regularly with an inverted phase-contrast microscope (Olympus CKX53, Japan). The outgrown cells at 70–80% confluence were detached using 0.25% Trypsin-EDTA solution and transferred to a 75-cm^2 flask without disturbing mother explant (E1). The mother explant was again maintained in DMEM supplemented with 20% FBS (E2). Same procedure as E2 was repeated for E3 to E7 [Figure 2]. The cells then continuously passed for further experiments. Cells from passage 2 were used for all experiments.

c. Population doubling time and growth curve

To determine the proliferative capability of DPSCs, 1×10^4 cells at passage 2 from each sample were seeded into 12-well culture plates and the count was taken every alternate day for 9 days. Population doubling times (PDT) was then calculated for 48 hours based on the following equations:

PDT = T log2/(logFCC – logICC)

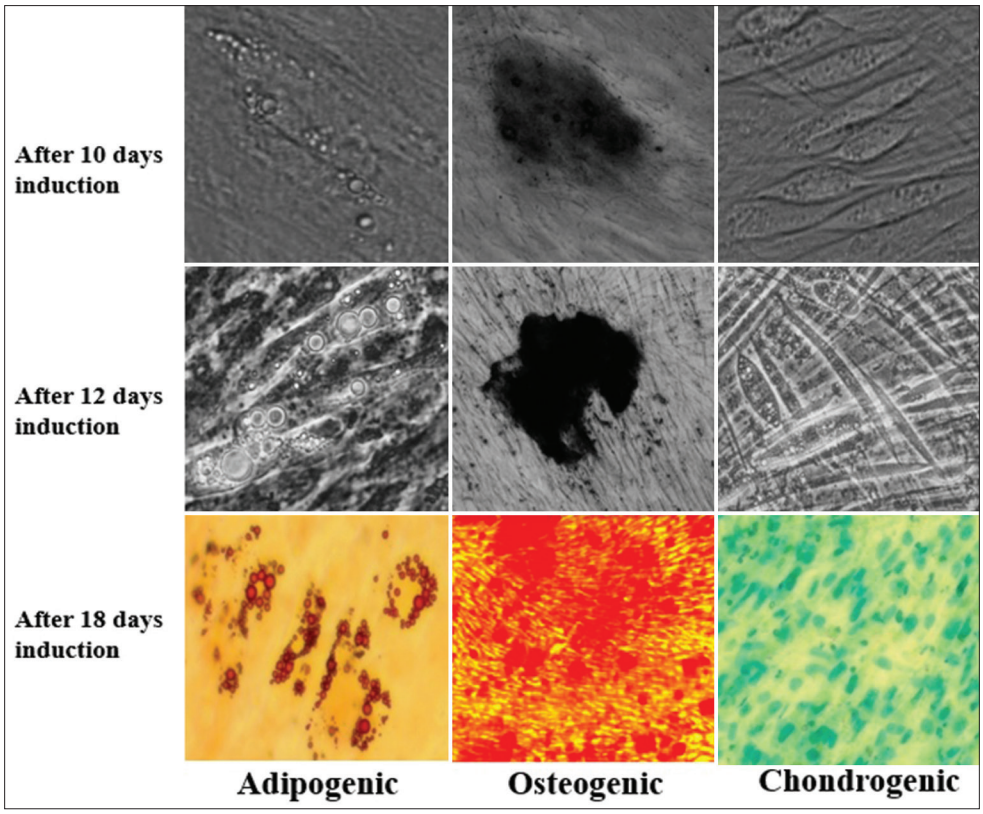


Figure 2: Trilineage differentiation of human dental pulp stem cells with explant culture method

where ICC is the initial cell count, FCC is the final cell count, and T is the incubation time (in hours). The growth curve for 9 days was plotted using cell numbers only.

d. Characterization using flow cytometry

For flow cytometry analysis, confluent DPSCs were harvested and washed with phosphate buffered saline (PBS) and incubated for 30 minutes at 4°C with CD73, CD90, CD105, CD34, CD45, HLA-DR (All from eBiosciences, USA) antibodies. Cells were blocked using appropriate isotype controls prior incubating with antibodies. After washing the cells with phosphate buffered saline (PBS), cells were analyzed on Attune NxT Flow Cytometer (Thermo Scientific, USA). At least 20,000 events were acquired per sample. The degree of positive staining was calculated as a percentage in comparison with the unstained controls.

e. Colony-forming unit fibroblast (CFU-F assay)

To assess the ability of cells to form colonies, a colony-forming unit-fibroblast (CFU-F) assay was performed. Cells were seeded onto 6-well plates at an initial density of 5×10^2 per well in culture media (DMEM with 10% FBS and 1% AA). After seven days of incubation, cells were washed with PBS and stained with 0.3% crystal violet.

f. Characterization of human dental pulp stem cells by trilineage differentiation

Trilineage differentiation such as osteogenic, chondrogenic, and adipogenic lineages were done as follows. In osteogenic differentiation, hDPSC was treated with osteogenic induction media containing DMEM with 1 µm dexamethasone, 10 mM glycerphosphate, and 2 Mm ascorbate-2-phosphate, 10 µg. To assess chondrogenic potential DMEM with 1 µM dexamethasone, 2 Mm ascorbate -2-phosphate, 10 µg/ml sodium pyruvate, 40 µg/ml L-proline, 1X ITS and 10 ng/ml TGF- 3. The adipogenic lineage media has DMEM with 1 µM dexamethasone, 0.5 Mm 3 isobutyl-5-methylxanthine, 200 µM indomethacin, and 1.7 µM insulin. The activation was done for 3 weeks with media change every 48–72 hrs. The final confirmation of the hDPSC into osteogenic, chondrocytes, and adipocytic cells were done with 0.1% Safranin O and 0.1% Alcian blue.

g. Cytotoxicity of RP and CU on hDPSC

The assessment of the cytotoxicity of RP (Astrazenca) and CU (Astrazenca) on hDPSC at concentrations of 5 µg/ml, 10 µg/ml, 20, 100, and 200 µg/ml. The hDPSC treatment was done with 50 µl MTT solution (dimethyl sulfoxide) which was incubated for 3 hrs, and it was removed, and then 100 µl dimethyl sulfoxide is added. The calorimetric absorbance measured at 560 nm using Thermo Scientific software was evaluated. The intergroup analysis was done by ANOVA, and intragroup analysis was done by *post hoc* Tukey's test.

Ethical Approval

Ethical clearance was obtained from Institutional ethics committee with ref no DYPV/EC /96/18 dated 5/06/2018 . Ethical clearance was also obtained from institutional stem cell research with ref no ICSCR/RMO5/18 dated 23/03/2018. Written informed consents was obtained in accordance to the above committees from all participants.

RESULTS

The hDPSC was cultivated with standard explant culture, trypsinized with great confluence till passage 4 and showed well subcultures and was utilized for the phases of the study. The flow cytometry analysis was positive for CD 73, CD 90, and CD 105 [Figure 3].

The hDPSC derived from the study sample differentiated into colony-forming units of cluster of hDPSC with chondrogenic differentiation for 21 days. The colony-forming units were done with completion of incubation period of 11 days with greater degree of fibroblastic morphology. The chondrogenic differentiation was confirmed by standard staining methods with Alcian blue and Safranin O. Chondrogenic proliferation was determined by 0.1% Safranin O stain and 0.1% Alcian blue stain and confirmed [Figure 4]. The intergroup analysis was done by ANOVA, and intragroup analysis was done by *post hoc* Tukey's test. The intragroup analysis showed P value < 0.05 for RP in comparison between the various preconditioning agents CU and RP. The calorimetric assay (MTT) quantitative analysis [Graph 1] of the chondrogenic cells with Safranin O stain is the standard deviation (SD = 0.017 for rapamycin), Alcian blue (SD = 0.49 for rapamycin [Graph 2] in comparison to DMSO (control) and CU. The concentration of 10 µg/ml of RP had significance statistically compared to CU and control thus concluding noncytotoxic and boost for the cell proliferation.

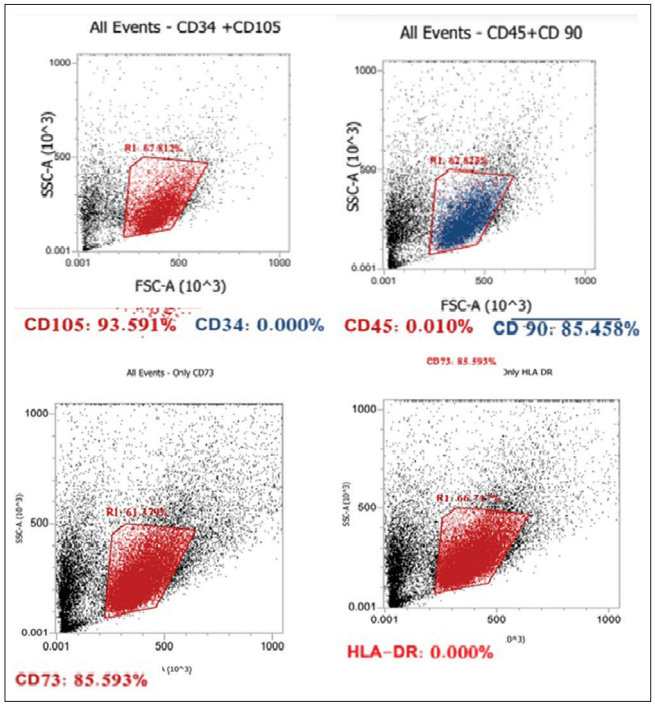


Figure 3: Flow cytometry

DISCUSSION

Mesenchymal stem cells derived from hDPSC have been a rich source for assessment of toxicity of chemical drugs including herbal formulations. Chemical preconditioning has never been used for dose quantification for three-dimensional construct for cartilage regeneration.

CU has always demonstrated as described by Liu *et al*.[8] that it has the capacity to protect stem cells in autologous Sprague-Dawley rat ADSC with increased therapeutic potential in myocardial recovery. In our study, we found that the dosage quantification of its effect was less compared to RP which was the comparative chemical preconditioning agent

RP as described by Maisse K[6] states that the mechanistic nature of RP toward stem cell helps in maintenance, proliferation, and differentiation for multiple disease models. The study on hDPSC and the dose of 10 µg/ml has been proved effective for chondrogenic proliferation and MTT assay proved nontoxic.

Kawakami Y *et al*.[9] demonstrated that RP with its inherent action on mTOR1 pathway demonstrated protection of age related changes on muscle derived stem cells in progeroid mice thus proving that our study would state the future for TMJ animal rabbit model with the optimum dose quantification.

Asgari N *et al*.[10] have demonstrated hydrogel with curcumin to be effective for cartilage regeneration in a cartilage model for microtissue chondrogenic regeneration. In our study, we found the quantitative analysis of curcumin for chondrogenic

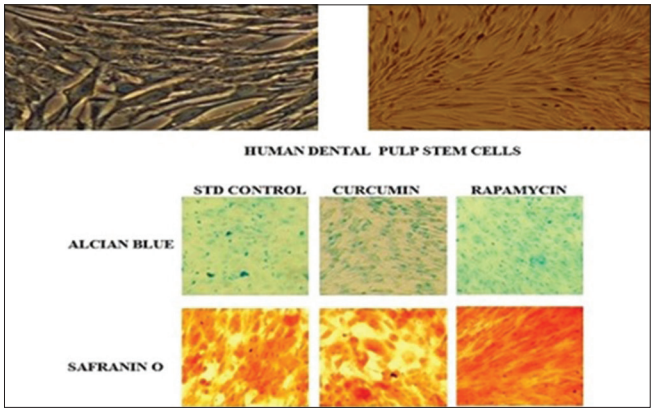
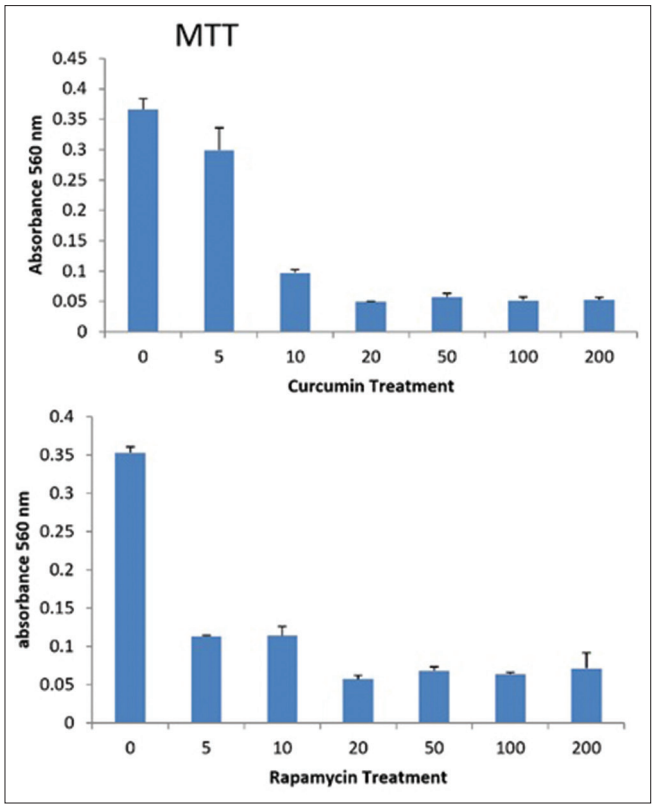


Figure 4: Chondrogenic proliferation with staining of the cells



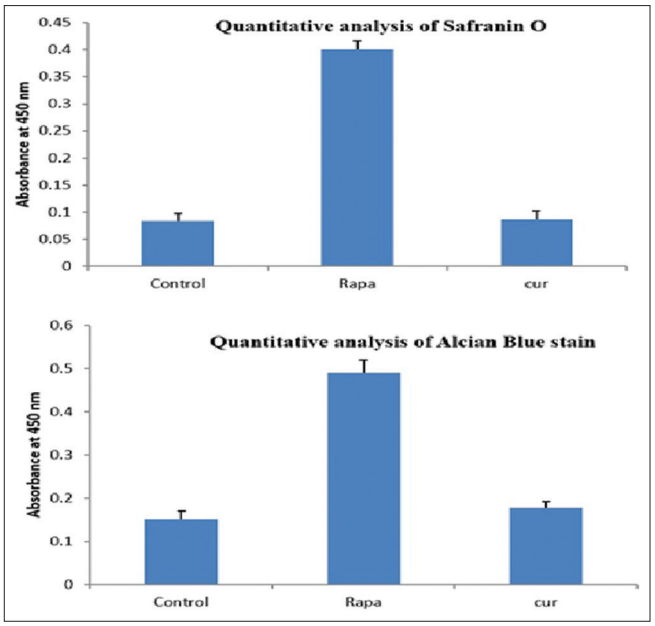
Graph 1: MTT calorimetric assay for dose standardization (Rapamycin 10∝g/ml)

differentiation of hDPSC to be less proliferative and hence will not be adapted for future TMJ regenerative cartilage model.

Buhrmann C[11] demonstrated that curcumin provides an microenvironment for chondrogenic proliferating and deactivates (IL-1β -induced activation of NF- KB and expression of apoptotic and pro-inflammatory genes) in chondrocytes. The chondrogenic cell proliferation with hDPSC as provided by CU at various doses of 5, 10, 20, 50, 100 µg/ml was not symbolic compared to similar concentration in rapamycin.

The hDPSC has the capacity to differentiate in different cocktails as asserted by Vater C,[12-14] and in our study we found the chemical preconditioning with RP to be effective to give chondrogenic cell proliferation and potential compared to standard chondrogenic cocktail and control, CU. Long-term explant culture as stated by Patil V *et al*.[15,16] which describes the new method without enzymatic preparation of human pulp derived stem cells by long-term safe explant culture, and we were able to adapt the same in our study for the said result.

MTT assay delineates the cytotoxicity of a drug, and here we have found for the first time the dose quantification of RP over chondrogenic lineage of human dental pulp stem cells



Graph 2: Quantitative analysis of chondrogenic proliferation

with effective dose calculation for future three-dimensional constructs for cartilage regeneration.

Jung Lu T *et al*.[17] described in their study that mTOR signaling pathway is required for chitosan film culture for chondrogenesis, and hence, our study highlights the propensity of rapamycin for future chitosan scaffold cultures for TMJ regeneration

Liu W *et al*.[18] demonstrated in his study that RP-induced autophagy increased chondrogenesis in synovium-derived mesenchymal stem cells in the temporomandibular joint by the effect on IL-1β . In our study, we have discovered that chondrogenic cells differentiation, and proliferation is maximum with RP and hence will be used in the phase 2 of this study on TMJ animal model.

The growth of viable chondrogenic differentiated human dental pulp stem cells on an three-dimensional construct will be the future for growth of cells in an animal model for cartilage formation. The importance of this study is that chemical preconditioning with RP has given the maximum cells for chondrogenic viability and hence can be cryopreserved to be used for further animal models toward *in vivo* studies. The site of future studies needs to be determined to implant these cells in a conducive scaffold material for future regeneration of cartilage in the animal model.

CONCLUSION

This study depicts the quantification for dose optimization

of chemical agent RP to be used for future chondrogenic proliferation for signaling toward cartilage regeneration in TMJ animal model.

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Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient (s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initial s will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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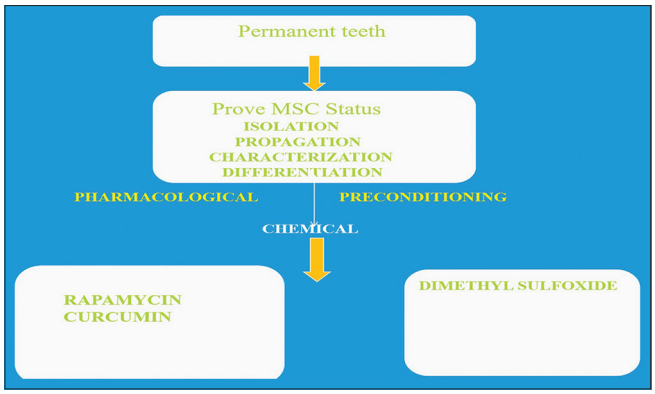

Suppl 1: Methodology flow design