EGYPTIAN DENTAL JOURNAL
www.eda-egypt.org
VOL. 69, 1119:1127, APRIL, 2023
PRINT ISSN 0070-9484 • ONLINE ISSN 2090-2360
ORAL MEDICINE, X-RAY, ORAL BIOLOGY AND ORAL PATHOLOGY
Submit Date : 20-01-2023 • Accept Date : 01-02-2023 • Available online: 01-04-2023 • DOI : 10.21608/edj.2023.188375.2415

# EFFECT OF PREMIXED BIOCERAMIC PUTTY AND MINERAL TRIOXIDE AGGREGATE ON HUMAN FIBROBLASTS (AN IN VITRO STUDY)

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## *ABSTRACT*

**Background:** There is few evidence about the biocompatibility of premixed bioceramic putty (NeoPUTTY) to be used in vital pulp therapy.

**Aim of the study:** in vitro evaluation of the effects of premixed bioceramic putty (NeoPUTTY) on human fibroblasts compared with a conventional MTA (ROOTDENT).

**Materials and Methods:** Human fibroblast cells were divided into 3 groups; group I: a negative control group that contained cells without treatment materials, group II: positive control group in which cells were treated with MTA and group III: test group in which cells were treated with the premixed bioceramic putty (NeoPUTTY). Cell viability was evaluated for the three groups using the following tests; Hematoxylin and Eosin (H&E) stain, MTT assay, Immunohistochemistry to detect caspase 3 (CASP3) after 24 and 72 hours and quantitative PCR (qPCR) for expression of CASP3 after 72 hours.

**Results:** Human fibroblasts examined under light microscope and exposed to MTA exhibited cell membrane blebbing and apoptotic bodies indicating late apoptosis. While In NeoPUTTY group, fibroblasts showed early apoptotic features and some cells exhibited normal mitotic figures suggesting more biocompatibility of NeoPUTTY than MTA. MTT assay and immunohistochemical analysis for CASP3 revealed higher cell viability in premixed bioceramic putty group after 24 hours and 72 hours compared with MTA and the results were statistically significant. qPCR revealed upregulation of caspase 3 gene expression in MTA group more than that obtained by Neoputty group.

**Conclusions:** Premixed bioceramic putty (NeoPUTTY) can be recommended as a superior biocompatible alternative to MTA.

**KEY WORDS:** Premixed Bioceramic, NeoPUTTY, Cell viability

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## INTRODUCTION

Successful vital pulp therapy strategies focus on the potential of dental pulp preservation and regeneration. An essential requirement for successful healing is the application of a material that offers a suitable environment, and supports the regenerative processes of dentin-pulp complex. From this perspective, treatment modalities should be capable of inducing odontoblastic differentiation. In this regard, calcium hydroxide and bioceramic materials are the most commonly used materials **(Li et al., 2021).**

Calcium hydroxide was commonly used nearly a century for direct and indirect pulp capping procedures. However, there is a paradigm shift in the preference of clinicians from calcium hydroxide to mineral trioxide aggregate (MTA) due to its favorable and predictable outcomes. Compared with calcium hydroxide, clinical research has confirmed the more efficient potential of MTA to induce reparative dentinogenesis. It exhibits higher structural stability and sealing ability than calcium hydroxide that suffers from the tunnel defective dentin ***(Okiji & Yoshiba, 2009)***

Attempts are being conducted to develop new bioceramic materials and to improve the properties of MTA such as the prolonged setting time, tooth discoloration potential, and difficulty in handling. Nowadays, a novel premixed calcium silicate low tack bioceramic putty (NeoPUTTY) is introduced with superior handling characteristics. Water required for hydrating this material is derived from a natural source in dentin ***(Torabinejad & Parirokh, 2010; Selvendran et al., 2022).***

In vitro cytotoxicity assessment protocols, executed to compare dental materials and predict their safety for clinical application, have been considered the initial tests before proceeding to in vivo trials **(Swetha et al., 2015).** Surveying the literature, there is few evidence testing the cytotoxic effects of premixed bioceramic putty (NeoPUTTY). Therefore, current study is conducted to evaluate the effects of this novel bioceramic putty on human fibroblasts compared with a conventional MTA.

## MATERIALS AND METHODS

The current in vitro study was approved by the ethical committee of the Faculty of Dentistry, Minia University.

### Cell culture *(Helgason & Miller, 2005)*

Human fibroblasts cell line (WI-38 cell line) was supplied from the Holding Company, Tissue Culture Department, for production of vaccines, sera, and drugs (VACSERA, Giza, Egypt). Cell culture and cell viability tests were conducted in Biology department, Faculty of Medicine, Al-Azhar University.

Cells were maintained in complete DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37°C in an atmosphere of 5% $CO_2$ (Jouan SA, Saint-herblain, Pays de la Loire, France). Growth medium was removed after 24 hours of incubation, and cells were then washed in phosphate buffered saline solution (Adwia Pharmaceuticals, El Sharkeya, Egypt) devoid of calcium and magnesium.

The wash solution was subsequently removed from the culture and cells were treated with dissociation reagent 0.25% trypsin enzyme and 0.05% (v/v) EDTA (GIBCO) then incubated at room temperature for 5 minutes. Cells were maintained according to manufacturing protocol. Detached cells were splatted according to need.

### Preparation of tested materials

MTA ROOTDENT (TechnoDent, Russia) and premixed bioceramic putty (NeoPUTTY, Avalon Biomed, USA) were purchased. Tested materials were prepared according to the manufacturers' instructions. Materials were mixed with distilled water. Each tested material was added to DMEM to be added to human fibroblasts for further testing.

Human fibroblast cells were divided into 3 groups; group I: a negative control group that contained cells without treatment materials, group II: positive control group in which cells were treated with MTA and group III: test group in which cells were treated with the premixed bioceramic putty (NeoPUTTY). Cell viability was evaluated for the three groups using the following tests:

**Hematoxylin and eosin staining of human fibroblasts**

Cells in the 3 groups were distibuated on three clean slides for each treatment after 24 and 72 hours. After being air dried, methanol fixed, and rehydrated in descending alcohol concentrations (100%, 90%, 75%, and 50%). Slides were cleaned for 5 minutes in distilled water then submerged in filtered hematoxylin stain for 3 min and washed with distilled water twice. Slides were submerged in filtered eosin stain for 5 seconds and washed with distilled water. Dried slides were dipped in xylene, mounted with Canada balsam then coverslips were placed and allowed to dry.

On each slide, ten microscopic fields were photomicrographed. A digital camera (Canon, Japan) mounted on a light microscope was used for this. Images were uploaded to the computer system to be analysed. Field selection was determined on the basis of the highest number of apoptotic cells. Evaluation of the photomicrographs was performed according to the morphological criteria of apoptosis.

**Cell viability and proliferation test (MTT assay) (Adan et al., 2016)**

MTT assay was used to assess cell viability after 24 and 72 hours by the capacity of the cells to cleave the yellow tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2.5-diphenyl tetrazolium bromid) via the activity of dehydrogenase enzymes, only in living cells, to produce the purple formazan dye.

Serial dilutions of cells in culture medium were prepared from 1 x $10^6$ to 1 x $10^3$ cells per ml. 100 µL of the dilutions were plated out, in triplicate, into wells of a microtiter plate and incubated at 37°C for 24 hours to achieve confluence. The tested biomaterial were prepared under sterile laboratory conditions according to the manufacturers' instructions and added to the wells. 90 mL of the DMEM culture medium containing 10% FBS and MTT solution (Sigma Aldrich, St. Louis, MO, USA) were added to plates in the dark. Plates were incubated at 37°C for 4 hours under 95% humidity and 5% $CO_2$ until purple precipitate was evident.

100 mL of detergent reagent were added to each well once the purple precipitate could be seen clearly. Plates were left with cover inside the incubator for 2 hours at 37°C in the dark to solubilize any formazan crystals that had formed. Using a microplate reader, optical absorption was evaluated at a wavelength of 570 nm and was found to be positively correlated with the number of metabolically active cells. Cell viability was expressed as percentage of optical density of experimental wells relative to that of control which is taken to represent 100% cell viability.

**Immunohistochemistry (Kaushal et al., 2014)**

Immunolocalization of Caspase 3 at 24 and 72 hours was used as a genetic marker for cell apoptosis. The adhered fixed slides were incubated in 1% H2O2 in PBS containing 0.1 % sodium azide for 10 minutes to reduce the activity of the endogenous peroxidase activity. Then slides were drained and incubated with caspase antibody at appropriate dilution (1:200) in antibody dilution buffer in a humidified chamber overnight at 4°C.

Slides were washed with PBS-T for 5 minutes, then incubated with secondary antibody that was conjugated to horseradish peroxidase for 1 hour at room temperature and in the dark. The slides were washed twice and incubated in the dark with horseradish peroxidase substrate solution which results in brown stain and seen under a light microscope. Finally, Slides were microscopically examined and photographed. Images were analyzed using ImageJ software.

**Real-time qPCR** for expression of CASP3 (a marker for apoptosis).

Total RNA was extracted from negative control, MTA and premixed bioceramic exposed wi-38 cells after 72 hours, respectively using RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to manufacturer's instructions. Using a Beckman dual spectrophotometer, the concentration of the isolated RNA was assessed (Beckman Instruments, Ramsey, MN, USA). Real-time PCR was used to measure the expression levels of the housekeeping gene ACTB (F 5'- AGCGAGCATCCCCCAAAGTT- 3'& R: 5'-GGGCACGAAGGCTCATCATT- 3) and CASP3 (F 5- CTCGGTCTGGTACAGATGTCGA & R: (5 CATGGCTCAGAAGCACAAAC-3). Changes in each target gene's expression were normalized in relation to the average critical threshold (CT) values of the housekeeping gene b-actin.

### Statistical methods

The collected data was tabulated, and statistically analyzed using IBM SPSS statistics (Statistical Package for Social Sciences) software version 28.0, IBM Corp., Chicago, USA, 2021. Quantitative data were tested for normality using Shapiro-Wilk test, then described as mean±SD (standard deviation) as well as minimum and maximum of the range, and compared using two-way repeated measure ANOVA test. The level of significance was taken at p-value ≤0.05 as significant, otherwise was non-significant.

## RESULTS

**Hematoxylin and eosin staining** (H and E, Original magnification 100X, Oil)

**In group I (the negative control),** untreated cells appeared regular with normal cellular and nuclear appearance. Cells exhibited intact cell membrane with normal chromatin distribution (Figure: 1a & 1b). **In group II (MTA group)** after 24 hours, swollen cells were most evident with nuclear karyolysis. Some cells showed early apoptotic features where they appeared shrunken with intact cell membrane (Figure: 1c). After 72 hours, most cells showed late apoptotic features. Cells were shrunken, hyperchromatic with chromatin condensation in most nuclei. The nuclei were found to be pyknotic and some cells showed membrane blebbing (Figure:1d).

**In group III (NeoPUTTY group)** after 24 hours, some fibroblasts were swollen showing nuclear karyolysis. Some fibroblasts showed normal mitotic figures (normal mitosis), while others were shrunken with slightly condensed chromatin and irregular cell membranes (Figure: 1e). After 72 hours, hyperchromatism was reduced, membrane blebs were clearly seen, and cellular debris were evident (Figure: 1f). These morphological features revealed by light microscope suggest more biocompatibility of NeoPUTTY over MTA.

### MTT assay

In NeoPUTTY group; viability percent was found to be decreased at 72 hours as compared to the corresponding 24 hours at most concentrations. Viability percent was recorded to be decreased by increasing the concentration. The viability percent differences were different across different concentrations at 24 and 72 hours. The same findings were observed in MTA group. Comparing NeoPUTTY and MTA results revealed that viability percent was higher in NeoPUTTY at most of concentrations at 24 and 72 hours and the results were statistically significant (Line graph: 1). Viability tested in both groups significantly decreased by time (time dependent).

### Immunohistochemistry to detect CASP3.

Group I cells showed negative immunostained cytoplasm for CASP3 and weak nuclear immunoreactivity (figure: 2a & 2b) while group II cells showed intense nuclear and cytoplasmic immunoreactivity for CASP3 (figure: 2c & 2d). Group III cells showed moderate cytoplasmic immunoreactivity for CASP3 (figure: 2e & 2f).

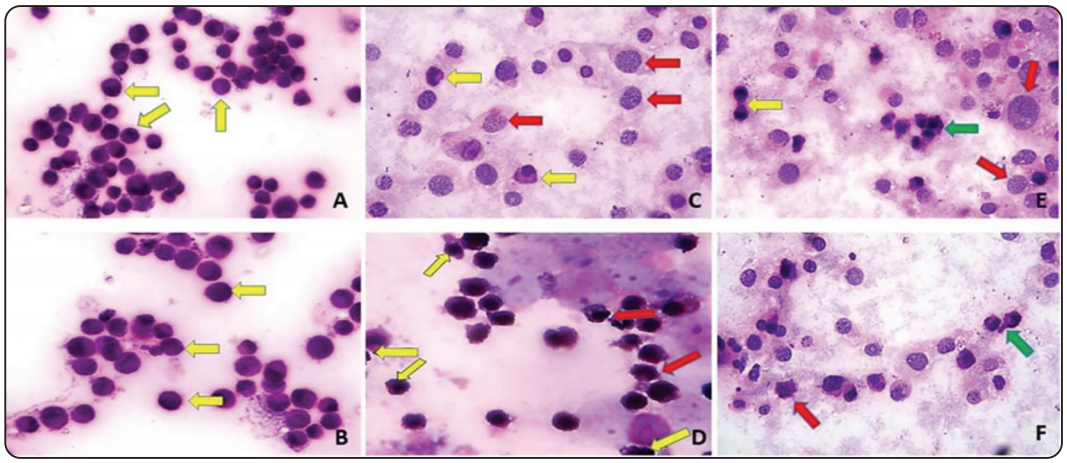
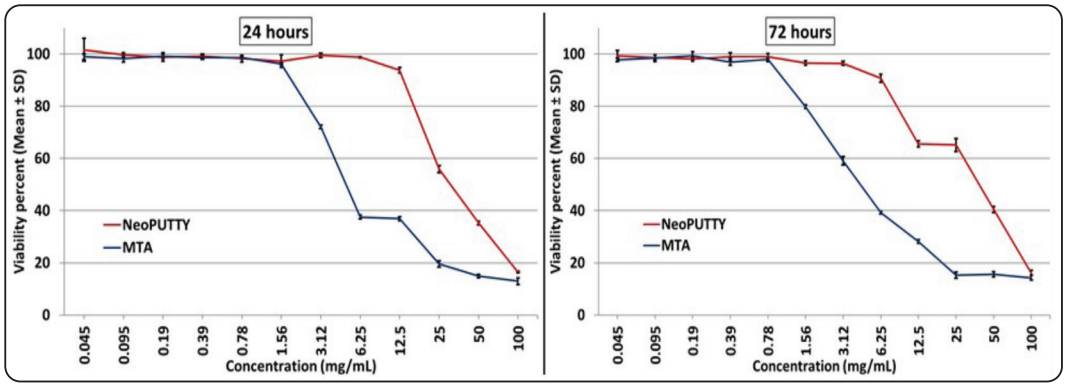



Fig. (1) Photomicrographs of human fibroblasts for the three groups. (**a & b):** group I after 24 and 72 hours, cells showed normal cell morphology with intact cellular membrane. Nuclei exhibited euchromatin and heterochromatin (yellow arrows) **(c):** group II after 24 hours showed swollen cells with nuclear karyolysis (red arrows) and cells with condensed chromatin (yellow arrows). (**d**): group II cells after 72 hours showing membrane blebbing (red arrows). Some cells exhibited irregular cell membrane (yellow arrows). (**e):** group III cells after 24 hours showed shrinkage with hyperchromatic nuclei and irregular cell membrane (green arrow), normal mitotic figures (yellow arrow) and some swollen cells with karyolysis (red arrows). (**F):** group III cells after 72 hours showed membrane blebbing (green arrow), and nuclear fragments (red arrow).




**Line graph (1):** Viability percent among study and positive control groups at hour 24 and 72 hours.

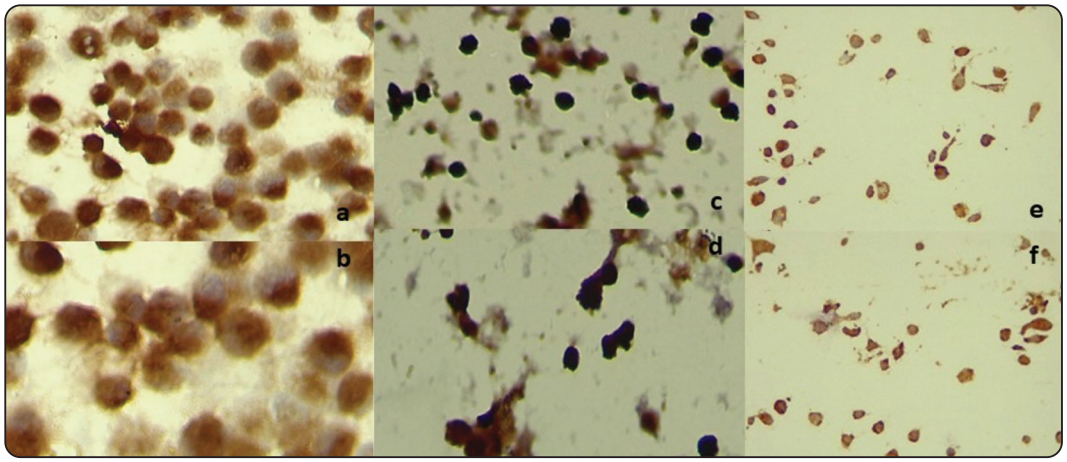



Fig. (2) Photomicrographs of immunohistochemistry for CASP3 for group I after 24 hours (a) and 72 hours (b), group II after 24 hours (c) and 72 hours (d), and group III after 24 hours (e) and 72 hours (f).

Measuring area fraction of CASP3 immuno-reactivity using the ImageJ software at both time intervals revealed that area percent was lowest in NeoPUTTY group (6.4±0.7; 7.2±0.7), followed by negative control group (10.9±1.5; 12.8±1.3) and highest in MTA group (15.0±1.0; 17.0±2.5c) and differences were statistically significant. In all groups, area percent increased at 72 hours as compared to 24 hours level. Area percent differences between 24 hours and 72 hours were different across study groups. Results of the tested groups revealed significantly decreased viability by time.

**qPCR for expression of CASP3**

At hour 72; Real-time qPCR for CASP-3 was expressed as folds of the negative control group. MTA group exhibited upregulation of caspase3 gene expression (4.1) more than that obtained by Neoputty group (2.6).

## DISCUSSION

MTA is considered the gold standard for vital pulp therapy. However, it suffers from certain limitations such as tooth discoloration and difficult handling **(Cintra et al., 2017).** NeoPUTTY is a novel bioceramic that received approval by the U.S. Food and Drug Administration (FDA) in 2020. This material doesn't exert tooth discoloration, ready to use without mixing, and possess similar mechanical and biological properties to MTA with improved handling characteristics **(Motwani et al., 2021).**

The popularity of premixed bioceramic putty has grown significantly, and the evidence supporting its biocompatibility as a vital pulp therapy material superior to MTA is still limited **(Haapasalo et al., 2015).** Therefore, the current study conducted to in vitro assess the cytotoxic potential of premixed bioceramic putty (NeoPUTTY) on human fibroblasts compared to MTA as a positive control and human fibroblasts without treatment as a negative control.

For the introduction of new dental products, biocompatibility assessment is an essential prerequisite before optimizing clinical performance. In vitro biocompatibility tests offer many advantages prior to in vivo test systems. These tests are capable of simulating biological reactions to materials when come in contact with or into living cells. These experimentally controllable tests provide more strictly standardized conditions than in vivo trials. These tests are more appropriate for studying novel products than costly and time-consuming animal studies as they are rapid and relatively simple, with no ethical issues **(Moharamzadeh et al., 2009).**

In this study, a cell culture technique of human fibroblasts was employed as it offers a significant cost effective repeatable tool. Human fibroblasts were selected due to their relevance to clinical circumstances. In addition, they have advantages of being simple to isolate and they can grow fast in normal culture medium. These cells are highly sensitive in cytotoxicity tests. Consequently, these cells are ideal to demonstrate potential adverse effects of different dental materials intended for restorative dentistry and vital pulp therapy **(Al-Haj Ali et al., 2014).**

DMEM was used as a main culture media, since it is the recommended mediim for in vitro cell culture experiments **(Tas, 2014).** Fetal bovine serum has been included to supplement the culture medium as it aids in cellular growth and tissue culturing **(van der Valk et al., 2018).**

Cell viability was assessed using four different methods to achieve accuracy. Light microscopy and histologic examination with hematoxylin and eosin stain were executed to detect distinct morphological characteristics of apoptosis **(Elmore, 2007).** MTT assay has been also performed as it is highly calibrated, sensitive and reliable assay **(Al-Haj Ali et al., 2014).**

Moreover, one of the most precise indications of the apoptotic process is the capacity to upregulate caspase 3 gene expression, and assays to detect or quantify caspase activity constitute a potent method for evaluating apoptosis, even before all

the morphological characteristics of apoptosis can be observed. In the current study, monitoring gene expression of CASP3 was performed using the extremely sensitive qPCR and the simple reliable immunohistochemistry technique **(Wlodkowic et al., 2011).**

Photomicrographs of fibroblasts stained with H&E and examined under light microscope revealed differences in the morphological characteristics among the different groups, in terms of apoptotic cells and apoptotic features.

Cells treated with MTA after 24 hours exhibited early signs of apoptosis which include cell shrinkage and chromatin condensation. With cell shrinkage, the cells are smaller in size, the cytoplasm is dense and the organelles are more tightly packed. Chromatin condensation is the most characteristic feature of apoptosis, the nuclear material characteristically aggregates peripherally under the nuclear membrane. After 72 hours, MTA treated cells showed late apoptotic features. Cells exhibited plasma membrane blebbing which is an irregular bulge in the plasma membrane of the cell. The bulge eventually blebs off from the parent plasma membrane taking part of the cytoplasm with it followed by separation of cell fragments into apoptotic bodies during a process called "budding." **(Elmore, 2007)**

Cells treated with NeoPUTTY at both time intervals showed scanty shrunken cells with slightly condensed chromatin, while other cells appeared normal with intact cell membrane and normal nuclei appearance. Additionally, some normal viable cells showed normal mitotic figures. Histologically, mitosis is the morphologically recognizable phenomenon that most closely reflect regenerative potential **(Leoncini et al., 2002).** These morphological features suggest superior biocompatibility of NeoPUTTY compared to MTA.

The results go in accordance with **Tsai et al. (2018)** who observed apoptosis of dental pulp stems cells in direct contact with MTA. On the contrary, **Kuru et al. (2021)** reported biocompatible characteristics of MTA. The difference could be explained on the basis that the positive control group in study of **Kuru et al. (2021)** was a calcium hydroxide based material while in the current study is MTA which is less biocompatible than NeoPUTTY that has more biocompatible ingredients.

It should be highlighted that these morphological results are descriptive non-statistical observations that are left to the interpretation of the investigators. Nevertheless, these types of biological assays are commonly used and complemented by quantifiable biological assays. **(Lozano-Guillen et al., 2022).**

MTT assay showed that viability percent was higher in NeoPUTTY as compared to MTA at most of concentrations at 24 and 72 hours and difference is statistically significant. Viability percent decreased by increasing the concentration and time in both groups. This goes in accordance with **Sun et al. (2021) and Lozano-Guillen et al. (2022).** Differences in biocompatibility could be clarified by differences in composition, hydration processes and setting reactions (**Yamamoto et al., 2016; Pelepenko et al., 2021**)

The increased cytocompatibility of tested materials by increasing the dilution goes in accordance with the results of **Rodriguez-Lozano et al. (2022)** since the dilutions simulate the clinical conditions, in which the tested materials can be placed on the remaining dentin or directly on pulp exposures **(Lozano-Guillen et al., 2022).** IC50 favors the use of NeoPUTTY rather than MTA.

Consistent with MTT assay findings, immunohistochemical analysis for CASP 3 revealed that NeoPUTTY was more biocompatible than MTA and difference was statistically significant. These results also go on harmony with qPCR for CASP-3 that revealed upregulation of caspase3 gene expression in MTA group (4.1) more than that obtained by Neoputty group (2.6).

These results of this in vitro study elucidate the biocompatibility of NeoPUTTY that has

the advantage of possessing a better window of maneuverability **(Sun et al., 2021)** suggesting it as a superior alternative to MTA in vital pulp therapy. However, it is difficult to translate the biological response observed in the in vitro tests to clinical situation **(Hosseinpour et al., 2022)**. Therefore, further animal studies, and clinical trials are needed to reinforce the existing evidence regarding the biological properties of premixed bioceramic materials and to test hard tissue formation potential and the quality of the produced tertiary dentin.

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