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Evaluation of Cytotoxicity of Diclofenac Sodium on L929 Fibroblasts - An *in vitro* Study

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Authors' contributions

This work was carried out in collaboration among all authors. Author MRS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors PA and SP managed the analyses of the study. Author SP managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The aim of the study is to evaluate comparatively the cytotoxicity of diclofenac sodium and calcium hydroxide on L929 fibroblasts. L929 fibroblast cells were cultured and grown on Dulbecco modified Eagle's medium. Intracanal medicaments tested were Diclofenac sodium, 5.0, 7.5, 10.0 mM/ml) and calcium hydroxide. The human fibroblast cell lines cultured in Dulbecco Modified Eagle's medium were used as control group. Cytotoxicity was evaluated by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The results showed that there was a significant difference in cell viability as compared with the control group ($P<0.05$). There was no significant difference in the group treated with diclofenac sodium and calcium hydroxide (1.0 mM/ml). However, diclofenac sodium at concentration more than 5 mM/ml was found to be cytotoxic. The study concludes that diclofenac sodium is cytotoxic at 5 mM/ml and above. Therefore, further studies are recommended to establish the antimicrobial efficacy of the medicament. Within the limitations of the study, Diclofenac sodium at concentration more than 5mM/ml was found to be cytotoxic for the cells. The inhibitory concentration (IC_{50}) of Diclofenac sodium at which the cells were viable was found to be 5.2 mM/ml. Further studies should be done to establish the antimicrobial efficacy of the medicament at these concentrations.

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1. INTRODUCTION

The main objective of root canal therapy is to render the root canal free of microbes. In order to achieve this objective, the root canal is often cleaned and disinfected thoroughly. The clinician should be aware of the biomechanical preparation as this is the most important step in endodontic treatment [1]. This step serves to customize the root canal for proper obturation by giving it a tapered and smooth preparation. Disinfection, cleaning and shaping steps ought to be performed in biomechanical preparation [1]. However, these steps take a second place in case of achieving an aseptic root canal without the presence of infection. In such cases, intracanal medicaments play a vital role in achieving controlled asepsis. The rationale behind placing medicament is to achieve asepsis in the root canal by killing microbes [2]. Any treatment capable of destroying the microbes without causing any irritation to the periradicular tissues could be chosen root canal therapy. Therefore, an effective treatment which can inactivate bacteria and their inflammatory consequences could be considered the best candidates for root canal therapy [3]. Studies have shown that the residual bacteria can still flourish in the root canal and reactivate even after obturation. Hence, necessitating the use of intracanal medicament [4].

However, irritants and medicaments often extrude through the apical foramen; necessitating the need to evaluate the cytotoxicity which is of great concern in choosing a medicament. Cytotoxicity can be described as outpouring of molecular and cellular events that cause unequivocal damage to the cells and tissues structurally and functionally by interfering with the macromolecular synthesis [5]. Materials, either medicament or irrigant, must be made biocompatible to make it suitable for use in the root canal. The major factor that determines biocompatibility is cytological effect: that is, effect on cell survival. However, it is a difficult process to characterize cytotoxic reactions because there are many mechanisms of triggering cytotoxic reactions [5]. However, in dentistry it is important and beneficial to maintain maximum tissue viability and prevent unpleasant cellular reaction. Thus, screening of the materials before placing it inside the canal is often made mandatory [5,6].

The most commonly used intracanal medicament is calcium hydroxide because of its broad-spectrum activity against microorganisms in the root canal, ability to form hard tissue and many others. The main mechanism of action of calcium hydroxide is due to its alkalinity and subsequent dissociation of calcium and hydroxyl ions [7]. But some microorganisms like *E. faecalis* and *Candida albicans* in the root canal are resistant to calcium hydroxide. Also, calcium hydroxide sometimes fails to exert its action against a complex biofilm [7]. Hence newer medicaments are being tested individually or in combination with calcium hydroxide. But the persistence of the ions and raised pH for a prolonged period raised the concerns of cytotoxicity of the surrounding tissues [8]. The present study focuses on a newer intracanal medicament which is a nonsteroidal anti-inflammatory drug, Diclofenac sodium. Dastidar et al demonstrated that diclofenac sodium exhibited its antimicrobial activity by inhibiting DNA synthesis [9]. Few studies also reported that diclofenac sodium, ibuprofen and amoxicillin have greater antimicrobial action compared to calcium hydroxide.

Therefore, having been identified as an alternative antimicrobial agent and a potential intracanal therapy, it is important to evaluate the cytotoxicity potential of the diclofenac sodium. We have numerous highly cited publications on well designed clinical trials and lab studies [10–25]. This has provided the right platforms for us to pursue the current study. This has provided the right platforms for us to pursue the current study. Hence the aim of this study was to evaluate comparatively the cytotoxicity of diclofenac sodium and calcium hydroxide on L929 fibroblasts.

2. MATERIALS AND METHODS

2.1 The L929 Cell Line Was Divided into the Various Groups as Follow

Group 1: Control (Untreated Cells); group 2: Diclofenac Sodium - 1.0 mM/ml; group 3: Diclofenac Sodium - 2.5 mM/ml; group 4: Diclofenac Sodium - 5.0 mM/ml; group 5: Diclofenac Sodium - 7.5 mM/ml; group 6: Diclofenac Sodium - 10.0 mM/ml; and group 7: Calcium Hydroxide 1.75 mg/ml of distilled water [Biogen laboratories]

2.2 Sample Preparation

Diclofenac sodium was purchased (Voltarol 50 mg DT, Novartis Pharma AG, Stein, Switzerland) and crushed to powder and mixed with distilled water until a paste like consistency suitable for intracanal medicament was obtained. Calcium Hydroxide was prepared in the ratio of 1.75 mg/ml of distilled water.

2.3 Chemicals

The materials used for the MTT test were 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide, 10% fetal bovine serum (FBS), 100 units/ml of fungizone, dimethyl sulfoxide (DMSO), human fibroblast cell lines (primary culture), Eagle's minimum essential medium (EMEM), kanamycin, and phosphate buffered saline.

2.4 Maintenance of Cell Lines

L929 fibroblast cell lines were purchased from NCCS Pune, and cultured in a humidified atmosphere at 37°C in the cell growth DMEM medium containing 10% fetal bovine serum, L–glutamine, 1% penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified CO_2 (5%) chamber and 95% air. The cells were detached using 0.25% EDTA trypsin. Neutralization of the trypsin was achieved using DMEM containing 10% FBS and PSGF, and cells were mechanically separated using a pipette. There were 96-well plastic culture plates filled with 200 µl of medium containing in each well. The plates were then incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air for 24 h to permit attachment of the cells to the plates

2.5 Cell Viability via MTT Assay

For cell viability assay, L929 cells were cultured in 96-well tissue culture plates. The microplates filled with 100 µl of cells with a density of 1×10^5 as negative control. The cells were allowed to adhere for 24 hours, and the growth medium (using micropipette and the monolayer of cells) was washed twice with MEM without FBS to remove the dead cells and excess FBS. Then 1.0 ml of the medium (without FBS) containing different dilution of diclofenac sodium (1.0, 2.5, 5.0, 7.5, 10.0 mM/ml) was added into the wells; 20 µl of MTT (5 mg/ml in PBS) were added to each well, and the cells were incubated for another 6-7 hrs in 5% CO_2 incubator. After removal of the medium, 1.0ml of DMSO was added to each well and tested. The supernatant was removed and 50 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The MTT gains access into the cells and enters the mitochondria where eventually it is reduced to an insoluble, coloured (dark purple) formazan product. The plates were placed on a shaker for 15 min and the absorbance was read on an enzyme-linked immunosorbent assay (ELISA) (MINDRAY90) reader at 570 nm. Each experiment was carried out in triplicate and the IC_{50} of the test samples as the percentage survival of the cells was calculated.

2.6 Statistical Analysis

Results were expressed as mean ± SD. Statistical significance was determined by one-way analysis of variance (ANOVA) and post hoc least-significant difference test by SPSS software (version22.0). $P < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Results

The results showed that there is no significant difference between the control group and Diclofenac sodium [1 mM/ml]. However, as the concentration of diclofenac sodium was increased, a significant difference was observed in the treated groups as compared with the negative control [Table 1]. The percentage of cell viability showed that there is no significant difference between negative control and Diclofenac sodium 1 mM/ml. Percentage of cell viability has no significant difference between DS 1.0 mM/ml, and calcium hydroxide ($p>0.05$). The inhibitory concentration (IC_{50}) of diclofenac sodium required to inhibit 50% of the cells was found to be 5.2 mM/ml.

Table 1: This table represents the mean and standard deviation of various test groups. It shows that there is no significant difference between the means of control group and Diclofenac sodium [1 mM/ml] ($p>0.05$) and as the concentration of the Diclofenac sodium increases it has been found that there is a significant difference between the means in comparison to negative control and Diclofenac sodium 1mM/ml ($p<0.05$).

Table 1. Mean and standard deviation of all the test groups

Test Groups	Conc. (mM/ml)	Mean ± SEM
L929 Untreated cells (control group)	-	0.485 ± 0.04
Diclofenac sodium	1.0	0.401 ± 0.03
Diclofenac sodium	2.5	0.312 ± 0.02*
Diclofenac sodium	5.0	0.256 ± 0.02*[a]
Diclofenac sodium	7.5	0.175 ± 0.01*[a]
Diclofenac sodium	10.0	0.112 ± 0.01*[a]
Calcium hydroxide	-	0.362 ±0.01*

**P<0.001, statistically significant as compared with Negative control. [a]P<0.001, statistically significant as compared with Ca(OH)2. The IC50 of the extract is 5.2 mM/ml*

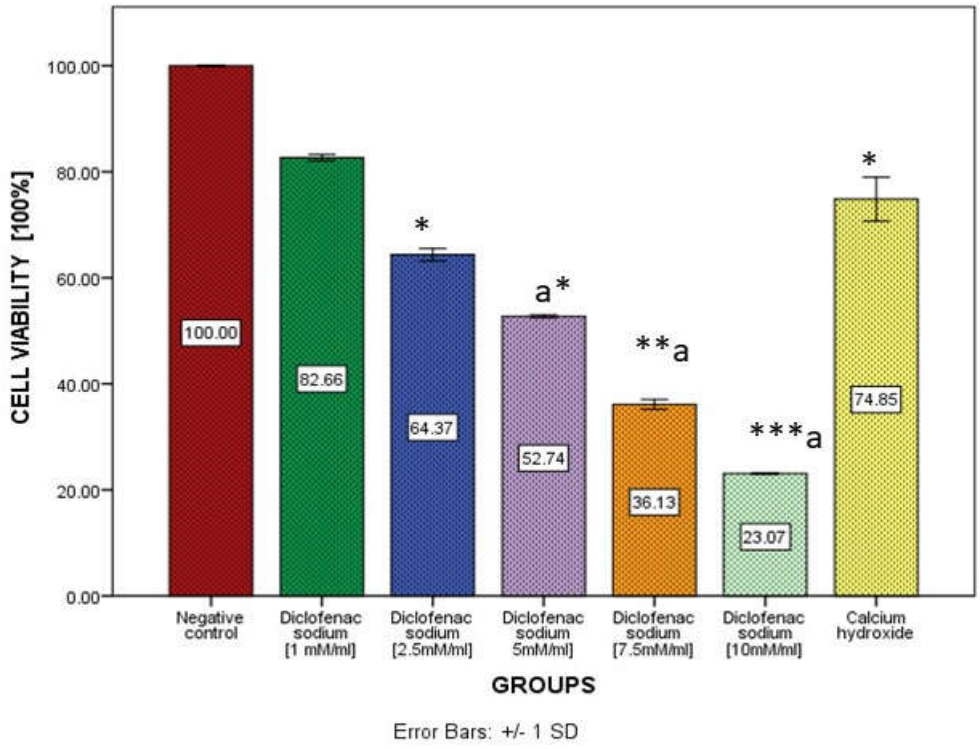

Fig. 1. Percentage of viable cells

Fig. 1 Bar chart shows that as the concentration of the Diclofenac sodium increases there is a significant difference in comparison to negative control and Diclofenac sodium 1 mM/ml ($p<0.05$). The inhibitory concentration(IC_{50}) of Diclofenac sodium at which the cells were viable was found to be 5.2mM/ml. [a]P<0.001 statistically significant as compared with Ca(OH)2. *P<0.1; **P<0.01 ***P<0.001, statistically significant as compared with Negative control.

3.2 Discussion

The main objective of root canal treatment is to eradicate microbes from the root canal by thoroughly performing the biomechanical preparation and disinfection that provide a fluid tight seal via canal obturation. However, in spite of the acclaimed measures, most of the endodontic procedures still fail [4]. The most common causes of root canal failure are reinfection and improper preparation of the canal. The main causes of reinfection are residual microbes within the canal that are failed to be eliminated by the clinician [4,5]. The presence of residual microbes can be attributed to the anatomical complexities of the canal which have some few inaccessible areas that do not permit the instrumentation. Hence the residual microbes residing in such areas can flourish and cause

reinfection thus, causing the endodontic failure. In spite of the availability of various techniques to mechanically and chemically debride the canal, it still remains a challenge to completely achieve a bacteria free root canal. The role of intracanal medicament is very crucial in reducing the microbial load.

The rationale behind placing medicament is to achieve asepsis in the root canal by killing microbes [26]. Biocompatibility and low cellular toxicity are the primary requisites of an intracanal medicament because they can extrude through the periapex and contact the soft and hard tissues of periapex which can cause tissue toxicity and delayed healing in case of cytotoxicity [7]. The most commonly used intracanal medicament is calcium hydroxide which was introduced in 1920 by Hermann. Some of the beneficial properties include antimicrobial efficacy, ability to induce hard tissue, high alkalinity, dissolution of microbial by-products and root resorption inhibition [9]. The antimicrobial efficacy of calcium hydroxide emanates from its ability to decompose into calcium and hydroxyl ions which creates a basic pH and an alkaline environment that subsequently inhibits the bacterial enzymes [27]. Also the hydroxyl ions can penetrate through the dentinal tubules and reach the root surface on the exterior; a process known to occur within seven days. Hence, it is necessary to place the medicament for seven days [28].

However, the prolonged period of alkalinity might cause cytotoxic effect on surrounding tissues which may cause destruction of soft tissues leading to necrosis and inflammation. Also calcium hydroxide is incapable of eliminating all the microbes attributed to its failure to reach the inaccessible areas in the root canal and buffering capacity of tissue fluids [9]. Data obtained from an *in vivo* study showed that $Ca(OH)_2$ paste induced an intense inflammatory reaction at the end of one week with necrotized areas surrounding the material due to the alkalinity of calcium hydroxide. However, the study also demonstrated that at the end of 30 days, the inflammatory reaction regressed and the number of collagen fibres and fibroblasts increased [29]. The necrotized tissue in turn, induced phagocytosis and activated tissue repair mechanism making calcium hydroxide a least cytotoxic agent. In order to overcome these potential limitations of calcium hydroxide, newer alternatives are being tested either individually or in combination to potentiate the effect of calcium hydroxide [3].

One such new material is diclofenac sodium. In this study, diclofenac is chosen as an alternative intracanal medicament and its cytotoxic effect tested. Diclofenac sodium, a NSAID [30], is known for its pain reduction on premedication with single dose and post extraction. The beneficial effects of NSAIDs are their local analgesic, antiinflammatory and possible antimicrobial efficacies. Dastidar et al, Dutta et al, Annadurai et al, have established the antibacterial efficacy of NSAIDs. [27,31] Salem et al also revealed in her study that Diclofenac sodium and ibuprofen were capable of eliminating *E. faecalis* and *C. albicans* compared to calcium hydroxide [32]. However even cytotoxicity of the material should be tested before its usage.

In this present study, cytotoxicity of diclofenac sodium was tested against L929 cells. L929 fibroblasts are recommended by ISO 10993-5 for cytotoxicity tests as they are suitable substitutes for oral keratinocytes and can be easily grown and passed indefinitely. The results showed that there is no significant difference between the negative control and the group treated with diclofenac sodium 1 mM/ml at $p>0.05$. As the concentration of the diclofenac sodium was increased, the percentage of cell viability was found to decrease significantly as compared with the control group. The IC_{50} of diclofenac sodium was found to be 5.2 mM/ml (Fig. 1).

Studies have shown that combination of diclofenac sodium and calcium hydroxide is not deleterious to the surrounding tissues [30,33]. The cytotoxic effect of NSAIDs may be attributed to their ability to inhibit cyclooxygenase pathways mediated by two isoforms COX 1 and COX 2. Inhibition of COX by NSAIDs was previously thought to be the main reason for their chemo-preventive effect; however, evidence now suggests that there are COX independent mechanisms by which chemoprevention effect occur [34].

In another *in vitro* study with hepatocytes, diclofenac sodium was found to synergise with cytokines to induce apoptosis [35]. It also causes an increase in the intracellular calcium, but the role of this in the cytotoxic synergy is yet unknown. Hence further studies are recommended to evaluate the exact mechanism of cytotoxicity of diclofenac sodium [36].

4. CONCLUSION

The study concludes that diclofenac sodium is cytotoxic at 5 mM/ml and above. Therefore, further studies are recommended to establish the antimicrobial efficacy of the medicament.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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