Comparative evaluation of remineralising and adsorption properties of regular cow milk and A2 milk using human enamel discs and hydroxyapatite discs: An in-vitro SEM study

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Abstract---Depending on the frequency and type of intake, milk can affect caries formation by virtue of its demineralisation-remineralisation properties. The effect of A2 milk on human dentition is still unexplored. Hence,the aim of the present study was to evaluate and compare the remineralising and adsorption properties of regular

cow milk and A2 milk using Human Enamel discs and Hydroxyapatite(HA) discs in an in-vitro model. Materials and methods: To evaluate the remineralising properties of milk, 24 human enamel discs and 24 Hydroxyapatite(HA) discs were divided equally into two different milk groups. Subsurface carious lesions were created and they were subjected to remineralisation-demineralisation cycles using twenty-one-day pH-cycling model. Baseline, post-demineralisation and post-remineralisation differences between the groups were studied by microhardness test (Vicker's Indenter) and surface roughness test (profilometer). Ca:P ratio of the subtrates was analysed using Scanned Electron Microscopy Energy Dispersive X-Ray (SEM-DEX) analysis of five HA discs, out of which two were treated with the different milk types and the remaining three with different controls. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) based analysis, followed by densitometric evaluation was performed to examine the adsorption of caseins from the two milk types on HA discs. Results: The results showed a trend towards increased remineralisation of both substrates with A2 milk (p>0.05) in the pH-recycling model. Ca:P ratios by SEM-EDX analysis also yielded a highly statistical difference (p<0.001) between the 2 groups, in favour of A2 milk. Adsorption of the three major caseins on HA disks was found to be comparable but was characterized by higher relative binding of kappa casein. Conclusion: Thus, our results demonstrate better remineralization potential of A2 milk compared to regular milk and we would like to propose that resulting variations in Kappa and Beta casein content in the miscelles of A2 milk may be responsible for this property.

Keywords---remineralisation, casein, adsorption, A2 milk, SEM-EDX, SDS-PAGE.

Introduction

Improving the oral health awareness of parents/primary caregivers is one of the most important aspects of Primary Prevention for Early Childhood Caries (ECC). Although the etiologic factors causing dental caries may be similar in adults and children, the hypoplastic defects in the newly erupted tooth surfaces of children pose a higher risk for caries. , In a recent systematic review and meta-analysis to find the risk factors in ECC by Kirthiga et al, the dietary factors, bottle feeding and enamel defects had a higher odd's ratio of 29,15 and 14.62 respectively. Thus, depending on the frequency and type of intake, milk can affect caries formation by virtue of its demineralisation- remineralisation properties.

Cow Milk is one of first weaning foods across the world and also forms an integral part of children's diet in the growing years. The anti-cariogenic properties, of cow milk can be attributed to the limited cariogenic potential of lactose in cow milk, besides milk being an excellent source of calcium and phosphates that prevent demineralisation and aid in remineralisation of enamel. The presence of casein phosphoproteins which represent about 87% of all proteins present in milk also

help this process The caseins in the milk exist in the form of micelles (similar to salivary micelles), which form a layer on the surface of the tooth, and it attracts more calcium and phosphate ions from the saliva thereby promoting remineralisation of the enamel.

Beta-casein forms 30% of the total protein content in milk. 13 different alleles of beta casein have been identified. The two most common are A1 beta and A2 beta casein. These are generally present in regular cow milk. However, A2 milk contains only A2 beta-casein. These two beta caseins differ only in terms an amino acid residue at position 67. In the A2 allele, the amino acid at position 67 is proline instead of histidine in the A1 allele. The Histidine containing A1 beta casein can lead to generation of 7 beta-casomorphin 7(BCM-), an opioid-like peptide having effects similar to that of morphine. It has been suggested that such peptides can trigger conditions like type 1 diabetes, cardiovascular diseases and neurological problems. Hence, use of A2 milk is being promoted as a healthier option to regular cow milk.

The effect of A2 milk on human dentition has not been examined so far. In the present study it was assumed that a difference in the casein subunits can alter the adsorption and thereby the remineralisation properties of the regular and A2 milk, which could have implications on the anti-cariogenic properties of the respective milk. Hence, the aim of the present study was to evaluate and compare the remineralising and adsorption properties of regular cow milk and A2 milk in an in-vitro experimental model.

Methodology

A clearance from Institutional Research and Ethics Committee (Registration File Number-EC/NEW/INST/2019/329) was obtained before commencement of the study (Supplemental Information_2_A) A brief overview of the methodology is shown in Figure 1.

Materials used

This study used the raw regular cow milk and raw A2 milk from the company ProvilacTM, India Pvt. Ltd. (FSSAI no. 11513039002735) (Supplemental Information_1_A). The pH cycling model of remineralisation used HA discs along with human enamel discs as substrates in order to obtain more uniform, reproducible and consistent results. The HA discs were used as supplied by the manufacturer 3D BiotekTM , USA. In order to prepare the human enamel discs, 24 Enamel cores, 3 mm in diameter and 1.6–2.0 mm thick from buccal surface of enamel were made from 15 extracted premolars which were stored in 0.10% thymol solution. These were mounted on acrylic and were polished by a 600 grit grinding disk and a slurry of 0.05 μm gamma alumina polishing gel and then placed in deionized water for 10 minutes to remove any residues of the polishing procedures. A total of 24 Human Enamel Discs made from 15 carious free premolars indicated for extraction and 32 (24+5+3) Hydroxyapatite Discs were used for the study as per the sample size calculated by the statistician (Supplemental Information_2_C). Other materials used were distilled water from 99% Pure Pvt. Ltd, demineralizing solution from Stratum Cosmeceuticals Pvt. Ltd,

India, Phosphate Buffered Saline (PBS) from Micromaster Laboratories Pvt. Ltd, artificial saliva from Wet Mouth, India and CPP-ACP Tooth mousse (GC India)

Creating Artificial Demineralisation Lesion

All specimens were placed individually in a 24-well plate for 72 hours at 37-degree C in demineralizing solution containing lactic acid as 0.1 molar, 0.2% carbopol 50% hydroxyapatite saturated in volumes and adjusted to PH 5.0 using NaOH. This procedure is known to result in lesions of approximately 35–50 µm in depth.

Parameters evaluated

The pH cycling model for Remineralisation Evaluation: The 24 Human Enamel Discs and 24 HA discs were divided using simple random sampling into 2 groups:

- GROUP A (Control Group): 12 specimens using regular cow milk as treatment material
- GROUP B (Experimental Group): 12 specimens using A2 milk as treatment material

In order to evaluate the remineralisation properties, the present study used the 21-day pH-cycling model treatment regimen designed according to Gocmen GB et al, 2016[12]. Each cycle of the 21-day pH-cycling model comprised of 3 hours of demineralization to simulate the daily acid challenges in oral cavity (shown in Table 1). All the time except applications, the samples were kept in artificial saliva. Each cycle was repeated for 21 days.Artificial saliva was changed each day. This model was expected to cause remineralisation of all specimen. Microhardness and Surface Roughness Tests for Remineralisation Evaluation: The Microhardness and Surface Roughness tests on the samples were carried out Praj Metallurgical Laboratory, Pune. The specimen in both the groups were subjected to microhardness tests (with Vicker's Indenter, Reichert Austria Make, Sr.No.363798, Load- 100 g, Reference Standard: ISO 6507) and surface roughness tests (with a Profilometer Mitutoyo, Japan. Model: SJ 210 Stylus Speed: 0.5mm/s, Cut off Length: 1.25mm) at baseline, post-demineralisation and post-remineralisation. Increased microhardness and decreased surface roughness indicated increased remineralisation of the substrates. The change in microhardness after use of two types of milk was evaluated by the mean (Remineralisation –Demineralisation; R-D) and by the mean (Baseline-Remineralisation; B-R). The change in surface roughness after use of two types of milk was evaluated by the mean (Demineralisation –Remineralisation; D-R) and by the mean (Baseline-Remineralisation; B-R). This was followed by statistical evaluation of readings of both the tests i.e. microhardness and surface roughness for both the substrates.

SEM-EDX Analysis for Remineralisation Evaluation: This analysis was carried out at Bombay Textile Research Association (BTRA), Mumbai. This was the second parameter used for remineralisation evaluation. 5 HA Discs were used for this analysis which were divided into 5 groups as shown in Table 2. All the

specimens were given 2 PBS rinses and were completely dried before they underwent SEM-EDX analysis (Figure 2,3). Before SEM analysis, they were coated with a gold sputter coating (JEOL, JEC-550 twin cutter, Japan) for 600 seconds. They were then analysed for surface morphology and EDX analysis (SEM Machine: JEOL, JSM IT 200 LV, Japan. EDX detector: EDAX from USA). This yielded the Ca:P ratio for every sample. Increased Ca:P ratio suggested better remineralisation properties of that agent. 5 readings each of every sample were taken for statistical significance. Mean of all 5 readings of every sample was then calculated. The details of this procedure are given in Supplemental Information_1_C.

SDS PAGE Electrophoresis for evaluation of adsorption properties of milk proteins on HA discs: The SDS-PAGE Electrophoresis was carried out at Central Dogma Pvt.Ltd. Biotechnology Laboratory, Pune and the densitometric analysis of the gel was carried out at IRSHA Research Institute, Pune.The initial gel analyzed the protein profile fractions of different caseins (alpha, beta and kappa) of milk. It involved use of different concentrations of both types of milk (1:10 and 1:20) alongwith GC Tooth Mousse as the positive control. A molecular weight marker run alongside, helped determine the molecular weight of the proteins in the given sample.1: 10 dilution in case of both milks gave a clear bands for all size proteins so it was finalized as a right dilution for further experiments. Molecular weight of the different milk casein types ranged between 19-25KDa. The positive control did not show any protein bands, hence it was excluded from further experiments. (Figure 4)

This was followed by analysis of HA-bound milk caseins on SDS-PAGE gels. Briefly, the HA discs were incubated with unprocessed fresh milk of the two types for a short period separately, to allow interaction with the milk proteins. Subsequently, the loosely, nonspecifically bound constituents were washed off gently using a buffer solution. The HA bound proteins were then solubilized and collected by incubating the discs with an appropriate buffer. Thus obtained HA-bound milk proteins were then analyzed on SDS-PAGE gels. (Figure 5a). The intensity of the staining further allowed semi-quantitative analysis (densitometric analysis) which estimated the binding of different types of caseins in the two types of milk.(Figure 5b). The details of the entire procedure have been explained in Supplemental Information 3.

Results

Statistical Analysis

Data entries were done in Microsoft Office Excel 2010 and analyses of results were done using Statistical product and service solution (SPSS) version 21 software. Descriptive statistics such as mean and standard deviation were calculated. The p value was fixed at 0.05. Data normality was checked using Shapiro Wilk test.

Comparative Evaluation of Remineralisation Property of Regular cow milk and A2 milk using pH cycling model

Mann Whitney 'U' test was used to compare change in Micro hardness and Surface Roughness of Human Enamel Discs and Hydroxyapatite discs before and after use of regular cow milk and A2 milk. As shown in Table 3 and Table 4, the results of the Micro Hardness test showed that the micro hardness of both types of discs increased on exposure to A2 milk. However, in terms of decrease in mean surface roughness, better mineralisation with regular cow milk was indicated with HA discs whereas the trend with human dental enamel discs was opposite. While none of these differences were statistically significant ($p>0.05$), three out of four comparisons performed using the pH recycling model suggested better mineralization with the use of A2 milk.

Comparative Evaluation of Remineralisation Properties of Regular cow milk and A2 milk by SEM-EDX Analysis: Figure 6 gives the graphical representation of comparison of Ca:P ratio of different groups of HA discs.

Over-all comparison of SEM-EDX Analysis of samples in relation to change in their Ca:P Ratio: One-way ANOVA Statistical test was applied for an over-all comparison of SEM-EDX Analysis in relation to Ca:P Ratio after treatment with different materials between 5 groups. The overall comparison of Ca:P between all the groups was highly significant. ($p<0.001$)

Pairwise comparison of SEM-EDX Analysis of samples in relation to change in their Ca:P Ratio: Tukey's Post Hoc Statistical test was applied for pairwise comparison of SEM-EDX Analysis in relation to Ca:P Ratio between 5 groups. On pairwise comparison, the mean difference (0.181) between Ca:P ratio of regular cow milk and A2 milk was highly significant ($p<0.001$). The mean difference (0.154) between Ca:P ratio of regular cow milk and positive control was also highly significant ($p<0.001$).However, the mean difference (0.027) between Ca:P ratio of A2 milk and positive control was not statistically significant ($p=0.870$). Thus, according to SEM-EDX Analysis, remineralisation potential of A2 milk is better as compared with regular cow milk, and the results are highly statistically significant.

Densitometric Analysis of the Gel cast by SDS-PAGE Electrophoresis

The densitometric analysis of the gels permitted a semi-quantitative measure of the casein binding where bands from total milk served as baseline (100%) quantity. A comparable binding pattern for three major caseins (Alpha, Beta, Kappa) to HA discs for both the milk types was seen. When compared in terms of the amount present in the total milk, it was observed that the relative binding of kappa casein to HA discs was highest (~50% of the total). This was followed by beta casein (30% of the total). The binding of alpha casein appeared to be least efficient with only 10% of the total could be recovered from the HA discs under comparable conditions. (Figure 5b)

Discussion

Human enamel discs and hydroxyapatite discs have both been individually used to evaluate the remineralising properties of different types of remineralising agents. Both the substrates have their own merits and demerits. According to Mellberg (1992) and Ogaard and Rolla (1992), with respect to clinical significance, human premolars are the most appropriate source for dental substrate. However, factors such as genetic effects, past caries challenge, fluoride exposure, diet, age (post-eruptive maturation and dentin sclerosis) may cause them to respond differently to acidic stresses.14 HA aggregates are significantly more physically and chemically homogeneous than enamel. However, they are thought to have extremely similar dissolution kinetics and can be utilised as a model for enamel in attempts to study caries or erosion production in vitro . Hence the pH cycling model of remineralisation used HA discs along with human enamel discs as substrates in order to obtain more uniform, reproducible and consistent results. For SEM-EDX Analysis and SDS-PAGE electrophoresis only HA discs were used as the substrates in order to get more consistent and uniform results.

The in-vitro pH-cycling models which were first described by Ten Cate and Duijsters (1982) and later modified by Featherstone, et al (1986), are one of the most commonly used methods in cariology research. The present study uses treatment regimen designed according to Gocmen et al, 2016 based on the one used by Dunipace et al, 1994 for evaluation of remineralisation properties of enamel. In this study, subsurface lesions were created on all specimens by placing the specimen individually in demineralizing solution prepared according to the procedure given by White et al. Partially saturating the demineralising solutions with hydroxyapatite crystals along with addition of surface protective agent like carbopol is known to induce sub-surface lesions, rather than erosion-like lesions.

Vickers Microhardness test was chosen over Knoop's hardness because the square indent formed with the former is easier to measure. The surface roughness measurement by contact stylus profilometer is considered one of the most precise methods and has been used previously in many studies for surface roughness evaluation. The microhardness of the substrates was decreased post-demineralisation and increased post-remineralisation with two types of cow milk. This established the effectiveness of the protocol used. These observations are in accordance with those reported by Dennis D et al., 2018 and Widanti et al., 2017. It may be noted that Widanti et al have used Knoop hardness test, instead of Vicker's test to evaluate microhardness. The surface roughness of the substrates was increased post-demineralisation and decreased post-remineralisation with the two types of milk. This was in accordance with results obtained by Al Naimi RJ et al., 2021 (used buffalo milk instead of cow milk). Of the four comparisons, better remineralisation potential of A2 milk was evident except for greater reduction in mean surface roughness seen with regular cow milk on HA discs. These observations together, though not statistically significant, are suggestive of overall better remineralisation by A2 milk. The results cannot be directly compared with any another previous study, as such a study hasn't been carried out in the past.

The role of CPP-ACP i.e. casein phosphopeptide-amorphous calcium phosphate paste (GC tooth mousse) as a remineralising agent is well established. , , It is for this reason that it was used as a positive control in this study to compare the remineralising properties of the two different types of milk. The A2 milk group showed the highest Ca:P ratio (1.64) which was comparable to the positive control group (1.6).The results from the overall and pairwise comparison of Ca:P ratios in all the groups clearly indicate that A2 milk caused remineralisation comparable to the positive control (i.e. CPPACP) which is considered to be the standard against which any remineralising agent can be compared. Also regular cow milk produced less remineralisation in comparison to A2 milk as well as the positive control. Thus, the SEM-EDX based study strengthened the trend observed with pH recycling model based studies indicating that the remineralisation potential of A2 milk is better compared with regular cow milk.

The binding of milk caseins to tooth surface and the remineralisation of tooth by milk can be considered as interdependent (Woodward M. et al., 2020). Therefore, it was of interest to examine binding of the three major caseins to hydroxyapatite, a putative substrate for the enamel that plays primary role in interaction with milk micelles. The SDS-PAGE gels revealed that the percentage of alpha, beta caseins in both types of milk were higher as compared to the kappa caseins. However, the relative binding of kappa casein to HA discs was the highest (~50% of the total). These findings were similar to those observed by was Tercinier L et al., 2014, who had analysed the binding of milk caseins on HA particles. The differences observed across individual caseins in terms of binding to HA disks are likely to arise via (1) ability of these caseins to dissociate into smaller complexes on exposure to HA disks, thus affecting their ability to interact with HA, (2) presence of charged residues in these proteins, alpha caseins being least charged entities and (3) location of these entities in the micelles or (4) combination of two or more factors above (Gorbunoff MJ et al.,1984)

Besides these considerations, it is of relevance to note that, the A2 beta-casein variant is known to form smaller micelles than A1 beta-casein (Raynes JK et al.,2015). The kappa-casein content of the milk is expected to increase with the decrease in the size of the micelle (Ekstrand B et al., 1980). Our adsorption study suggests that kappa casein bound more efficiently to HA disks. Together, on the basis of these findings we would like to propose that the small micellar structure of A2 milk provides an increased surface area for binding of higher proportion of kappa casein. Thus, the A2 containing micelles would interact more efficiently in higher quantity, with HA in the enamel contributing to better deposition of Ca P and subsequent remineralisation. Our studies provide important leads for further investigations in this context.

Conclusion

In summary, we would like to conclude that,

- The remineralisation potential of A2 milk is better as compared with regular cow milk.
- Kappa casein fraction of regular as well as A2 milk shows highest binding to HA Discs amongst others.

- Higher remineralisation potential of A2 milk observed in the present study may be a result of the relatively higher amount of kappa casein available in its smaller micelles for interaction with substrate leading to their more efficient binding to Hydroxyapatite and better deposition of Calcium and phosphate.

Limitations of this study

- Due to limited resources we were unable to use multiple HA disks for different treatment combinations for SEM-EDEX studies. This was compensated by ensuring maximum coverage of the surface area of the individual HA discs with five different readings for each specimen. Further studies with a greater sample size can be undertaken.
- The human enamel discs were made from permanent extracted premolars. The primary molars of children, which are exposed to dietary milk in equal or even greater quantities than permanent teeth are known to be more sensitive to pH (acid) induced demineralization (Wang LJ et al., 2006). Hence, it would be only appropriate to further investigate these results using extracted primary teeth.
- Finally, due to limited sensitivity, the SDS-PAGE based analysis may have been unable to detect difference between the casein binding patterns from the two milk types. This would be especially relevant with regards to the binding of kappa casein to HA which is presumably present on the micelle surfaces and would be expected to show higher binding in case of A2 milk.

Further Directions

Apart from remineralisation via casein micelles which increase the calcium: phosphate ratio in milk, the anti-cariogenic properties of milk can also be attributed to other bio-protective factors like lactose, fat and whey proteins. The regular cow milk and A2 milk used in the present study were comparable in terms of amount of lactose and fat percentages (Supplemental_Information_1_A). However, the role of whey proteins influencing the remineralising properties of both types of milk remains to be investigated.

Clinical Significance of this paper

- The frequency and method of ingestion of milk can affect caries formation by virtue of its demineralisation- remineralisation properties and is considered one of the major risk factors of ECC.
- Cow Milk is one of first weaning foods across the world and also forms an integral part of children's diet in the growing years. Use of A2 milk is being promoted as a healthier option to regular cow milk. The effect of A2 milk on human dentition has not been examined so far.
- This is the 1st study to compare both, the remineralising potential and adsorption properties of caseins from A2 milk versus regular cow milk using two different substrates, which may further affect the cariogenic potential of milk.

Conflict of Interest Statement

Funding Sources

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Tables

Table 1
21-day pH-cycling model used in the study

Time	Treatment Material
7:10-8:10	Demineralization Solution
8:10-08:15	Treatment material
8:15-12:15	Artificial saliva
12:15-13:15	Demineralization Solution
13:15-13:20	Treatment material
13:20-18:20	Artificial saliva
18:20-19:20	Demineralization Solution
19:20-19:25	Treatment Material
19:25-7:10	Artificial Saliva

Table 2
Groups for SDM-EDX analysis for remineralisation evaluation

Group	Treatment Material
Group 1 (A1 regular milk)	1 HA Disc using regular milk as treatment material & underwent 21 day-pH cycle
Group 2 (A2 milk)	1 HA Disc using A2 milk as treatment material & underwent 21 day-pH cycle

Group 3 (No treatment group)	1 HA Disc which did not undergo any treatment
Group 4 (Demin group)	1 HA Disc which underwent only initial demineralisation and no remineralisation cycle
Group 5 (Positive control)	1 HA Disc using positive control (GC Tooth Mousse) as treatment material & underwent 21 day-pH cycle

Table 3

Comparison of change in Micro hardness and Surface Roughness on Human Enamel Discs after regular use between regular cow milk and A2 milk

Human Enamel Discs	Change in Microhardness (R-D) scores (Mean SD)	Change in Microhardness (B-R) scores (Mean SD)	Change in Surface Roughness (D-R) scores (Mean SD)	Change in Surface Roughness (B-R) scores (Mean SD)
Group Regular Milk	40.57(27.75)	10.6(2.7)	0.054(0.048)	O.292(0.185)
Group A2 Milk	44.28(34.64)	10.2(1.78)	0.099(0.065)	0.289(0.036)
Mann Whitney 'U'	U=23.0	U=11.5	U=16.0	U=17.0
p value, Significance	P=0.848	P=0.831	P=0.277	P=0.337

p>0.05 – no statistically significant difference

Table 4

Comparison of change in Micro hardness and Surface Roughness on HA Discs after regular use between regular cow milk and A2 milk

| HA Discs	Change in Microhardness (R-D) scores (Mean SD)	Change in Microhardness (B-R) scores (Mean SD)	Change in Surface Roughness (D-R) scores (Mean SD)	Change in Surface Roughness (B-R) scores (Mean SD)
Group Regular Milk	8.0 (3.53)	2.6(3.91)	0.149 (0.129)	0.142 (0.07)
Group A2 Milk	9.2 (5.21)	1.0(3.67)	0.01 (0.375)	0.279 (0.272)
Mann Whitney 'U'	U=11.5	U=10.5	U=12.0	U=8.0
p value, Significance	P=0.834	P=0.675	P=0.917	P=0.347

p>0.05 – no statistically significant difference

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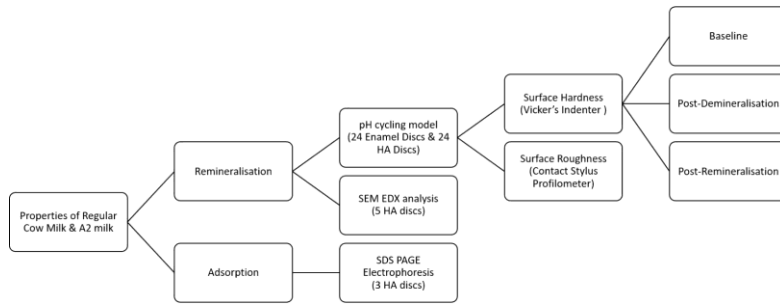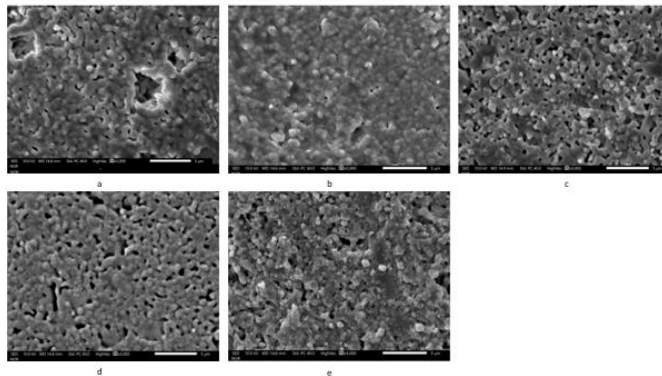
Figure 1

Figure 1. A brief overview of the methodology

Figure 2. SEM images of HA discs under 5000 magnification: (2a) HA discs treated wih regular milk, (2b) HA discs treated with A2 milk, (2c) Untreated HA discs, (2d) HA discs treated with demineralisation solution only, (2e) HA discs treated with positive control
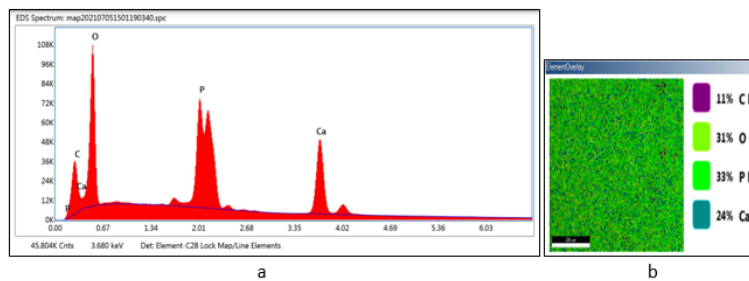

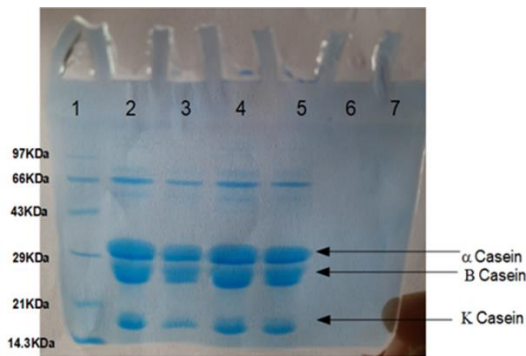Figure 3. Sample image of EDX Spectrum (6a) and element overlay (6b) of HA disc treated with A2milk

Figure 4. SDS-PAGE gel showing Protein Profile of two types of milk. On Lane1: Protein molecular weight marker, Lane2: A2 milk (1:10), Lane3: A2 milk (1:20), Lane 4 Normal milk (1:10), Lane 5: Normal milk (1:20), Lane 6: GC Tooth Mousse (10ul), Lane 7: GC Tooth Mousse (15ul) loaded
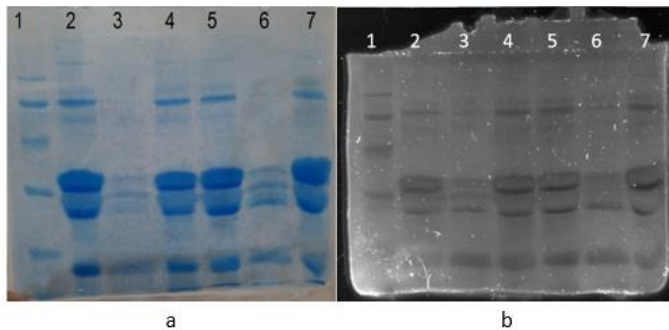

Figure 5. (5a) SDSPage Gel Analysis: Lane1: Protein marker, Lane2: A2 milk, Lane3: A2 milk protein bound to HA disk extracted, Lane 4: A2 milk removed post HA disk treatment, Lane5: Normal milk, Lane6: Normal milk protein bound to HA disk extracted, Lane 7: Normal milk removed post HA disc treatment (5b) Densitometric image of same gel
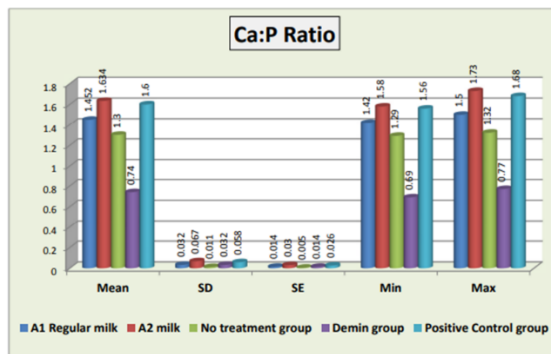

Figure 6. Graphical representation of comparison of Ca:P ratio of different groups of HA discs