Evaluation of The Efficacy of Diode Laser, Chlorhexidine Digluconate Gel and Calcium Hydroxide Paste In The Disinfection Of Candida Albicans Infected Root Canals, In Vitro Study

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ABSTRACT

the study aims to evaluate the efficacy of CHX-gel, $Ca(OH)_2$ and diode laser in eliminating C.albicans from the root canal system and hence to eliminate the chances of reinfection and minimize treatment time.

Materials and methods: 180 single rooted anterior teeth were prepared and divided into 4 equal groups, the first was prepared to ISO 60 while the 2nd-4th were prepared only to ISO40, all the teeth were then sterilized and inoculated with C.albicans suspensions for 14 days, followed by evaluation of the starting bacterial counts in each tooth by means of colony forming units (CFU/ml), after this the groups 2-4 were prepared to ISO 40 MAF, using NaCl 9% in the 2nd group, NaOCl 1% in the 3rd and CHX 0.2% in the 4th. Finally each of the 4 groups was divided into three subgroups (n=20), the first irradiated with diode laser, the second with injected with $Ca(OH)_2$ paste and the third injected with CHX-gel for 7 days, followed by estimation of the end bacterial counts in CFU/ml in canal lumen and in dentin.

Results : the reduction of C.albicans was higher in all the groups after $Ca(OH)_2$ and CHX gel injection than with diode laser irradiation ($p<0.001$, Chi-square test). After chemomechanical preparation a higher effectivity of the laser disinfection was shown and more specimen could reach the lower limit values, the effectiveness was also increased by the use of NaOCl or CHX as irrigants ($p<0.001$).

INTRODUCTION:

A preliminary condition for successful root canal therapy is the reduction of microorganisms before filling of the root canals (Sjogren et al. 1997, Sundqvist et al. 1998), in spite of the reduction achieved by the chemomechanical preparation and the utilization of intracanal medicaments, some microorganisms stay in the irregularities of the canals and the dentinal tubules (Peters et al. 2000) which can be a reason for minimizing success rates and reinfection (Sjogren et al. 1997) this is not only caused by individual failures but by existence of resistant bacterial species (Sundqvist 1994), usually the primary endodontic infections are associated with a mixed anaerobic population, while secondary infections are mostly caused by enterococci species or candida albicans (Waltimo et al.1997, Sundqvist et al. 1998, Pinheiro et al. 2003), the effectivity of the classic calcium hydroxide against these species has been found limited (Waltimo *et al.* 1999, Siqueira *et al.* 2004, Siren *et al.* 2004), thus many alternatives have been tried to optimize the disinfection of the root canal system and to enable a one session therapy. Laser light have antimicrobial properties and can achieve an effective microbial reduction in vivo (Moritz *et al.* 1997), till now little evidence based studies have focused on their effect in achieving effective one session therapy.

The aim of our study is to find the effectivity of laser application in comparison with the use of $Ca(OH)_2$ and CHX- gel in the reduction of C. albicans from the root canal system.

MATERIALS AND METHODS

Study design

As a part of our study three types of treatment were carried on, which required classifying the teeth into three subgroups, in the first subgroup diode laser application was done (GENTLEray 980, Kavo, Germany), in the second a 7 day intracanal medication of Calcium hydroxide paste (UltraCal XS, Ultradent, USA) was injected and in the third subgroup a 7 day intracanal medication of CHX (Chlorhexamed, 1% gel, Galaxosmithcline) was injected.

Every one of these treatments was examined alone and in combination with the other therapy methods which included mechanical preparation/ no preparation conditions and NaCl or NaOCl or CHX irrigation conditions, a total of 12 study groups were built , each study group had a total probe count of 15 specimen, so a total of 180 probes was examined.

Table.1. description of the study groups:

Subgroup (n=15)	*Main group (n=60)*			
	1	*2*	*3*	*4*
	Without preparation	*Preparation with NaCl irrigation*	*Preparation with NaOCl irrigation*	*Preparation with CHX irrigation*
Laser	1.1	2.1	3.1	4.1
$Ca(OH)_2$	1.2	2.2	3.2	4.2
CHX-Gel	1.3	2.3	3.3	4.3

n: number of teeth (probes).

Before beginning of the study sequence a complete preparation of the specimens of main group 1 was done to ISO 60(MAF) while in the groups 2-4 initial preparation to ISO 40 was done (MAF), the complete preparation of the canals in these groups was done during the study, in the main group 2 with physiologic normal saline (NaCl 1%), in the main group3 with sodium hypochlorite (NaOCl 1%) and with CHX-gel 0.2% in the 4th main group. The subgroups 3 and 4 served as positive controls, the negative controls were the bacterial counts of 1st subgroup, the following table describes in details the study procedures:

Table.2; description of the study procedure.

Main group1	*Main group 2*	*Main group 3*	*Main group 4*
Irrigation and dryness Sterilization Placement in tubes			
Preparation to ISO 60 NaCl irrigation	Preparation to ISO 40 NaCl irrigation		
Sterilisation Implantation of C.albicans Incubation			
Starting bacterial count (CFU/ml)			
No further preparation	Further preparation to ISO 60		
	NaCl irrigation	NaOCl irrigation	CHX irrigation
1.1	2.1	3.1	4.1
1.2	2.2	3.2	4.2
1.3	2.3	3.3	4.3
End bacterial count 1 (CFU/ml) End bacterial count 2 (CFU/mg Dentin)			

Preparation of the specimens:

A total of 180 single rooted extracted teeth were collected, after full debridement with ultrasound endo tips (SONICFlex, Kavo),they were sterilized with ethylene oxide and the crowns cut with diamond burs and all the specimen were brought to a length of a 19mm, Fig.1,

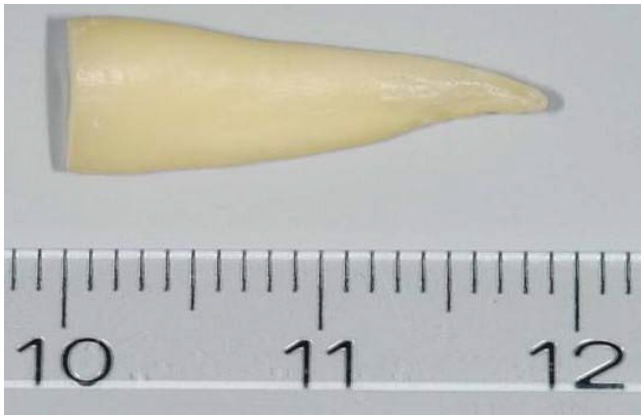


Fig.1; root length determination.

During the period of preparation the samples were kept in physiological normal saline and the preparation of all teeth was unified to a length of 18 mm. Specimen of the first main group were prepared to ISO 60 and of the other groups to ISO 40 as listed above (Flexmaster System VDW), during the preparation debris were removed using normal saline (NaCl 0.9%) and EDTA (FileEze, Ultradent) was used to wash away the smear layer. To ensure a unified preparation depth, composite stoppers were designed on the files, and the orifices of the canals were assured to be cone shaped using 2.9mm Ø, orifice former diamond (Riitano access bur kit, Ultradent, USA). To ensure ease of access. After preparation each of the specimen were kept in sterile normal saline containers for 14 days and the solution was changed everyday.

After preparation the teeth were painted with nail polish followed by embedding in fast setting resin, Fig.2,

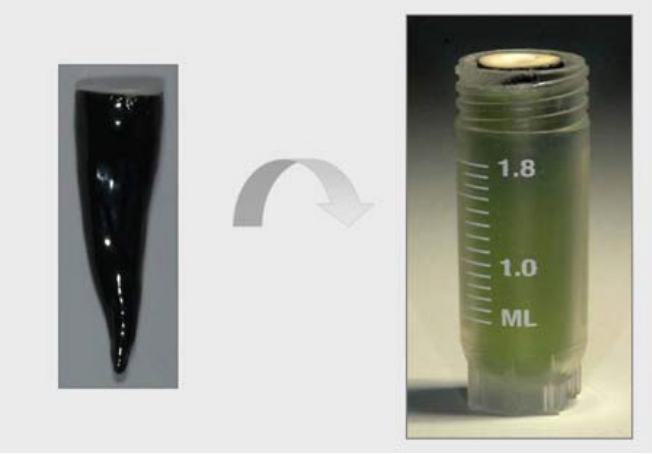


Fig.2; nail polish painting and embedding of specimen.

To ensure sterilization ten specimen were selected randomly and injected with universal nutrition solution (Tryptone Soya broth) and incubated for 14 days at 37°C the resulting solution was then implanted over columbia agar with sheep blood and incubated for 24 hrs at 37°C. after proving the sterility a 30µl suspension of C.albicans in Sabouraud Dextrose solution injected in the canals of all the teeth in the specimen, the starting concentration was 10^5 CFU/ml, the specimen was incubated for 14 days at 37°C and a sterile nutritive medium was daily given (Sabouraud liquid medium, Oxoid), the cover of the embedding tubes was kept loose. Before beginning of the main experiment To obtain the intracanal bacterial count , 5µl of the solution was drawn with paper points and with a dilution of 1:4000000 implanted, after an incubation for 24hrs at 37˚C, the resulting colonies were counted and the count of microorganisms in the canal per ml of liquid (CFU/ml) against the dilution factor was calculated.

To be able to calculate the exact bacterial count pro milliliter of solution in the canal we need to know the exact volume of fluid taken by the paper point and hence to evaluate this, paper points (ISO 40 VWD) were immersed in previously pipetted volumes of 5, 10 and 15 µl of liquid to the depth of 18 mm. we found that an average paper point can fully absorb up to 5µl of the solution. To reach the dilution value , paper points were immersed in sealable tubes with 1,995ml normal saline, the tubes were placed in vortex mixer for 30 seconds, from this solution 10µl were pipetted and placed in 0.99ml sterile normal saline tubes then vortexed for 30s, 10µl were pipetted from this solution and implanted in (Sabouraud-Glucose-Hefeextract selective media, Oxoid) and so a dilution of 1:4000000 was reached which enables us to find the exact bacterial count pro ml.

Pipetted volume	Dilution factor
5µl paperpoint in	X 400
10µl solution in 990µl NaCl	X 100
10µl solution	X 100
	= 4.000.000

Calculation of bacterial count was done on a selective media for fungi (Sabouraud- Glucose-Hefeextract selective media, oxoid), in addition three specimen from each subgroup were examined for contamination on universal nutritive media (Columbia agar with sheep blood, oxoid) during the start and the end bacterial count procedures.

Procedure:

Immediately after calculation of the starting bacterial count, the experimental procedure started with the preparation of the specimen of groups 2-4 to ISO 60 with 9% NaCl in group 2 and with 1% NaOCl in group3 and with 0.2% CHX in the 4th group. Candida solution was washed with 2ml 9%NaCl and each canal was dried with 3 paper points ISO 40, corresponding to their classification in subgroups, the specimen were treated after that as follows:

The first subgroup was irradiated with diode laser (Epic, Biolase, USA) with maximum output power of 7W,200µm fiber, inserted into 17mm length, the fiber was inserted in spiral movement along the canal wall in 3mm/s for 10s from apical to coronal, Fig.3;

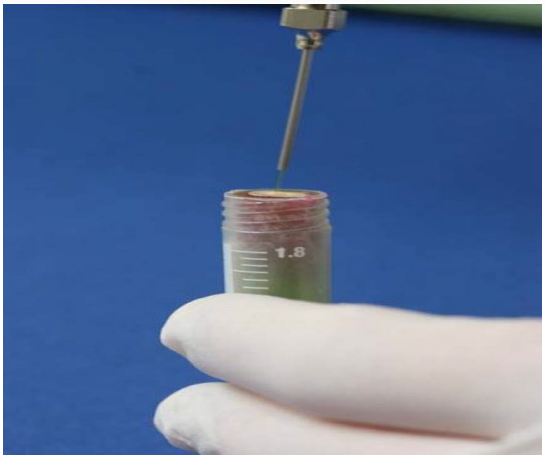


Fig.3; laser application procedure.

In each canal the procedure was repeated 4 times for overall 40s.

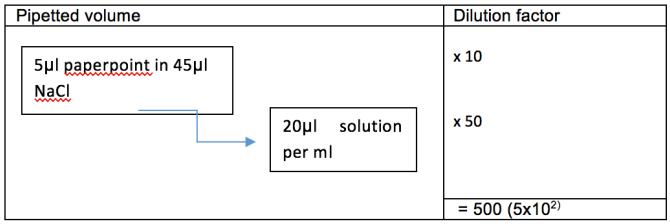
In the 2nd subgroup, chlorhexidine digluconate gel (1%) was injected in the canals and they were incubated for 7 days, a disposable endo injection tip was used (NaviTip, Ultradent, USA) to inject the material slowly from apical to coronal to avoid airbubble formation and adequately fill the canal then canals were covered and incubated for 7 days at 37°C. Similarly in the 3rd subgroup the canals were injected with calcium hydroxide paste (UltraCal XS(Ph 12.5), Ultradent, USA) with endo tips (NaviTip, Ultradent, USA) from apical to coronal in a slow movement then covered and incubated for 7 days at 37°C.

To obtain the end bacterial count, all canals were washed with 2 ml NaCl (0.9%) solution. In the first subgroup this was done after laser irradiation while in the 2nd and 3rd subgroups after the incubation, then paper points (ISO 40) were used to take the bacterial specimen (CFU/ml) and Hedstrom files (ISO 40) were used to obtain dentinal specimen from the root canal wall (CFU/mg).

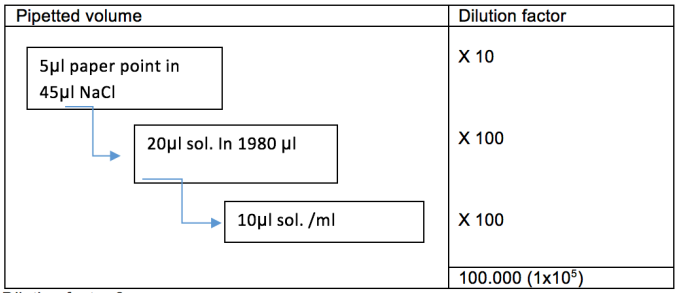
Evaluation of the end bacterial count:

a.End bacterial count of the canal :

Because of the previously investigated C.albicans growth sequence in literature , the dilution factor and the starting average bacterial count could be evaluated so that an average bacterial count of 10^8 CFU/ml could be accepted and the dilution factor of 1:4000000 was considered (Sen et al. 1999-2000, Valera et al. 2001, Ferguson et al. 2002, Siqueira et al. 2003, Menezes et al. 2004, Brandle et al. 2008) . For the end count this was not possible since we have no expected value for the CFU/ml. In case we consider a smaller dilution factor so the result is higher number of colonies >1000 which are difficult to count and their growth behavior is limited on the media, a smaller dilution however gives the possibility to lower the lowest confirmed bacterial count to $5x10^2$ CFU/ml, so to keep the lowest limit as small as $5x10^2$ and to have the possibility to quantify specimen with higher bacterial concentrations, 2 dilution factors were produced and implanted so that any concentration from $5x10^2$ to 10^8 CFU/ml could be quantified.



Dilution factor 1



Dilution factor 2

b.Evaluation of the end bacterial count of the canal wall dentin:

ISO 40 Hedstrom file was inserted 3 times along the canal wall to obtain the dentinal specimen, files were then placed in sealable tubes (Safeseal Microtube 2ml, Germany) with 50 µl sterile normal saline and vortexed for 30s then 20µl were pipetted and implanted on (Sabouraud-Glucose-Hefeextract, Oxoid) selective media. After an incubation for 24hrs at 37°C colonies were counted. First 10 files with and without dentin specimen were weighted and an average 0.0003g specimen weight was obtained. Since 20µl were implanted so multiplying the counted colonies by 2.5 gives the bacterial count in 50µl, this corresponds the count in a 0.0003 g specimen and the division on 0.3 gives the count per milligram (CFU/mg).

Statistical analysis:

For the quantitative evaluation, three variables were considered:

a.End bacterial count 1 (end bacterial count in canal) CFU/ml

b.End bacterial count 2 (end bacterial count in dentin) CFU/mg

Then all the values of the starting bacterial count were considered as baseline and tested with Kruskal-

Wallis test for difference (p <= 0.05). since the end bacterial count inside the same subgroup varied either below or above the limits, we built three classes for the end count values , the classes were (under the lower limit, middle value , high value), presence of these classes for all the 12 subgroups was summarized in cross tables and graphs were produced for each main group. The study groups were compared concerning the end bacterial count in canal and in dentin, each single treatment group against different preparations and different preparation against treatments were compared with Chi-square test, all the previous tests were carried out using SPSS software 16.0 (SPSS, Germany). 2 factor ordinal logistic regression analysis were carried out to evaluate the three levels of end bacterial count and the relative bacterial count reduction using the software SAS 9.1 (SAS Institute, USA). The start and end bacterial count in dentin was compared (Chi-Quadrat-test) and the presence of a relation between the end bacterial count in canal and in dentin was examined.

RESULTS

Quantitative evaluation:

Before starting the experimental work, a random specimen of the experimental teeth starting from 10^5 CFU/ml, could reach a concentration of 10^8 CFU/ml after 14 days, in this power the concentration remained until 21 days, so the incubation period of C.albicans suspension was fixed on 14 days.

Starting bacterial count:

At the beginning of the procedure the starting count was estimated in all the canals, the median value was $3.66x10^8$ CFU/ml, where the lowest value was $8x10^6$ and the highest $1.28x10^9$ CFU/ml. the starting count in dentin was estimated by selecting 4 more canals , implanting them with C.albicans and incubation for 14 days, the starting counts in the canals and in the dentinal wall were:

Table 4; starting bacterial counts in the canal and in the dentin of the selected specimen.

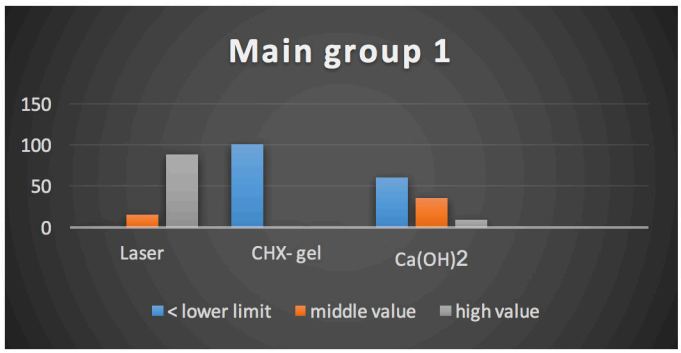
Specimen	*Starting count in canal*	*Starting count in dentin*
1	1 3,84 × 10^8 CFU/ml	7,10 × 10^3 CFU/mg
2	2 2,04 × 10^8 CFU/ml	1,02 × 10^5 CFU/mg
3	3 9,60 × 10^8 CFU/ml	9,83 × 10^4 CFU/mg
4	4 2,56 × 10^8 CFU/ml	6,80 × 10^4 CFU/mg
Average	4,51 × 10^8 CFU/ml	8,48 × 10^4 CFU/mg

End bacterial count 1:

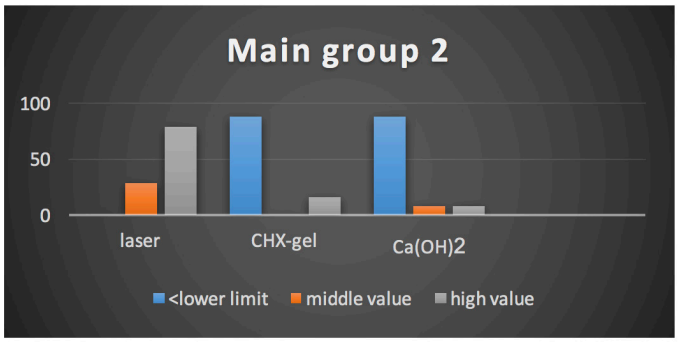
The end bacterial count was estimated in all the specimen and since the values varied but either were lower than a lower limit or higher than an upper limit, 3 classes of values were considered for statistical analysis.

Lower than the lower limit	< 5 × 102 CFU/ml
Middle value	5 × 102 - 4 × 103 CFU/ml
High value	> 4 × 103 CFU/ml

Classification of the end bacterial counts in the value fields was done for all the specimen and the results were tested against one another (Chi square test, α= 0.05), to simplify the results, percentages (of 100%) of each value were clarified using bar charts for each main group:



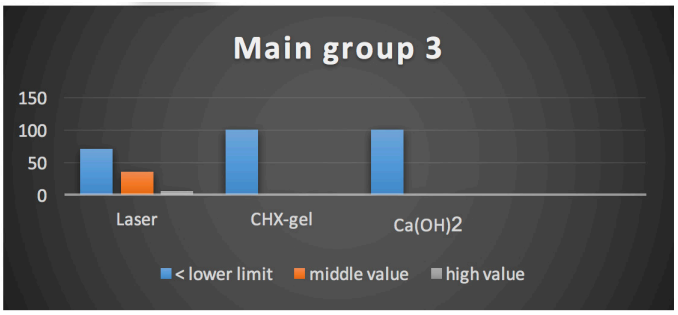
By the use of $Ca(OH)_2$, 60% of the cases were in the lower limit field and 33,3% were in the middle field, using diode laser no values were in the lower value field and 86,7% were in the high value field, the utilization of Ca $(OH)_2$ had significant effect from the laser treatment (p<0.001) and the use of CHX-gel caused significant decrease more than $Ca(OH)_2$, (p=0.017) were all the specimen were in the lower value field. .



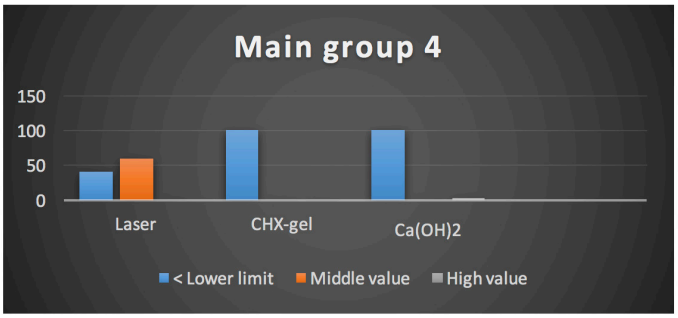
through the additional preparation and use of NaCl the effect was clear on having specimen with lower limit values for the laser and $Ca(OH)_2$ groups, utilization of $Ca(OH)_2$ and CHX-gel did not differ significantly , both reached 86,7% bacterial count under the lower limit, while laser treatment did not have any counts below the lower limit.

Utilization of NaOCl as irrigant in group 3 , caused an increase in the number of teeth without C.albicans after laser and $Ca(OH)_2$, only the laser

group showed values higher than the high value. The combination of chemomechanical preparation and NaOCl irrigation together with CHX-gel or $Ca(OH)_2$ resulted in bacterial count reduction below the lower limit in all the treated teeth, however there was a significant difference between CHX-gel and $Ca(OH)_2$ from the laser therapy (p=0.042).



In group 4 the chemomechanical preparation with CHX-irrigant resulted in more specimen in the subgroups of laser and $Ca(OH)_2$ below the lower limit, this was clear for about 70% of the laser and 100% of $Ca(OH)_2$ specimen.



The results show that the lower limit values of < $5x10^2$ CFU/ml could be 100% achieved through application of CHX gel without further preparation using CHX irrigant, NaCl or NaOCl.

End bacterial count 2:

Since the values varied they were classified in three value fields according to the following tables:

Table.6. classification of the end bacterial count 2 values in main group 1;

No preparation (CFU/mg)	*preparation*			
	Laser	*CHX-gel*	*$Ca(OH)_2$*	*Total*
< lower limit	0	15	11	26
8,33-4,999	0	0	4	4
> 5000	15	0	0	15
Total	15	15	15	45

Table.7. end bacterial count 2 values in main group 2;

NaCl (CFU/mg)	*preparation*			
	Laser	*CHX-gel*	*$Ca(OH)_2$*	*Total*
< lower limit	0	15	13	28
8,33-4,999	1	0	2	3
> 5000	14	0	0	14
Total	15	15	15	45

Table.8. end bacterial count 2 values in main group 3;

NaOCl (CFU/mg)	*preparation*			
	Laser	*CHX-gel*	*$Ca(OH)_2$*	*Total*
< lower limit	11	15	15	41
8,33-4,999	4	0	0	4
Total	15	15	15	

Table.9. end bacterial count 2 values in main group 4;

CHX (CFU/mg)	*preparation*			
	Laser	*CHX-gel*	*$Ca(OH)_2$*	*Total*
< lower limit	11	15	15	41
8,33-4,999	3	0	0	3
> 5000	1	0	0	1
Total	15	15	15	45

DISCUSSION

We examined C.albicans in our research as one of the most causative factors of endodontic revisions (Siqueira et al.2004). After a standatdised procedure the starting situation could be unified in all the probes, the standard deviation ($2,728x10^8$) was due to difference in dentin morphology between the teeth and was considered biologically unnoticeable since the average value of all the probes had an exponent of 10^8. The starting bacterial count in our specimen as average of $4,51 \times 10^8$ CFU/ml is near to that found as an average count of $4x10^5$ in canals by Bystrom (Bystrom et al. 1981) and that in dentin of $8,48 \times 10^4$ CFU/mg is somewhat lower than that found in infected canals by Peters $5x10^6$(Peters et al. 2001). The comparision of the end bacterial count1 to the starting counts showed an indication for the treatment, in main group 1 the treatment with CHX-gel was not significantly different from that of $Ca(OH)_2$,(α<= ٠,٠٠٤٢, Chi square test) they both were significantly different from the laser group. More frequently there was a significant difference between the CHX-group

and the laser group ($p<0.001$), the results were similar for $Ca(OH)_2$ as the bacterial reduction was frequently higher than that of the laser group ($p<0.001$). considering the bacterial count reduction in dentin the results were similar to the previous and the reduction was more by CHX-gel then $Ca(OH)_2$ then laser. The end bacterial counts of the free (canal) and adherent bacteria are highly related to this sequence as laser irradiation has not the same strong effect on biofilm bacteria as intracanal medicaments and in order to eliminate the chances of recurrent infection bacterial elimination is inevitable, however Peters reported that remaining microorganisms below the concentration of 10^2/ml at the time of canal filling has no negative effect on the treatment outcome (Peters et al. 2002). According to our study this concentration could be 100% reached by the application of CHX-gel alone or in combination with preparation and irrigation with NaOCl or CHX solution, placing in mind the aim of manual or mechanical root canal preparation besides eliminating the necrotic and dentinal debris is to remove the bacterial attachments to the canal wall and provide a wider lumen for the action of intracanal medicaments but never to fully eliminate bacterial colonization's since many anatomical irregularities, ramifications and accessory canals cannot be reached (Orstavik et al. 1991), the concentration also could be reached after $Ca(OH)_2$ application combined with chemomechanical preparation with NaOCl or CHX solution. The most effective treatment recommended according to our study is by the suggested intracanal medicaments with chemomechanical preparation with NaOCl or CHX solution. However we cannot give a clear recommendation to use the above treatments since our in vitro study is excluded from contamination factors and through the preparation and application of irrigants contamination may occur through oral microorganism or through the instruments themselves and this increases with the number of treatment sessions (Weiger et al. 2000), however our results are similar to those obtained by Ercan et al. whom investigated the effects of 2% CHX-gel on C.albicans and E.faecalis in comparision to $Ca(OH)_2$. Some studies state minimal effectiveness of $Ca(OH)_2$ against C.albicans (Waltimo et al. 1999, Ferguson et al. 2002), however these methods varied greatly in their examination procedure and probe preparation and also in the method of medicament application. In our study we did not place the medicament solution in direct contact with the medication outside the canal but followed the usual in vivo situation in which resistance occurs through adhesion and colonization of the dentinal surfaces and accessory canals, on the contrary to the clinical situation a higher amount on intracanal medicament is applied through the larger root canal lumen, however through sufficient amount of root filling this can be simulated. Still few studies are present about the canal disinfection with diode lasers. Gutknecht et al. 2000 and Moritz et al. 1997, could reach through the intracanal application of diode laser a reduction of streptococcus and staphylococcus to 10^3-10^4CFU/ml reached, in our study the unique laser utilization resulted in reduction to 10^6 CFU/ml which corresponds when converted to percentage to 99%. In vitro studies show many attempts to eliminate intracanal microorganisms especially E.faecalis which can reach up to 100% (Moritz et al. 1997, Gutknecht et al. 2000/ 2004, Schoop et al. 2006, de Souza et al. 2008) however the results obtained without previous preparation and medicaments are subjectively high. Sennhenn-Kirchner et al. investigated the effectivity of Er:YAG and 810 diode lasers against C.albicans biofilms attached to glass and titanium plates with 4x20s irradiations and 30s pause after each irradiation. The differences in irradiation periods, microbiological procedure and wavelengths used (810-980nm) make the direct comparison to our study invaluable. Chan et al. stated that different diode wavelengths can affgect their bactericidal activity, however this or presence of another bacterial species in the canal has a little scientific value since the bactericidal effect depends on the excessive heating and rupture of the bacterial cells and not on specific cellular elements. Even against heat resistant Bacillus stearothermophilus Nd:YAG laser irriadiation can be effective to achieve below 10^2 CFU/ml reduction (Hardee et al. 1994). However pigmentation affects bacterial sensitivity to great extent due to increasing the absorption coefficient , even for resistant bacteria a methylene blue pigmentation will increase their sensitiy to diode laser irradiation (Chan et al. 2003).

CONCLUSION:

CHX –gel and $Ca(OH)_2$ had perfect effectiveness againt C.albicans in root canals when combined with chemomechanical preparation and NaOCl and CHX irrigation , however laser disinfection showed less effectiveness and should be further investigated.

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