RESEARCH ARTICLE

EFFECT OF CPP- ACP, POLARIZED ENAMEL USING HYDROGEN PEROXIDE ON REMINERALIZING POTENTIAL OF BOVINE - AN *EX-VIVO* STUDY

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ABSTRACT

Demineralization of enamel surrounding orthodontic brackets is a serious clinical problem and these conditions are treated by the use of remineralization creme. The effectiveness of the regimens can be determined by measuring the Bone Mineral Density (BMD) and Bone Mineral Content (BMC). In general these parameters are studied using polarized light, desktop micro CT. In the present *ex vivo* study, effectiveness of remineralization creme namely Casein Phosphopeptide Amorphous Calcium Phosphate (CPP-ACP), and enamel surface of bovine anterior teeth treated with hydrogen peroxide was analyzed using dual X-ray absorbtiometry (DXA). 52 samples of bovine anterior teeth were collected from slaughter house and cleaned as per the standard procedures. The collected samples were subjected to demineralization using a demineralizing buffer solution. Subsequently, the teeth samples were remineralized with CPP-ACP or hydrogen peroxide treatment and curing with light cure unit, stored in artificial saliva. The BMD and BMC in control and experimental group samples were measured using non-invasive DXA at regular intervals. The BMC of the sample treated with hydrogen peroxide was more when compared to the sample treated with CPP-ACP. However, no significant changes in BMD contents were observed among the experimental groups. No significant changes were seen in terms of their lean body mass and total fat content among the teeth of control and experimental groups. The remineralization potential is more when teeth were treated with hydrogen peroxide and light cure when compared to CPP-ACP.

INTRODUCTION

Enamel demineralization is a common sequela to orthodontic treatment with fixed appliances. The increased number of plaque retention sites created by orthodontic appliances makes optimal oral hygiene a challenge, resulting in elevated *Streptococcus* mutant levels and a lower resting pH of dental plaque. White spot lesions are early smooth surface carious lesions, which occur when the acidic environment results in subsurface demineralization of enamel (Benjamin *et al*., 2012). A comparative study between non-orthodontic patients and orthodontic patients revealed that orthodontic patients are much more vulnerable to the demineralization of enamel with a rate of 4.9% to 84% (Guotao *et al.,* 2010). Therefore, it is a crucial task for orthodontists to minimize the occurrence of enamel demineralization. Few studies have reported that, a significant increase in the prevalence and severity of demineralization after orthodontic therapy compared with the

controls, and the overall prevalence amongst orthodontic patients ranges from 2 to 96% (Chang *et al*., 1997; Artun and Brobakken, 1986). The most commonly affected teeth are molars, maxillary lateral incisors, mandibular canines and premolars (Maki *et al*., 2007). The problem of enamel demineralization is well addressed by the usage of remineralizing regimens like Recaldent, Fluoride regimens, Bio glass etc. The extent to which the surface has remineralized is estimated by measuring the BMD and BMC using micro computed tomography, Transmission electron microscopy, Quantitative laser or light induced fluorosence (Benjamin *et al*., 2012). Compounds like Casein Phosphopeptide Amorphous Calcium Phosphate (CPP) as a remineralizing agent and ACP technology started in the early nineties (Chang *et al*., 1997). The anticariogenic potential of CPP-ACP nanocomplexes in laboratory, animal and human *in-situ* models has been well documented. Further, CPP-ACP has also been shown to remineralize enamel with a mineral that is more resistant to acid challenge than normal enamel mineral. Hydrogen peroxide (HP) and carbamide peroxide (CP) treatments on the enamel and dentine were used primarily for

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their bleaching effect or for their etching properties. The HP and CP containing products even if used in highest concentrations have no significant deleterious effects on enamel and dentine, surface morphology (Gorelick *et al.*, 1982; Joiner, 2007). The idea of using HP for remineralizing on the extracted premolars is well described by Reina Tanaka (Tanaka *et al.*, 2009). The non-invasive DXA instrument has been widely used to determine the BMD and BMC of the skeletal structures like jaw and other bones of the human body (Yucang Li *et al.*, 2012). For the first time we have used DXA to measure the BMD and BMC of the anterior dentition of bovine. At the same time we have carried out an *ex-vivo* study and compared the effect of remineralization potential of CPP-ACP and HP using the method described by Reina Tanaka (Tanaka *et al.*, 2009).

MATERIAL AND METHODS

52 bovine anterior teeth were obtained from a slaughterhouse and stored in deionised water after extraction. They were examined for cracks and erosion. The crown sections of the teeth were separated using diamond disc bur, subsequently they were cleaned using ultrasonic scaling procedure. The crowns were arranged as per the groups and initial levels of BMD and BMC content of the teeth was measured (T1). The teeth were soaked in demineralization buffer for 2 weeks to produce an artificially demineralized area. Two weeks later, the anti-oil varnish was washed and measured (T2) BMD and BMC. These teeth were stored in artificial saliva and the composition of the artificial saliva and the dimenieralizing buffer used are given in the Tables 1 and 2.

Table 1. Composition of Demineralizing Buffer

S.No	Components	mMol (m mol/L)
1	$Ca(NO_3)_2$	2.2
2	KH_2PO_4	2.2
3	HAc	50
4	NaN_3	1.0
5	NaF	0.1
6	NaOH	---

$Ca(NO_3)_2$- Calcium nitrate; KH_2PO_4 –Potassium dihydrogen phosphate; HAc- Acetic acid; NaN_3 – Sodium azide; NaF- Sodium Fluoride; NaOH- Sodium hydroxide.

Table 2. Composition of Artificial Saliva (pH 6.8)

S.No	Components	Wt/vol% (g/L)
1	NaCl	0.4
2	KCl	0.4
3	$CaCl_2.2H_2O$	0.795
4	$NaHPO_4.2H_2O$	0.78
5	$Na_2S.2H_2O$	0.005
6	Urea	1.0
7	H_2O	1.0

NaCl – Sodium chloride; KCl-Potassium chloride; $CaCl_2$ – Calcium chloride; $NaHPO_4.2H_2O$ – Disodium phosphate; $Na_2S.2H_2O$ – Sodium sulphide; H_2O – Water.

Teeth were randomly divided into three groups Viz., A, B and C for enamel remineralization. The groups were as follows:

Group A consists of 9 teeth acting as control and were subdivided into CONTROL-1, CONTROL-2, with 6 and 3 teeth respectively. The teeth were stored in artificial saliva at 37° C. The artificial saliva was refreshed every 3 days.

Group B consists of 21 teeth and were sub divided in to EXPERIMENT-1A, EXPERIMENT-1B, EXPERIMENT-1C, EXPERIMENT-1D consisting of 6 in each group and 3 in the last group. The demineralized area was coated with a thin film of CPP-ACP tooth mousse every day for 5 minutes in artificial saliva. The remineralization treatment was carried out 5 hours every day and the CPP-ACP tooth mousse was washed off with distilled water. After the treatment, samples were soaked in artificial saliva at 37°C. The duration of remineralization was for 10 days.

Group C consists of 21 teeth and were subdivided into EXPERIMENT-2A, EXPERIMENT-2B, EXPERIMENT-2C, EXPERIMENT-2D consisting of 6 in each group and 3 in the last group. 35% Hydrogen peroxide with appropriate mixture of sodium, calcium salt of poly (MVE/MA) was applied on the teeth and activated using halogen lamp for 3min. The treatment was repeated twice before submerging in artificial saliva (Lynch, 2006) for 5 hours daily. The samples were stored in artificial saliva for 10 days. Group C BMD and BMC were measured at the end of 1 week (T3). The difference in the bone mineral composition of the samples were evaluated at the T1, T2 and T3 time intervals using DXA and compared between the groups.

Procedure

The freshly extracted bovine anterior teeth are examined under the polarized light for elimination of cracks. The teeth are cleared of any tissue remnants by using scalpel and not by hydrogen peroxide as it can change the mineral composition. The crowns of the tooth are separated using diamond disc bur and stored in deionised water. The chemical solutions, demineralization buffer and artificial saliva are prepared according to Artun and Brobakken (1986). The bone mineral composition is measured at time intervals of T1, T2 and T3 and evaluated using Hologic QDR series discovery model DXA equipment. DXA instrument calibration included an infant step bar phantom (Hologic P/N 010-0757, Rev.004) made up of Plexiglas and used for all scans. The platform was of the same height as the lowest step of the calibration bar, putting the teeth samples in the range of the calibration of the machine. The scanner was calibrated using control phantom, and its performance was monitored as per the quality assurance protocol. No sign of scanner drift was observed during the study period. The *ex-vivo* precision (coefficient of variation) was 1-2% for all the measurements. Bovine anterior teeth samples were placed on the platform and were scanned with DXA using Hologic ultra high resolution small animal software. The scans were then analyzed with ultra high resolution analysis software, and values were recorded.

Statistical Analysis

Statistical analysis was carried out using SPSS, version 15.0. One-way ANOVA and Durnett test of homogenecity was employed to determine significant difference between the mean values of BMC and BMD parameters in different groups. Further, paired 't' test and Wilcoxin ranked test resulted in a statistically significant difference between initial and final values in each of the groups.

Table 3. Bone mineral composition in control and experimental bovine teeth. (N=52)

Groups	BMC			BMD		
	Normal	Demineralization	Remineralization	Normal	Demineralization	Remineralization
Control	0.39± 0.07	0.29± 0.07	0.31± 0.07	0.28± 0.04	0.26± 0.04	0.28±0.04
Experiment-1	0.35± 0.05	0.32± 0.05	0.36± 0.05	0.31±0.03	0.29±0.03	0.31± 0.03
Experiment-2	0.86**± 0.11	0.36± 0.05	0.56± 0.05	0.82**±0.11	0.32± 0.04	0.48± 0.03

BMC – Bone mineral concentration; BMD – Bone mineral density.
Experiment – I – CPP-ACP tooth mousse treated.
Experiment – II – 35% Hydrogen peroxide treated.
** Values were significant at *P<0.01*.

Non-parametric Kruskal-Wallis and Man Whitney tests were used when the data for particular group was non-Gaussian in nature. All the values given were mean ± standard error of mean (SEM). A probability level of less than 5% (P<0.05) was considered statistically significant. The study was reviewed and approved by the Institutional Animal Ethical Committee and was conducted in accordance with the internationally accepted principles for laboratory animal use and care.

RESULTS

The bone mineral concentration and bone mineral density values analyzed by DXA of normal group and teeth treated with CPP-ACP and 35% hydrogen peroxide was given in Table 3. Overall there is an increase in the area and mass of the teeth with increase in the size, however no significant changes were seen among the parameters like lean body mass, total fat and fat% of teeth between the groups. The BMC and BMD levels were significantly increased in normal teeth of experiment-2 compared control and experiment-1(P<0.01). No significant differences were seen in the values of BMD of normal, demineralization and remineralization. However, there is an increased trend is in terms of BMD contents is seen in the teeth treated compared to control. This trend was more pronounced in teeth treated with hydrogen peroxide.

DISCUSSION

The present *ex-vivo* study mainly showed that the remineralization potential of CPP-ACP and polarized Hydrogen Peroxide are different for subsurface lesions. Furthermore, polarized Hydrogen Peroxide displayed more remineralizing effects on the surface lesion of the sample. Rationales for using bovine enamel and dentine specimens have been discussed previously, and this source represents an accepted substitute for human dental hard substances (Kielbassa *et al.*, 2006; Tschoppe *et al.*, 2009 and Iijima *et al.*, 2004). The opaque white appearance of the enamel lesion induced in the present study is due to subsurface demineralization with an increase in porosity and consequential changes in the optical properties of the enamel. The surface may appear chalky and there may also be direct erosion of the surface. The lesion itself occurs through a series of repeated episodes of mineral loss, with mineral from the surface being lost into the plaque fluid and saliva and mineral from the subsurface reconstituting the surface. This is interrupted as the dynamics of repair and destruction alter according to the oral environment (Chang *et al.*, 1997 and Silverstone, 1983). CPP-ACP is pH responsive and works effectively as a remineralizing agent at all pH range.

CPP-ACP provides a highly effective means for elevating calcium levels in dental plaque fluid, something which is desirable for enhancing remineralization, but is difficult to achieve by using calcium in other forms (Reynolds, 1997). The remineralizing capacity was greater for the solutions with the higher levels of CPP-stabilized free calcium and phosphate ions. Remineralization was not significantly correlated with either the CPP-bound ACP of the degrees of saturation for hydroxyapatite, octacalcium phosphate, or ACP. However, remineralization was significantly correlated with the degree of saturation for dicalcium phosphate dihydrate (CaHPO4.2H2O), but this was attributed to the significant correlation of remineralization with the activity gradients from the solution into the lesion of some calcium phosphate ions and ion pairs, in particular the neutral ion pair CaHPO4(0) (Maki Oshiro *et al.*, 2007). Energy absorption of the deeper enamel region would be allowed by activation of permeated hydrogen peroxide using an energizing source because this would increase the rate of decomposition of oxygen to form oxygen free radicals in the region. It is also observed that the remineralizing potential of the enamel increased by many folds with application of Hydrogen peroxide and curing it with light cure unit (Tanaka *et al.*, 2009). In the present study, there is a possibility that similar mechanism might have been involved and this needs a detailed study.

Conclusions

A *ex vivo* study was carried out to assess the effectiveness of CPP-ACP or hydrogen peroxide as a good remineralization agents. From the data it was found that bone mineral concentration was significantly increased in hydrogen peroxide treated teeth compared to CPP-ACP. Further study in larger teeth sample and longer duration is required to confirm the above obtained resulted.

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