



In Vitro Comparison of the Effect of Ledermix® Paste and Green Tea Extract on the Concentration of Inflammatory Mediators

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ABSTRACT

Background and Aim: The purpose of this study is to compare the effect of Ledermix® paste and green tea extract on the concentration of inflammatory mediators.

Methods and Materials: In this in-vitro experimental study, first, the noncytotoxic concentrations of Ledermix® medicament and green tea extract were determined by MTT assay. Afterwards, the concentrations of IL-1 β , IL-6, and TNF- α inflammatory mediators were assessed by ELISA test in the four experimental groups and also in the control group at different time intervals of 2, 24, and 48 hours and after 7 days. One-way analysis of variance (ANOVA) was used for statistical analysis of the obtained raw data followed by Tukey's post-hoc test for pair comparisons.

Results: The noncytotoxic concentration of green tea extract and Ledermix® paste equaled 12 μ g/ml and 0.25 μ g/ml, respectively. Time interval had no significant effect on the concentration of the mediators. Generally, both substances significantly decreased the concentration of IL-1 β and IL-6 inflammatory mediators in comparison with the control group.

Conclusion: Green tea extract is less cytotoxic and more biocompatible than Ledermix® paste. Both Ledermix® paste and green tea extract expressed anti-inflammatory effects.

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Introduction:

Progression of pulpitis to the periapical area and accumulation of microorganisms in the root canal system trigger innate and acquired immune responses and consequently, inflammatory mediators such as IL-1 β , IL-6, and TNF- α are released in the periapical area. ⁽¹⁾ The release of these cytokines can lead to alveolar bone destruction and formation of periapical lesion. ⁽²⁾

The basis of a successful endodontic treatment which leads to ideal reparation is the identification and elimination of factors that induce apical periodontitis. Generally, mechanical and chemical debridement of the root canal and local application of intracanal medicaments (such as calcium hydroxide, Ledermix® paste and antibiotics) help eliminate the inflammation and repair the periapical tissues. ⁽¹⁾ Considering the controversies, none of these methods seems ideal and there is no consensus regarding their use. Nevertheless, reducing the inflammation to the minimum level is necessary to ensure successful treatment outcomes. ^(1, 2)

Studies have displayed that intracanal medicaments such as calcium hydroxide and Ledermix® paste can have side effects on vital tissues and since these substances are in direct contact with periapical tissues, it is important to consider the biocompatibility of intracanal medicaments upon selection. ⁽³⁾ Various studies have evaluated the antibacterial and bactericidal properties of green tea, as an intracanal medicament, on different bacterial species. The results proved that green tea extracts are probably beneficial as medicaments in infected root canals. ⁽⁴⁾ But the anti-inflammatory effects of Ledermix® and green tea have not been compared. Therefore, in the present study MTT assay was conducted to determine the noncytotoxic concentration of Ledermix® paste and green tea extract in monocyte cell culture medium. Also, the effects of Ledermix® paste and green tea extract on the level of IL-1 β , IL-6, and TNF- α inflammatory mediators in the cell culture medium were compared.

Methods and Materials:

In this in vitro experimental study, U937 mono-

cyte cell line culture media in 96-well plates were assessed (96 samples including 3 samples for each inflammatory mediator and one sample as the control), and purposive sampling method was used. In addition, the concentrations of different inflammatory mediators (IL-1 β , IL-6, and TNF- α) were measured at different time intervals of 2, 24, and 48 hours and after 7 days in the presence of Ledermix® paste (Sigma, Australia) and green tea medicament (Soha Jissa, Iran).

All the experimental steps were performed by one operator after comprehensive training. All the samples were incubated at 37°C and 95% relative humidity in compliance with ISO standard 6876 (This international standard specifies the requirements and test methods for root canal (endodontic) sealing materials which set with or without moisture, and are used for permanent obturation of the root canal with or without the aid of obturating points/cones. It only covers the sealers that are intended for orthograde use i.e. a root filling placed from the coronal aspect of a tooth).

In the first phase of the study, MTT assay was repeated three times to evaluate the effect of Ledermix® paste and green tea extract on monocyte viability. U937 monocyte cell lines obtained from the cell bank of Pasteur institute of Iran were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS (Fetal Bovine Serum), 2 mmol/l of L-glutamine and 1,000,000 U/L penicillin/streptomycin at 37°C, 5% CO₂ and 95% humidity. After 24 hours of incubation with green tea extract at the concentrations of 12, 25, 50 and 100 μ g/ml and Ledermix® at the concentrations of 0.25, 0.5, 1 and 2 μ g/ml, the cells were washed twice with Phosphate Buffered Saline (PBS) according to the study by Taylor et al. ⁽⁵⁾ and were incubated for one hour in fresh MTT culture medium at a dilution of 1 gr/l at 37°C. Afterwards, an equal volume of Lysis Buffer (pH=4.7) containing 200 μ g/ml, 50 % (v/v) N,N-Dimethylformamide was added to the culture medium and incubated for 24 hours. Finally, the amount of formazan absorbance was measured by spectrophotometer (SHIMADZU, UV 2100) at 570 nm wavelength, and the non-

cytotoxic concentrations of green tea extract and Ledermix® were determined. The highest concentration of the medicament with an optical density (OD) similar to that of the control group was determined as the final concentration, which indicated the maximum cell activity unaffected by the medicaments in the culture medium.

In the second phase of the study, U937 monocyte cell lines were cultured in DMEM culture medium inside 96-well plates, containing 10% FBS and 2 mmol/l of L-glutamine and 1,000,000 U/L penicillin/streptomycin and were incubated at 37°C, 10% CO₂, and 95% humidity. Four to five cell passages were transferred to fresh culture medium in 96-well plates with the capacity of 20000 cell/well.

In the first group (control), lipopolysaccharide (LPS) from E.coli K12 bacteria procured from Sigma-Aldrich Company (St. Louis, MO, USA) at the concentration of 1µg/ml was added to the monocyte cell culture medium and incubated. (6) In the second group (case), the same concentration of LPS from E.coli K12 bacteria was placed on monocyte cell culture medium and after one hour of incubation, 1ml of green tea extract at the concentration of 12 µg/ml was supplemented (0.25 µg/ml concentration of Ledermix® and 12 µg/ml concentration of green tea were selected due to the similarity of the optical density with that of the control group). In the third group (case), the same concentration of LPS from E.coli K-12 bacteria was placed on the culture medium and after one hour of incubation, 1ml of Ledermix® at a concentration of 0.25 µg/ml was added. In the fourth group (case), initially 1ml of green tea extract at a concentration of 12 µg/ml was added to the culture medium and incubated. After one hour, (7) 1µg/ml LPS from E.coli K12 bacteria was added. In the fifth group (case), 1ml of Ledermix® at a concentration of 12 µg/ml was added to the culture medium and incubated and one hour later, 1µg/ml LPS from E.coli K12 bacteria was supplemented. The samples were incubated and subsequently, the floating liquid surface over the culture media of the five experimental groups was collected at the time intervals of 2, 24, and 48 hours and after 7 days, and the concentrations of IL-1β, IL-6,

and TNF-α inflammatory mediators were measured by ELISA kit (Minneapolis, R&D systems, MN, USA). One-way analysis of variance (ANOVA) was used for statistical analysis of the obtained raw data followed by Tukey's post-hoc test for pair comparisons.

Results

The basic objective of this experiment was determination of the proper concentration of the medicaments to be used in the second phase of the study. Therefore, MTT Cell Viability Assay was performed on U937 monocyte cell line. The results of the MTT Cell Viability Assay are summarized in Table 1. The final concentrations of green tea extract were 12, 25, 50 and 100 µg/ml while for Ledermix® paste the concentrations equaled 0.25, 0.5, 1 and 2 µg/ml. It was shown that green tea extract at the concentration of 100µg/ml and Ledermix® at the concentration of 0.5 µg/ml have the ability to destroy half the cells in the culture medium, and can cause 50% reduction in the formation of formazan and 50% reduction in the optical density (OD) at 570 nm (measured by spectrophotometer) compared with the control culture medium. These values represent the cytotoxic concentration (Inhibitory Concentration₅₀ or IC₅₀) of these medicaments. 0.25 µg/ml of Ledermix® paste and 12 µg/ml of green tea extract are the noncytotoxic concentrations, which were used in the subsequent phase of the experiment.

Table 1- Results of the MTT Cell Viability Assay performed on Ledermix® paste and green tea extract

Medicament	Concentration (µg/ml)	Optical density (OD) at 570 nm
Control	-	45.1
Ledermix® paste	0.25	45.9
	0.5	22.3
	1	16.8
	2	3.6
	12	45.3
Green tea extract	25	42.3
	50	34.6
	100	22.9

In the second phase of the study, the concentrations of IL-1 β , IL-6, and TNF- α inflammatory mediators at the time intervals of 2, 24, and 48 hours and also after 7 days were assessed by ELISA kit in the four case groups and also in the control group. The concentrations of IL-1 β , IL-6, and TNF- α inflammatory mediators are shown in Tables 2, 3 and 4, respectively. The levels of significance (p-value) of the results of one-way ANOVA in comparing the concentrations of IL-1 β , IL-6, and TNF- α inflammatory mediators in the control group and the four study groups at the time intervals of 2, 24, and 48 hours and after 7 days are also presented in these tables.

Table 2- Average concentrations of IL-1 β inflammatory mediator at different time intervals

Groups	2 hours	24 hours	48 hours	7 days	p-value
LPS Control	8.9 \pm 1.5	9.5 \pm 1.17	8.79 \pm 1.44	9.03 \pm 2.3	
LPS-G	7.2 \pm 0.3	6.1 \pm 0.5	6.2 \pm 0.5	5.7 \pm 0.2	0.08
LPS-L	7.7 \pm 0.4	6.9 \pm 0.8	6.5 \pm 0.5	5.9 \pm 0.4	0.24
G-LPS	6.6 \pm 0.4	6.3 \pm 0.6	6.8 \pm 0.9	5.7 \pm 0.4	0.88
L-LPS	7.1 \pm 0.3	6.4 \pm 0.4	6.6 \pm 0.3	5.6 \pm 0.5	0.16
p-value	0.04	0.029	0.019	0.013	

LPS: lipopolysaccharide, G: green tea, L: Ledermix® paste

Table 3- Average concentrations of IL-6 inflammatory mediator at different time intervals

Groups	2 hours	24 hours	48 hours	7 days	p-value
LPS Control	194.21 \pm 11.34	123.38 \pm 218	115.64 \pm 7.31	154.63 \pm 22.48	
LPS-G	120 \pm 8	76 \pm 8	75 \pm 2	102 \pm 11	0.22
LPS-L	106 \pm 10	69 \pm 5	103 \pm 3	100 \pm 7	0.23
G-LPS	176 \pm 7	92 \pm 2	130 \pm 7	94 \pm 8	0.53
L-LPS	150 \pm 6	77 \pm 8	83 \pm 8	98 \pm 4	0.39
p-value	0.01	0.001>	0.001>	0.001>	

LPS: lipopolysaccharide, G: green tea, L: Ledermix® paste

Table 4- Average concentrations of TNF- α inflammatory mediator at different time intervals

Groups	2 hours	24 hours	48 hours	7 days	p-value
LPS Control	8.9 \pm 1.3	885 \pm 1.47	854 \pm 1.2	8.72 \pm 1.53	
LPS-G	5.5 \pm 0.6	6.1 \pm 0.5	6.5 \pm 0.3	5.2 \pm 0.2	0.49
LPS-L	6.9 \pm 0.5	5.7 \pm 0.3	5.6 \pm 0.7	4.6 \pm 0.3	0.38
G-LPS	5.7 \pm 0.7	6.4 \pm 0.6	4.7 \pm 0.1	6.4 \pm 0.1	0.08
L-LPS	6.8 \pm 0.5	68 \pm 0.9	5.9 \pm 0.3	5.1 \pm 0.1	0.24
p-value	0.03	0.015	0.016	0.015	

Discussion:

MTT Cell Viability Assay is a customary technique for assessing the biocompatibility of medications and different materials used in various medical fields, (8) and also in endodontics. (9, 10) Various researches have estimated the cytotoxic effect of green tea and its polyphenol derivatives such as Catechin on different cell lineages including carcinogenic cells. (11) Studies have shown that intracanal medicaments such as calcium hydroxide and Ledermix® can have side effects on vital tissues and since these substances are in direct contact with periapical tissues, it is important to consider the biocompatibility of the intracanal medicaments upon selection. (3, 12)

Currently, Ledermix® paste is a combination of 9% demeclocycline hydrochloride (antibiotic), and 1% and 2% triamcinolone acetonide (corticosteroid) in a polyethylene glycol base. The corticosteroid ingredient is used for mediating the pain and inflammation and suppressing the local immune responses, while the antibiotic part is used for preventing the bacterial growth. The therapeutic ingredients of Ledermix® have the ability to permeate through the dentinal tubules and cementum to reach periodontal and periradicular tissues. (13)

Catechins (flavan-3-ols) are the main polyphenols of green tea. The four main catechins are: epigallocatechin-3-gallate (EGCG) (59% of total catechins), epigallocatechin (EGC) (19%), epicatechin-3-gallate (ECG) (13.6%) and epicatechin (EC) (6.4%). (14) Beside their direct role as antioxidants, the polyphenols have supplemen-

tary mechanisms by which they reduce oxidation level: 1- Binding of metal ions such as iron and copper and preventing their participation in the oxidation reactions (leading to the formation of hydroxyl radical). 2- Preventing the activation of redox-sensitive transcription factors that serve as mediators of inflammatory reactions. 3- Suppressing the oxidation stimulants such as induced nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2), lipoxygenase 2 (LOX-2) and xanthine oxidase. 4- Inducing the antioxidant enzymes such as glutathione S-transferase and superoxide dismutase. ⁽¹⁵⁾ In the first phase of this study, MTT assay was performed to determine the cytotoxicity of Ledermix® medicament and green tea extract on U937 monocyte cell line. 100µg/ml green tea extract and 0.5 µg/ml Ledermix® have the ability to destroy half the cells in the culture medium, and indicate the cytotoxic concentration (Inhibitory Concentration₅₀ or IC₅₀) of these medicaments. Considering the obtained results, higher concentrations of green tea extract in comparison with Ledermix® are needed to destroy half the cells in the culture medium. In other words, green tea extract is less cytotoxic and more biocompatible than Ledermix®. 0.25 µg/ml of Ledermix® and 12 µg/ml of green tea extract are the noncytotoxic concentrations which had an OD similar to that of the control group, and were used in the subsequent phase of the experiment. In 1989, Taylor and colleagues evaluated the effect of Ledermix® paste on mouse fibroblasts *in vitro*. Ledermix® at the concentration of 0.001 µg/ml terminated cell mitosis, while 1 µg/ml concentration caused cell death.⁽⁵⁾ In the present study, Ledermix® paste at the concentration of 1 µg/ml caused 50% cell death, while based on the study by Taylor et al, 10-4 mg/ml concentration caused more than 80% of the cells to fail mitosis.⁽⁵⁾ These differences can be attributed to the use of different cell lines and different methods for the assessment of medication's impact. In the study by Taylor et al, mouse fibroblasts were observed while in the present study, monocyte cell lineage was assessed.

Han et al in 2009 assessed the cytotoxic effect of green tea polyphenols (GTPs) by MTT Assay. IC₅₀ of GTPs extract in hamster pulmonary cells equaled 50 µg/ml, while on human leukemic cells, this value was over 100 µg/ml, which indi-

cates low cytotoxicity.⁽¹¹⁾ Overall, these findings concur with the results of the present study.

Lee et al in 2004 assessed the impact of Epigallocatechin-3-Gallate, a type of catechin derived from green tea, on human gastric epithelial cell line by the aid of MTT Assay. The results of this research showed that Epigallocatechin-3-Gallate has low cytotoxicity and it even has cytoprotective influence on the *Helicobacter pylori*-induced cells.⁽¹⁶⁾

In the second phase of the present study, the anti-inflammatory properties of Ledermix® and green tea extract were estimated. The concentrations of IL-1β, IL-6, and TNF-α inflammatory mediators of the control group and four study groups were measured by ELISA kit at the time intervals of 2, 24, and 48 hours and after 7 days. Overall, based on the obtained results, time interval has no significant influence on the level of the mediators released from the monocytes that have been induced by different medicaments. Generally, both substances significantly decreased the concentration of IL-1β, IL-6, and TNF-α inflammatory mediators at the mentioned time intervals in comparison with the control group. Nevertheless, the efficacy of these substances decreased gradually over time, and showed no significant difference from the control group after one week. The anti-inflammatory effect of these two substances has been assessed in previous studies, especially in the field of endodontics. The findings are generally in line with our results. The impact of Ledermix® has not been directly evaluated on Interleukins but Chen et al in 2008 showed that immediate application of Ledermix® intracanal medicament prevents root resorption of replanted dog teeth.⁽¹⁷⁾ Considering that IL-1β, IL-6 and TNF-α have a major role in hard tissue resorption,⁽¹⁸⁻²⁰⁾ it can be presumed that Ledermix® may affect the released concentration of these interleukins.

A research by Thong et al in 2001 revealed that Ledermix® prevented inflammatory root resorption of replanted monkey teeth, and that its ability to induce healing in periodontal tissues is slightly higher than that of calcium hydroxide.⁽²¹⁾ In an article published by Nakanishi and colleagues in 2010, the anti-inflammatory effect of catechin on human dental pulp cells was studied.

They exposed the cells to bacteria-derived factors such as LPS, and afterwards the effect of EGCG and ECG (which are the main ingredients of green tea catechins) on the expression of pre-inflammatory cytokines was evaluated. The expression of IL-6 and IL-8 in the dental pulp cells exposed to LPS or PG was significantly decreased in the presence of EGCG and ECG. ⁽⁷⁾

In 2009, Lee and colleagues stated that a green tea extract, epigallocatechin-3-gallate, reduces the size of periapical lesions by inhibiting the expression of cysteine-rich61 in osteoblasts, and decreases the infiltration of macrophages into the lesions and prevents the development of apical periodontitis. ⁽²²⁾ Since the studied Interleukins can induce bone resorption, green tea extract may be useful in healing the periapical lesions according to the study by lee et al. ⁽¹⁶⁾

A research by Hirao et al in 2010 showed that tea catechins reduce inflammatory reactions in stimulated dental pulp cells. ⁽²³⁾ They also showed that the expression of IL-8 and PGE2 in the fibroblasts of stimulated human dental pulp cells is blocked by catechins. ⁽²³⁾

In the present study, both Ledermix® and green tea extract showed distinctive anti-inflammatory effects compared with the control group. However, no significant differences were identified between these two substances.

Ledermix® paste inhibits phospholipase and PGE2 pathway and consequently impedes the production of other cytokines, probably due to its corticosteroid ingredient (triamcinolone). ⁽²⁴⁾ Ledermix® reduces the activity of clast cells and ends root resorption process. ⁽³⁾ Likewise, green tea contains active ingredients such as variety of catechin polyphenols which can probably block Cyclooxygenase-2 pathway and discontinue the production of inflammatory mediators especially TNF- α . It can also reduce the activity of osteoclasts. ⁽²⁵⁾ Accordingly, it seems that in addition to controlling the secretion of IL-1 β , IL-6, and TNF- α mediators and hard tissue resorption, green tea can also mediate pain by blocking the byproducts of Cyclooxygenase-2.

Contemporary studies have proven that Ledermix® can induce tooth discoloration due to its demeclocycline ingredient, but no tooth discoloration induced by the regular concentrations of green tea extract has been reported. Perhaps,

green tea lacks some of the disadvantages of Ledermix® paste such as higher cytotoxicity and probable tooth discoloration. ^(26, 27) Therefore, green tea extract can be proposed as a suitable and highly biocompatible intracanal medicament to reduce inflammation. Nevertheless, more comprehensive studies of the other important characteristics of intracanal medicaments should be performed on green tea extract.

Conclusions:

The results of the present research indicate that green tea extract is less cytotoxic and more biocompatible than Ledermix® paste. Moreover, both Ledermix® paste and green tea extract show anti-inflammatory effects. It is noteworthy that in particular cases, green tea extract shows higher anti-inflammatory effects in comparison with Ledermix®.

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