Comparison between triple antibiotic paste & dental pulp stem cells potentials in regenerative endodontics: a systematic review.

Supervisor

Dentist (specialist in endodontics), Monika Vanagaitė

Kaunas, 2018
## EVALUATION TABLE OF THE MASTER'S THESIS
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**SUMMARY**

**Background:** Regenerating pulp-like tissues after a pathological process is shown to be a challenging and debatable topic in regenerative endodontics. This review highlighted two methods of dental pulp regeneration: one using the triple antibiotic paste and another using the dental pulp stem cells.

**Methods:** Different scientific databases and dental journals (Pubmed, Researchgate, Journal of Endodontics, etc) were investigated in order to collect the studies for this review. 14 articles met the inclusion criteria of it and the search was bordered by publications from the last 10 years only. The Prisma 2009 checklist for systematic review organisation was referred to in order to make this study accurately represented.

**Results:** Both methods showed advantages as well as some limitations but this didn’t prevent using them in dental field in the same time keeping them under experimentation for further improvements and explanations.

**Conclusion:** TAP is safe enough to keep managing as a dental pulp regeneration solution until DPSCs are better investigated and till they become more stable, safe and affordable for clinical use.

**Keywords:** Dental pulp stem cells, Triple antibiotic paste, regenerative endodontics, dental pulp regeneration.
ABBREVIATIONS

- **DMEM**: Dulbecco’s Modified Eagle Medium, is a cell culture medium developed by Harry Eagle that can be used to maintain cells in tissue culture.
- **DPSCs**: Dental pulp stem cells
- **EDTA**: Ethylenediaminetetraacetic acid
- **ELISA**: enzyme-linked immunosorbent assay (ELISA) is a test that uses antibodies and color change to identify a substance.
- **FBS**: Fetal Bovine Serum, is the most widely used serum-supplement for the in vitro cell culture of eukaryotic cells. This is due to it having a very low level of antibodies and containing more growth factors, allowing for versatility in many different cell culture applications.
- **MTT assay**: The MTT assay is a colorimetric assay for assessing cell metabolic activity
- **NaOCL**: Sodium Hypochloride
- **OM**: Osteogenic medium
- **PBS**: Phosphate-buffered saline, with EDTA is also used to disengage attached and clumped cells.
- **Real time PCR**: also known as quantitative polymerase chain reaction (qPCR). It monitors the amplification of a targeted DNA molecule during the PCR, i.e. in real-time, and not at its end, as in conventional PCR.
- **TAP**: Triple antibiotic paste.
INTRODUCTION

Throughout the history of dental medicine, scientists have always believed that conserving a vital structure of the teeth is the most beneficial for their longevity. Despite the validity of this statement, maintaining the vitality of the tooth was an uneasy challenge. Worldwide, dental caries are the most prevalent chronic disease among both children and adults affecting 92% of the population [1], which makes the dental pulp regularly exposed to a high risk of inflammation leading to an irreversible necrosis. It wasn’t until the intracanal medication pastes were tested by Sato et al [2] when a hope was given to the necrotic pulps and partially formed roots that led to some sort of resuscitation giving back a considerable value to the regenerative endodontics. Triple Antibiotic Paste (TAP) made a positive feedback in forming dentin and pulp-like tissues combining between the antimicrobial activity, a simple method of application and a narrowed local effect instead of a systemic one [3]. This convincing trio didn’t prevent this method of having some down points: canal calcification, crown discolouration and even failure of the procedure for unknown reasons was reported in some cases. Lately, seeking to find a safer more homogenous way to regenerate pulpal tissues, stem cells research came to the surface in hope to open wider horizons in regenerative endodontics. It was shown that the dental pulp stem cells (DPSCs) are able to produce tissues similar to the dental pulp in structure in vitro as well as in vivo. Based on the hypotheses that (i) triple antibiotic paste treatment method has some inconveniences and (ii) dental pulp stem cells treatment method came with an innovative outcomes concerning dental pulp regeneration, the aim of this review is to explore in details both protocols and highlight the progress that DPSCs has reached and if they are ready to be used in clinical practice.
1. SELECTION CRITERIA OF THE STUDIES, SEARCH METHODS AND STRATEGY

1.1) The systematic review organization:
PRISMA requirements/checklist for publication of systematic review of scientific literature (2009) was followed in order to arrange the parts of this review adequately (source: http://www.prisma-statement.org/)

1.2) Search methods:
Internet based search engines where used (Google, Google Scholar, etc) as well as articles and journals databases (PebMed, Researchgate, clinicalkey, journal of chemical and pharmaceutical research, journal of endodontics). The search was done in English language and only English article were taken. Search terms such as “dental pulp stem cells”, “regenerative endodontics”, “triple antibiotic paste”, “dental pulp regeneration”, “immature dental roots”, “dental pulp revascularization” where used in order to audit the searching method and to get to more accurate information about the reviewed topic.

1.3) Data collection process:
All the studies collected from the different databases and included in this systematic review were selected as free full texts, thus accessible to everyone. “Researchgate.net” was the only database that allowed us to request the full text content of a paid article from the authors themselves.

1.4) Inclusion and exclusion criterias

1.4.1) Inclusion criterias:
- Population: children, young adults, adults
- Etiology: odontogenic, trauma to oral cavity only
- Interventions: regeneration with pastes (triple antibiotic paste only), stem cells studies (DPSCs only)
- Studies subjects: humans (in vivo/in vitro)
- Dentitions: primary and permanent
- Literature type: confined to clinical trials only
- Year of publication: last 10 years

1.4.2) Exclusion criterias:
- Animals
- Other sources of stem cells then dental pulp
- Systematic reviews (such texts were only used an references for some of the discussion part or introduction)
- Case reports

1.5) Quality assessment:
In order to ensure a adequate quality level for this review, it was decided to follow two phases of assessment:
- 1st the student writing the systematic review was responsible for searching and collecting the included studies
• 2nd a supervisor (endodontics specialist dentist) reviewed and approved these studies.

1.6) Risk of bias:
Briefly, this systematic review is exposed to a certain amount of bias. This is due to the absence of articles that discussed specifically the comparison of both methods to each other in one single study. That’s why we had to include several articles that considered one of both methods to be studied and evaluated as a contributor to dental pulp regeneration and then compared individually in order to form a fair, well organised discussion.

1.7) Contacting the authors for missing data:
Some of the included studies about stem cells treatment didn’t show the accurate number of patients that underwent the procedure, above that there was a low accessible amount of articles about that topic, for that, e-mails were sent to the respective authors in order to get informed about the missing inputs but none of them was reachable.

![Flow diagram](image)

**Fig. 2.** Flow diagram showing the selection process of articles from different databases.

(PRISMA 2009 flow diagram)
2. SYSTEMATIZATION AND ANALYSIS OF DATA

2.1) Search results:
6812 articles with related topics to this review subject were collected for this systematic review. After the elimination of duplications we were dealing with 350 studies. A total of 40 abstracts that centered on the studied subject were accepted. 12 articles were chosen. After comparing the literature to the inclusion and exclusion criteria needed. Only those articles giving informations about the use of stem cells to regenerate the dental pulp or the clinical application of triple antibiotic paste in clinical cases where selected. The patients from the selected studies had an age range between 7-55 years old and a total amount of over 148 teeth were included.

2.2) Characteristics of studies:
The review included 10 randomized control clinical trials, 1 clinical study and 1 double blinded randomized control clinical trial. The studies were published in a range of years between 2011 and 2017. Country wise, 1 study was taken from India, 2 from Iran, 2 from the USA and 1 from each Australia, Japan, Belgium, Brazil, Malaysia, Syria and China. In order to properly organize and for better mentioning of informations, a table of characteristic was made for this purpose (Table.1), in which the authors, year, country, study design, age, number, gender of each study are displayed.

2.3) Triple Antibiotic Paste (TAP):

2.3.1) Protocol used for triple antibiotic paste (TAP) treatment method:
In a part of the litterature included in this systematic review, TAP treatment technique was used to induce a regenerative endodontic mechanism. Generally, all studies except G.H.Yassen 2015 [22] followed the protocol described by Hoshino et al [2] and each study contained some variables dealing mainly with wether a mechanical instrumentation was used to extract the dental pulp, the size of the instrument used to induce periapical bleeding, type of permanent restoration and the concentrations of the irrigation solutions. Starting with anesthesia, two studies used local injections with lidocaine 2%, one study used lignocaine 2% injection and two studies experimented on extracted teeth thus did not used anestheisla. Access cavity was done by a round diamond endo bur mounted on a high speed handpiece in four studies and one study used teeth cuts instead of cavity access. For the irrigation process the main irrigator was NaOCl where in some studies extra irrigation with Chlorehexidine or Saline was added. The NaOCl concentrations ranged from 1.5% to 6% with a quantity ranging between 1ml to 20ml among the included studies. The antibiotic mixture was the same in all of the studies including a mix of Ciprofloxacin, Metronidazole and Minocycline and the application period ranged between 7 and 21 days before removing it from the canals. In three studies K-files were used to induced periapical bleeding letting the blood reach 3mm below cemento-enamel junction and
teeth were left to form a clot for a period ranging from 5 to 15 min. The post treatment restorative material used were dentine bonded resine composite, Colostol, glass ionomer cement (double seal) and Cavit. Follow up periods for four studies ranged between 1 week to 19 months and one study did not associate any informations about that point.

Althought the modifications that took place in the different studies included, it doesn’t seem that there is a proportional relationship between the steps taken to secure the canal environment from bacterial invasion and the insurance of positive results in TAP treatment method. In other words, the studies that used higher NaOCl concentrations than others didn’t proved that it’s a way to ensure a better outcome of treatment than the ones that used lower concentrations. But it was noticed in one of the studies [6] that the authors removed the TAP mix from the canal after 2 weeks instead of 1 week which might have been responsible for the crown discolouration in 10 out of 12 treated teeth still without altering the treatment success.

Etiology, composition of the TAP as well as the results of each study included which involved TAP treatment are found in details in (Table.2).

2.3.2)Triple antibiotic paste mode of action and efficacy:

Along an adequate instrumentation and copious irrigation of the root canal with a potent desinfectant solution, TAP has shown to be a good choice for pulp regeneration and the development of immature permanent teeth roots. Generaly the TAP is a mix of three main antibiotics: Metronidazole is a nitroimidazole compound that exhibits a broad spectrum of activity against protozoa and anaerobic bacteria (Hoshino et al). Minocycline is a semisynthetic derivative of tetracycline with a similar spectrum of activity. Ciprofloxacin, a synthetic fluoroquinolone, has a bactericidal mode of action (Windley et al). Some studies suggested that as any antibiotic side effect on the organism, TAP might be causing a bacterial resistance toward the medication via the typical triad of antibodies production, that’s why regenerative endodontics is always on the track of finding new solutions in order to get to a more secure treatment protocol.

Even while having some disadvantages such as crown discoloration, risk of bacterial resistance to the antibiotics and altering the composition of the roots dentine, pulp regeneration with TAP is a method to go for treating immature permanent teeth. The included studies showed acceptable results when it comes to the pulp regeneration and the development continuation of the roots as well as positive pulp sensibility testing after the treatment.

Many factors seems to come advise the antibiotics effect itself to achieve a successful treatment: a copious irrigation using a well concentrated NaOCl and an adequate bleeding time that allows a blood clot to take place in a bigger surface area inside the root where also necessary to achieve positive results.
2.4) Dental Pulp Stem Cells (DPSCs) :

2.4.1) Protocol used in dental pulp stem cells (DPSCs) treatment method:
In the reviewed articles dealing with the DPSCs method in regenerative endodontology, a similar steps were used. Variables specific to every study are further described in (Table.4). A precise protocol describing how to harvest and culture dental pulp stem cells isn’t yet officialy cited, that’s why here is a brief overview of the main steps of this method :

1. Cleaning of tooth surface
2. Teeth are cracked open to expose pulp
3. Removing pulp and mincing it to small pieces
4. Placing pulp parts in flask containing a favorable culture medium and at a specific temperature and CO2 concentration
5. Changing medium every (n) days
6. Cell harvesting after exposure to extraction medium until a certain passage
7. osteogenic medium (OM)
8. Cells proliferation and reproduction and viability examination (Table.3)

2.4.2) Stem cells and Dental pulp stem cells (DPSCs):
Stem cells are undifferentiated biological cells that can differentiate into specialized cells and can divide via mitosis to produce more stem cells. There is three main sources of stem cells from which they can be extracted, isolated and then cultured :

- Bone marrow via a process called harvesting
- Adipose tissues which requires liposuction
- Blood via withdrawal from a donor.

In the dentistry field, stem cells are taken from many sources:

- From dental pulp as dental pulp stem cells (DPSCs)
- From human exfoliated deciduous teeth (SHED)
- From apical papilla (SCAP)
- From periodontal ligaments (PDLSCs)
- From dental follicle precursor cells (DFPCs).

The accuracy of the DPSCs culture procedures seen in the included studies (Table.4) shows the complexity of such process. Starting with the culture medium where the cells should be embeded in, the mix of DMEM, FBS and antibiotic solution shows to be the proper environment for the stem cells vitality based on the success witnessed in these experiments. Additionally, in order that the DPSCs become shifted into the oral environment while maintaining their proliferative features, they must be safely extracted and detached : the Trypsine-EDTA solution showed good results in adopting that function which makes it an adequate conveyor of cells from the experimental location to the oral one.
MTT assay is one of the important method that help determining the metabolic activity of any cultured cells, it was used in the studies analyzed (Table.3) and showed increasing metabolic action of cells in the regular culture medium as well as a constant metabolism when the cell were exposed to osteogenic medium [29]. Alizarin red staining is one of many method to detect cells activity, in such case we are intrested in determining the quantitative calcific deposition of the DPSCs and here where Alizarin red function lies. It was shown that DPSCs can produce calcific depositions even under abnormal situations [9].

DPSCs showed as well that they share with the human body cells the same karyotype [23][28] which might indicate that it could be easier for such cells to fuse with there biologic surroundings easily. In a recent study, according to R.Kabir : “The dental pulp offers a source of stem cells (in post-natal phase) that is readily available, with minimally invasive process that results in minimal trauma” [4]. This prove us that the stem cells and specialy DPSCs can be obtained by simple and very conservative means.

In the same study as well it was mentioned that : “Dental pulp tissue engineering is a promising field that can potentially have a major impact on the oral health...depending on specific signals from their environment, DPSCs can either regenerate new stem cells or undergo a differentiation process”,this enlighten us about the potential therapeutic applications of the stem cells in regenerative dentistry and emphasis how adaptive are such cells with their surroundings being able to change according the needs of the medium they are placed in.

3.DISCUSSION

Since the existence of odontology,the pulpal regenration was and still one of the most discussed riddles in this field. Many points of view and opinions were discussed among the scientific industry in order to reach a better understanding of the dental pulp and its potency of regeneration in case of a pathology. In this systematic review, two techniques of dental pulp regeneration were debated : the first dealt with a frequently used protocol of regeneration using the triple antibiotic paste and the second one highlighted a newly introduced protocol which might be leading us to a new era in the regenerative endodontics medium, the dental pulp stem cells culture. In the following, pros and cons of both methods are discussed in order to clarify how useful they can be in endodontics.

3.1) TAP

3.1.1) TAP treatment efficacy against bacterial resistance to antibiotics :

Nowadays antibiotics are used almost as the first choice treatment method for most of the bacterial infections in the organism. This course of application has led to the formation of bacterial resistance against these antibiotics which means that specific bacterias are now able to defend themselves against the healing action of the antibiotics thus making such drug non functional.
Such bacterial reactions should be taken in consideration when it comes to the TAP treatment that might be inhibited by some resistance forming bacteria and what should let us think of better antibiotic-free ways to form pulp-like tissues.

In a study that compared the efficacy of TAP versus other antibiotics-free agents on the elimination of *E. faecalis* from the root canal (one of the most abundant pathologic gram + facultative anaerobes in infected canals) it was shown that: “2% CHX gel is the most effective medicament against *E. faecalis* in infected root canals” [5]. Although this study cannot by itself only be taken as a concrete reference that ensures the bacterial resistance to the TAP but it definitely alerts to the necessity of finding other methods to treat root immaturity.

### 3.1.2) Crown discoloration induced by TAP treatment:

One of the main disadvantages of this method is that it makes the tooth crown more prone to discoloration, a result of the effect of TAP on the dentinal tubules. One study showed that 10 out of 12 teeth that underwent TAP treatment ended up with having crown discoloration no matter if the treatment was successful or not [6]. Another study published in 2009 suggested a modified protocol of the conventional TAP method described by Sato et al. 1996 [2], Banchs & Trope 2004 aiming to eliminate any crown discolouration. Following the canal instrumentation and before filling it with TAP, the crown dentinal walls were etched and bonded for 20 sec for each solution and then in order to maintain patency a K-file was projected inside the canal, then the coronal dentine was filled with a layer of flowable composite thus sealing the dentine tubules and preventing the TAP from entering [7]. In this way the dentinal tubules were sealed and TAP didn’t affect them, as a result there wasn’t any crown discolouration and still they managed to induce TAP in the canal.

### 3.2) DPSCs

#### 3.2.1) DPSCs treatment efficacy:

In the studies included about DPSCs in this review, the authors submitted the cells samples to strict evaluative tests that assessed the presence, viability, activity and genetic expression of the stem cells. The dental pulp stem cells showed positive results in all the included studies although the changes and variables between each study, the success of the experiment was always maintained. DPSCs are with time, proving that they are an autologous and easily affordable mean of dental pulp replacement. In other words, a simple culture medium, short period of time and an effective extraction solution demonstrated that they are enough for a successful stem cells culture.

#### 3.2.2) DPSCs comparison among other stem cells sources:

A study done by Huang et al in the University of Maryland, USA proved that “DPSCs do share a similar
pattern of protein expression with BMMSCs *in vitro* (BMMSCs are the bone marrow mesenchymal stem cells and they are known as the most studied stem cell population due to their great potential in regenerative medicine). In the same study it was mentioned that “Due to certain shortcomings of obtaining the BMMSCs, including pain, morbidity, and low cell number upon harvest, alternate sources for MSCs have been sought”. It came as well that “the neurogenicity of dental stem cells may be more potent than that of BMMSCs, most probably due to their neural crest origin” [8]. A positive neural response and stimulus transmission is a main dental pulp function that might be signaling a start of pathological process, that’s why the greater potential of DPSCs in forming neural tissues is very important and gives this type of cells an uplift against other sources.

### 3.2.3) DPSCs activity in hypoxic environment:

The conditions of the medium were a cell is cultured have a major role in the affirmation of the strenght of these cells to withstand different environmental changes, thus proving that they might be more potent then others in the cell culture field. Cultured cells can strongly prove their proliferative and regenerative ability if, under hypoxic condition they maintained a similar activity levels compared to the normal condition where the Oxygen concentration is within the normal value. According a recent study in which DPSCs stemness was put under evaluation in normoxic and hypoxic (with 2%O and 5%CO, with balance of 93%N at 37°C) conditions. Cellular hypoxia was confirmed by the evidence of hypoxia-inducible factor 1-alpha (HIF1A) after decreasing oxygen levels in the culture medium. It was emphasised that: “hypoxic microenvironment can maintain proliferation capacity, enhance pluripotency marker expression, and promote differentiation potential of PDLCs and DPCs” [9]. This statement form a strong argument that highlights the success of DPSCs, ensuring that a change in oxygen concentration didn’t affect the ability of these cells to proliferate normally. In another meaning, presumably, patients suffering from conditions characterised by decreased blood flow to different parts of the organism and undergoing DPSCs therapy might not be worried about the results of there treatment due to the resistance of the DPSCs shown in hypoxic conditions. The following diagram extracted from the same study prove this hypothesis.

![Fig.3. Diagram showing a comparison of cell proliferation in DPC between normoxia and hypoxia. (Copyright © 2014 Yinghong Zhou et al)](image-url)
3.2.4) DPSCs viability in the oral cavity:

When we want to prove that DPSCs can regenerate to pulp-like tissues, we need to make sure that as well such cells will behave inside the teeth canals in a similar way that a normal dental pulp will starting from the mechanism of defence against pathologies all the way to the nature of the nervous response that it will produce and send.

A recent study done in Japan introduced the cultured DPSCs from five patients back into the oral cavity and specifically inside the teeth root canals in order to evaluate their activity in case of clinical usage. According to the authors: “the safety of MDPSC transplantation in pulpectomized teeth was demonstrated. The efficacy of the combinatorial regenerative therapy of MDPSCs with G-CSF for pulp/dentin regeneration was also suggested by EPT, MRI, and cone beam computed tomography” [10].

A table was made as well to prove the efficacy of these implanted DPSCs. Almost in all patients a positive electrical pulp testing and a lateral dentine formation confirmed by CBCT radiography was found after several days which primarily demonstrate that some characteristics of the normal dental pulp were found in the pulp-like tissues formed by DPSCs.

![Fig 4. Safety and efficacy of DPSCs after implantation inside 5 patients oral cavities (Nakashima et al. Stem Cell Research & Therapy 2017)](image)

3.2.5) Cryopreservation effect on DPSCs:

DPSCs pulp-like tissues are not necessarily always needed intraorally as soon as their culture is done, in many
cases DPSCs are isolated and cultured then kept deep frozen in special locations until the time of treatment comes, this procedure happens via a technique called cryopreservation. A systematic review published in the Brazilian Dental Journal, 21 studies were included in which the authors compared different cryopreservation techniques using DMSO (10%-20%) cryoprotector. It was concluded that "DSC could be cryopreserved, mainly with DMSO [10% - 20%], for periods up to 2 years maintaining their high proliferation rate, multipotency, karyotype and stem cells surface markers... On the other hand, the cryopreservation of the intact pulp tissue seemed to constitute an attractive and reliable source to isolate DPSC. It could be valuable since avoids the immediate stem isolation before cryopreservation... just 19% of the included studies have been evaluated the cryopreservation for periods longer than one year. Thus, the behavior of DSC in long times storage cannot be securely predicted. The conclusions presented in this systematic review should be interpreted with caution" [11].

In simple words, stem cells extracted from dental sources showed a safe interaction with cryopreservation essentially because until up to two years they maintained all there functionality characteristics which by this make them ready to use in dental field even after being frozen.

4. CONCLUSION

Finally, going through this systematic review and analyzing the included studies, we can conclude that the TAP treatment method has showed fair success in dentistry, as well the use of DPSCs proved impressive positive results. But to be more clear, we should understand that stem cells studies are still under experimentation and many hidden faces of such method are to be revealed in the future, although DPSCs might have a great chance to get be on top of the regeneration pyramid due to its efficacy, reproducibility and ability to regenerate under different environmental conditions but for now mostly they are still under laboratory investigations which make them so far not suitable for clinical use in everyday life. In the other hand, TAP is able temporarily to stay a safe way to regenerate the dental pulp tissues since its easily affordable in the clinical practice as well as that its disadvantages are still bounded and limited.

The culture of DPSCs in necrotic teeth root canals is so far proving an affirmative distinction in the world of regenerative endodontics, but until further studies and experimentations are done to eliminate any doubts in there success and in addition to find the ideal way to contain them ergonomically, TAP will stay the ideal replacement.

5. PRACTICAL RECOMMENDATIONS

If DPSCs managed in the near future to become clinically affordable and more stable in the dentistry field and less experimental, it could be adopted as the next pulp regeneration method which gives us a better understanding on how to keep the teeth vital as long as possible and preventing weakening it with artificial
materials. But while waiting this to happen, TAP method of treatment is a sufficient way to recusitate hopeless roots and pulps.

6.CONFLICT OF INTEREST

The author of this study reports no conflict of intrest.

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