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In vitro evaluation of biocompatibility of two intracanal medicaments on stem cells from apical papilla – a comparative study

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Abstract

The study explore some of the factors showing the biocompatibility and cellular metabolism of stem cells from apical papilla after their treatment with hapten solutions of two intracanal medicaments - Hydrocal and Acroseal. The MTT test and Annexin V test are used and the levels of HMOX-1 and IDO monitored after 48 hours of incubation in hapten solutions. In addition to proliferation and apoptosis, the possible sensitizing potential of these dental materials is assessed. The results show that after 48 hours, the groups treated with dental haptens found significantly fewer living cells than in the control group, which is especially indicative in the case of Acroseal. The proliferative activity of stem cells is increased, but without significant differences. HMOX-1 was statistically elevated in the Acroseal-treated group, indicative of its sensitizing capacity. IDO is statistically elevated in the group of cells treated with Hydrocal, which unequivocally speaks of the serious oxidative stress up to 48 hours and the need for protective action of this enzyme.

Running title: Biocompatibility of intracanal medicaments on SCAPs

Keywords: intracanal medicaments, calcium hydroxide, epoxy based sealer, dental haptens, apoptosis

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Introduction

The success of endodontic treatment is dependent on the obturation of the root canal with gutta--percha and sealers which can seal the entire root canal, thereby preventing microleakage and the reinfection of the root canal. Over time, sealers and gutta-percha have become the standard approach to obturating root canals. Sealers are needed to seal the gutta-percha core material and prevent microleakage. In the absence of sealer, gutta-percha cannot adequately seal root canals and prevent microleakage and reinfection. Gutta-percha is most commonly used to obturate the root canal because it can be placed relatively easily and also be removed relatively easily if the tooth requires retreatment.

The biocompatibility requirements for endodontic sealers are somehow connected with their antimicrobial activity. A number of studies have shown that the antimicrobial activity of commonly used endodontic materials is often closely related to their cytotoxicity [1]. Materials that are antimicrobially effective and toxic only briefly after mixing may be a good alternative. The bioactive properties of sealers are only acceptable if endodontic treatment completely seals the root canal for a long period of time. The most widely used root canal sealers have sensitizing potential. In this context, the biocompatibility of intracanal drugs with the macroorganism is an important factor in the choice of material. Biocompatibility is "the ability of a material to elicit an appropriate biological response in a given application" [2,3]. Toxicity of a material is the ability to damage a biological system by chemical means. It is a dose related potential of a material to cause cell or tissue death [4].

Dental materials must be assessed through several toxicity and biocompatibility steps. ISO 7405 is especially developed for devices used in dentistry, and adopted the following sequence of tests for evaluation of dental materials i.e in vitro tests (cytotoxicity, mutagenicity); in vivo tests (sensitization, implantation tests, mucosal irritation) and usage - clinical tests [5].

In this research we consider the effect of different materials on the stem cells of the apical papilla SCAP. One is for temporary obturation of the root system for the treatment of acute and chronic periodontitis, pulpotomy, biological treatment with direct or indirect coating of dental pulp - **Hydrocal** and the other for permanent obturation - **Acroseal**.

Hydrocal PPH Cercamed is a mix of calcium salts (calcium hydroxide, hydroxyapatite, calcium salts, anhydrous sodium carbonate, magnesium oxide). Is used during dental treatment as material for making pastes and dental fillings. All the above components are applicable for biomedical and dental use and have hydrophilic properties. Although $Ca(OH)_2$ shows excellent biocompatibility, the commercial addition of other substances to this compound may affect its biological properties [6].

Acroseal is an epoxy-based canal filling that is prepared ex tempore before being introduced into the root canals. Epoxy resins are biologically active substances, and the monomer Bisphenol A diglycydil ether (BADGE) is an important contact allergen. The base contains methylenamine and enoxilone and the catalyst contains $Ca(OH)_2$ and BADGE.

The studied intracanal materials were prepared in the form of hapten solutions in which the stem cells were incubated for 48 hours. The hapten form allows making an additional conclusion about the sensitizing potential of these dental materials. Haptens are considered low molecular weight compounds up to 1000 daltons, which are defective allergens with very low immunogenicity. Almost all dental materials - metal alloys, photopolymers, canal fillers, immersed in the oral cavity in the right conditions - constant temperature, abrasion during chewing, biochemical corrosion - from haptens can become complete allergens when bound to glycoproteins and their structure turns more complicated [7].

The aim of this study was to identify some of the main effects of Hydrocal and Acroseal at the cellular level.

Stem cells from dental apical papilla (SCAPs) were first identified and characterized by Sonoyama et al. in human permanent immature teeth and are described as adherent clonogenic with mesenchymal stem cell features [8]. This makes them extremely suitable for tracking changes in parameters in the present study. Intracanal medicaments also affect the survival and proliferation of mesenchymal stem cells.

Cell viability was measured using the MTT assay, and apoptosis was measured using the Annexin V-binding assay.

Human **mesenchymal stem cells** might exert their immunomodulatory function through production of indoleamine 2,3-dioxygenase (IDO) [9], and oxydative stress through HMOX-1.

IDO is a heme enzyme that initiates the oxidative degradation of the essential amino acid l-tryptophan along the kynurenine pathway. IDO also represents an important immune control enzyme. Cells expressing IDO are capable to suppress local T cell responses and to promote immune tolerance under various physiological and pathophysiological conditions of medical importance [10].

HMOX-1 is a member of the oxidoreductase family and catalyses the degradation of heme in carbon monoxide, divalent iron and biliverdin. It is then converted in bilirubin, the most abundant endogenous antioxidant in mammalian tissues, responsible for a number of antioxidant activities due to the action of multiple oxidation factors and contact sensitasers, including monomer BADGE [11].

The aim of the research is to study some parameters of biocompatibility of two root canal materials on STAMs.

Materials and methods

Preparation of material specimens

According to the utility model with patent №3493/23.01.2020 from Patent office of the Republic Bulgaria, the dental haptens for patch test containing root canal materials are in gel form with vaseline and in 1 to 5 wt. % concentration. For the needs of the study in the laboratory at the Department of Chemistry and Biochemistry – Medical Faculty, Medical University - Sofia, a 4% solution in dimethyl sulfoxide (DMSO) of each root canal powders were prepared-Hydrocal 4% and Acroseal 4%.

SCAP Culture and Passage

SCAPs were isolated from freshly extracted immature teeth, by enzyme digestion method in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase. The cells were cultured with Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C in 5% CO_2. When the cells reached 80% of confluence, they were subcultured, using 0.05% of trypsin. For the purposes of this study, cells from passages 2 to 4 were used. The cells were incubated for 48 hours in DMSO solutions of both intracanal medicaments; cells cultured without haptens solutions in the same condition were considered control group.

Cell Proliferation and Cytotoxicity Detection

Novus' MTT Cell Proliferation Assay Kit (Catalog # NBP2-54883) was used for quantification of viable cells in proliferation and cytotoxicity assay. The method is based on the conversion of water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) compound to an insoluble formazan product. Viable cells with active metabolism convert MTT into formazan, dead cells lose this ability. Thus color formation serves as a useful and convenient marker of only the viable cells. The measured absorbance (590 nm) is proportional to the number of viable cells. The assay was performed according to the manufacturer's instruction.

Cell Apoptosis Detection

The apoptosis in SCAPs and was detected by using the Novus' Annexin V Apoptosis Kit [FITC] (Catalog # NBP2-29373-100Tests). The assay works in the following manner: cells that are viable are both Annexin V-FITC and PI negative; cells that are in early apoptosis are Annexin V-FITC positive and PI negative and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive. The assay was performed according to the manufacturer's instruction. A flow cytometric analysis was conducted by Navios EX Flow Cytometer - Beckman Coulter.

Cell Metabolic Activity

SCAPs culture supernatant concentrations of natural and recombinant human Indoleamine 2,3-dioxygenase (IDO) and HO-1/HMOX1 were me-

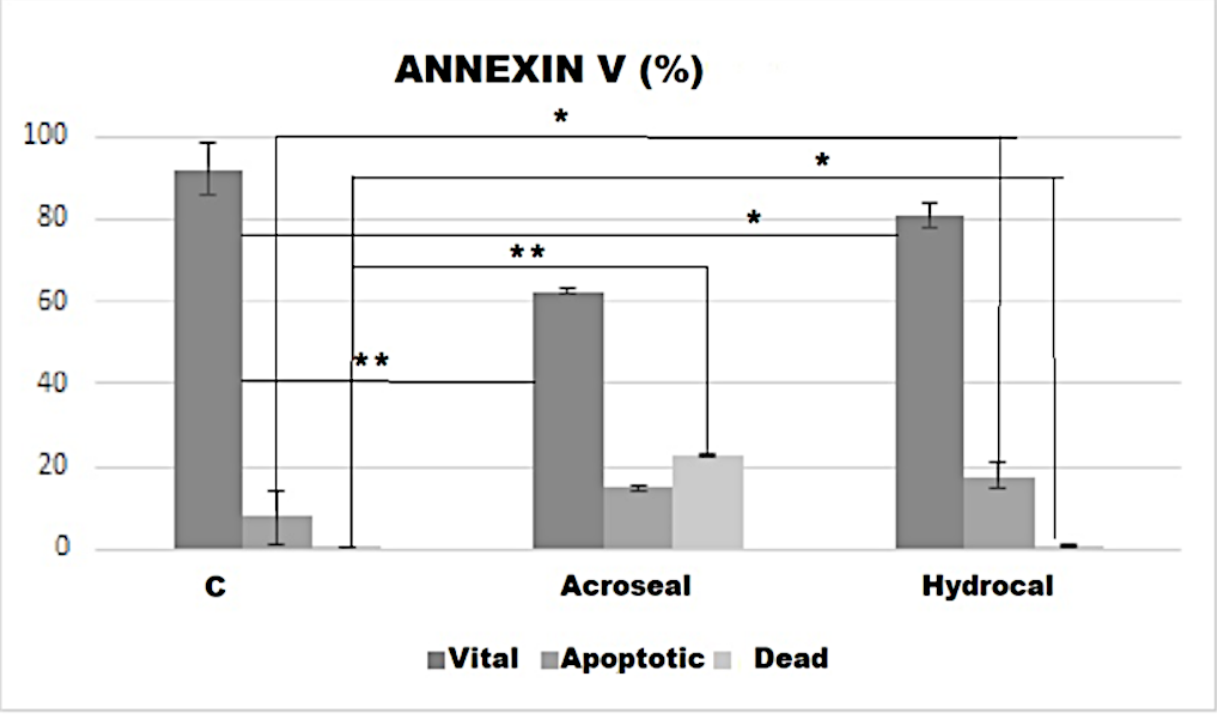


FIGURE 1 Results from the Annexin V test in the groups (* $p < 0.05$, ** $p < 0.01$)

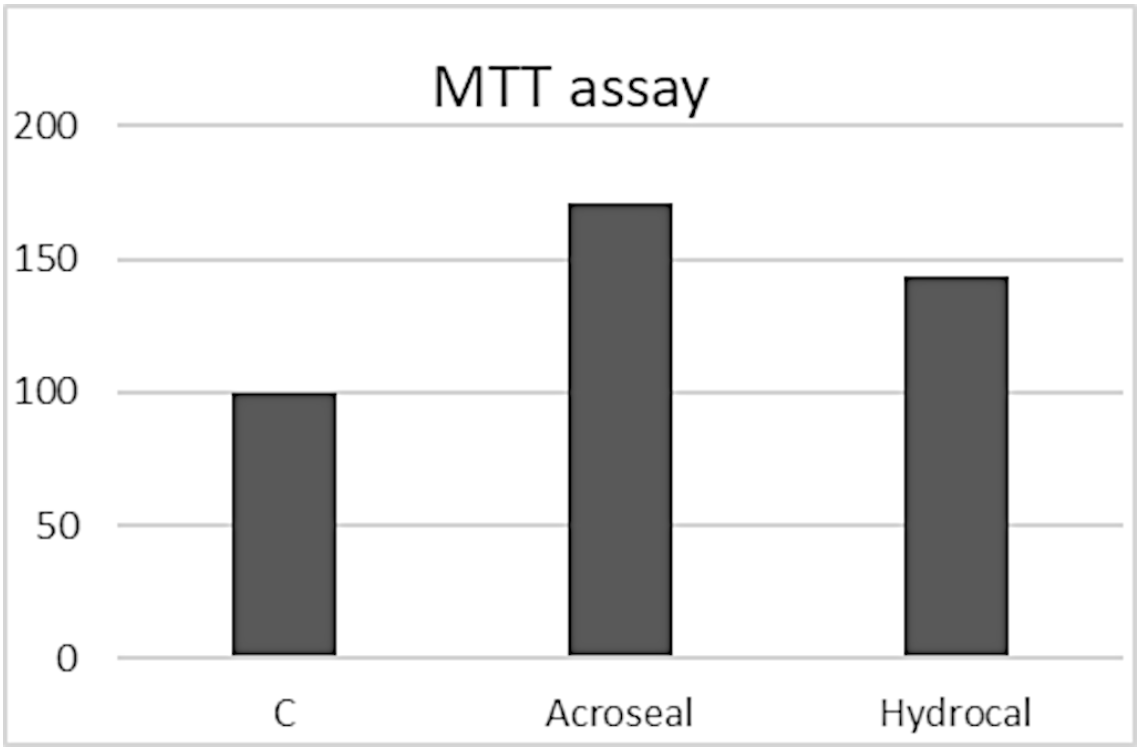


FIGURE 2 Proliferation of SCAPs in both groups

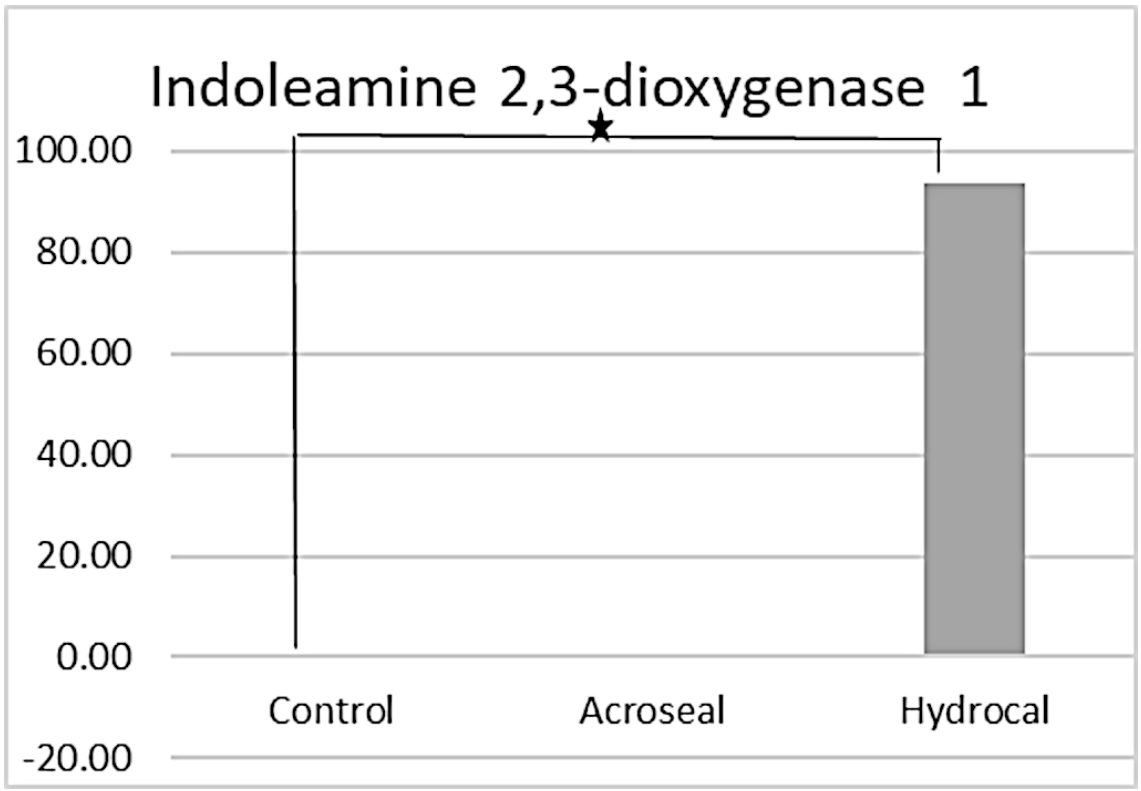


FIGURE 3 IDO concentration in the groups (p= 0.0001)

asured using Novus' Human Indoleamine 2,3-dioxygenase/IDO DuoSet ELISA kit (DY6030-05) and Human Total HO-1/HMOX1 DuoSet IC ELISA kit (DYC3776-2). The assays employ the quantitative sandwich enzyme immunoassay technique. They were performed according to manufacturer's instructions.

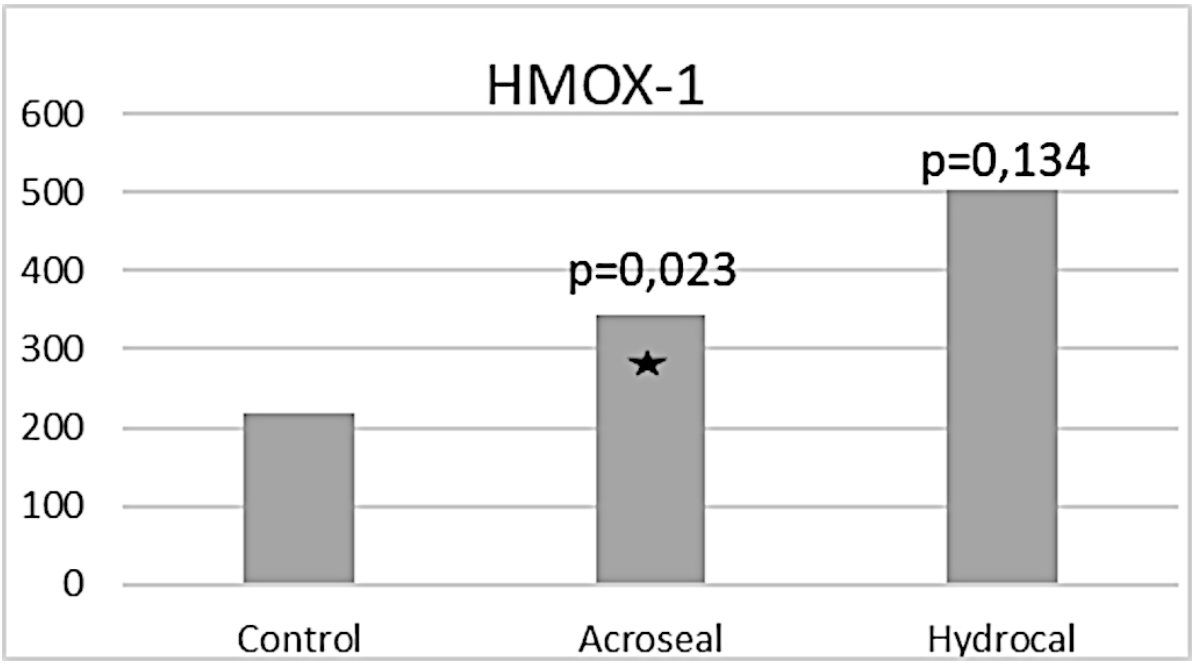


FIGURE 4 HMOX-1 concentration in the groups

Data Analysis

Data was analyzed with parametric t-test for the difference in mean values at 95% significance level, and $p < 0.05$ was taken as significant (* $p < 0.05$ and ** $p < 0.01$), using statistical software SPSS – 17.

Results

Both dental haptens showed a statistically significant lower value of live cells after 48 hours compared to the control group, as was particularly pronounced in Acroseal. In the same group, a higher percentage of dead cells was found, which indicates a direct toxic effect in the period up to 48 hours (**Fig. 1**).

A comparison of the percentage of apoptosis of SCAPs showed significantly higher apoptosis with Hydrocal. Cells prone to greater proliferative activity were those incubated in haptenic Acroseal solution, but without a statistically significant difference (**Fig. 2**).

Cell Metabolic Activity

We could suggest that the increased IDO production statisticaly significant from SCAPs treated with Hydrocal could present a protective response against the oxidative stress induced by the increased release of $(OH)^-$ and Ca^{2+} ions (**Fig. 3**).

The cells incubated in the Acroseal hapten solution stimulate the cell population to secrete HMOX-1, which is evidence of both: its antioxidant and sensitizing effects for at least the first 48 hours (**Fig. 4**).

Discussion

Endodontic treatment of teeth, regardless of the clinical diagnosis that led to the need for its application, ends with obturation of the root canals. The exact performance of root canal shaping and cleaning procedures, followed by obturation of endodontic space, does not reveal any further complications. The prognosis is good and the tooth does not act as an active field of disturbance in the body. The field of disturbance can be both bacterial and immunogenic. The latter is largely influenced by the used channel filling material and its composition.

In our study, Hydrocal and Acroseal exposure up to 4% concentration affect significantly the viability of stem cells of the apical papilla after 48 h. The main active ingredient in Hydrocala is calcium hydroxide, but we must not neglect the participation of other components. In contrast, a previous study showed that calcium hydroxide is not toxic for stem cells of the apical papilla (SCAPs) and increases cell survival and proliferation [12].

Prasetyo et al. (2021) study the effect of calcium hydroxide on umbilical cord stem cells. They found that Apoptotic Protease-Activating Factor-1, Caspase-3 and Caspase-9 increased significantly on days 3 and 7 [13].

In our study, the measurement for apoptosis was 2 days. Again, the values are statistically significantly higher compared to the control.

Lei Dou et al. conducted a study on five dental pulp capping agents found that calcium hydroxide $Ca(OH)_2$ inhibited cell proliferation and the percentages of dead and apoptotic cells were relatively higher in the group of $Ca(OH)_2$ on days 1, 3 and 7 compared with those in the other groups [14]. Researched by them, is in line with our results.

This is in contrast to the results of Bhandi et al., Who found that after 48 hours calcium hydroxide has no significant effect on cell vitality, we find that Hydrocal has a statistical effect on their survival [15].

In the study by Camargo et al. Acroseal has been found to have toxic effects on mammalian cells, which coincides with our results in the Anexin V test, which found direct cell death of statistical significance without being observed in apoptotic cells [16].

The cellular metabolism characterized by the enzymes HMOX-1 and IDO provides information not only about oxidative stress, but also about the possible sensitizing effect of the studied dental haptens. HMOX-1 is the most consistently found genetic marker induced by skin sensitizers [17].

The dental hapten Acroseal has the most pronounced effect on the concentration of HMOX-1, which defines it as a strong sensitizer in the studied period of 48 hours. Hydrocal dental hapten leads to a statistically significant increase in IDO, which proves its strong protective role against oxidative stress.

Coclusions

This study examined some biocompatibility effects of two intracanal medicaments at one and same concentration 4% on the viability and survival of SCAPs for 48 hours.

We found that Hydrocal hapten induced the highest percentage of apoptosis in the SCAPs.

The proliferation that occurs in the cells is not statistically significant compared to the control group.

Elevated HMOX-1 levels with Acroseal confirm previous studies on the possible sensitization of epoxy-based sealants. Hydrocal dental hapten leads to increased statistically significant synthesis of IDO.

Ethical approval
The conducted research is not related to either human or animal use.

Acknowledgement
Not applicable.

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Conflicts of interest
All authors declare that they have no conflicts of interest.

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