Cariogenicity and Acidogenicity of Human Milk, Plain and Sweetened Bovine Milk: An *In Vitro* Study

Prabhakar A R* / Ameet J Kurthukoti** / Pranjali Gupta***

*The **objective** of the present study was to determine the acidogenicity and cariogenicity of human breast milk and plain and sweetened packaged bovine milk.*

***Study Design:** First all milk specimens were inoculated with a cariogenic strain of Streptococcus mutans (SM). The culture pH and number of colony forming units (cfus) was assessed. Second, the buffer capacity of all milk specimens was evaluated by mixing with acid. Finally, enamel windows were created on extracted primary maxillary incisors and colonized with SM. Enamel demineralization and caries progression were assessed visually, histologically, and radiographically at the end of twelve weeks. **Results:** Plain and sweetened packaged bovine milk (BM) supported greater bacterial growth and caused more fermentation than human breast milk (HBM). The buffer capacity values for plain and sweetened bovine milk were highest; HBM, however, had poor buffering capacity. The progression of the carious lesions into the dentin was most severe for the sweetened bovine milk. **Conclusions:** HBM and plain bovine milk are relatively cariogenic in an in vitro caries model in the absence of saliva. However, supplementation with sugar exponentially enhances the cariogenic potential of the natural milk.*

***Keywords:** Human breast milk, bovine milk, bacterial growth and fermentation, buffer capacity, caries progression, early childhood caries.*

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INTRODUCTION

Milk has been defined as "a complex colloidal mixture of proteins, fat, lactose, minerals, and many other constituents, some in suspension and some in solution."[1] It is an emulsion of fat globules and a suspension of casein micelles (composed of casein, calcium, and phosphorus), all suspended in an aqueous phase that contains solubilized lactose, whey proteins, some minerals and salts.[2]

From the very first day of life, breast milk forms the staple diet of children until the weaning period. Humans feed their infants with not only their own milk, but also animal milk and have also devised ways for the artificial procurement of milk to cater to the needs of infants.[3]

Extensive research has reported diverse and compelling advantages for infants, mothers, families, and society from breastfeeding. These advantages include health, nutritional, physical, immunological, developmental, psychological, social, economic, and environmental benefits.[4]

To date, information about the cariogenicity or cariostatic properties of milk is surprisingly sparse.[1] It has been suggested that both bovine and human breast milk can cause dental caries in infants if consumed frequently and retained for long periods in the mouth.[5] Prolonged and excessive breastfeeding has also been suspected as a causative factor of early childhood caries. However, controversy exists regarding the cariogenicity of human breast milk. Human breast milk has a higher carbohydrate content and lower calcium, phosphorus, and protein levels than bovine milk, thus making it potentially more cariogenic.[6]

It is a common household practice to add sugar to bovine milk to make it more palatable for infants. Recent studies indicate that milk containing sucrose is more cariogenic than milk alone.[1] As a fermentable carbohydrate, sugar acts as food for caries-producing microorganisms, thus enhancing their growth and colonization on infants' newly erupted teeth, making them more susceptible to acid decay and caries initiation.[7,8]

The purpose of the present study was to evaluate the aci-

* Prabhakar A.R, MDS, Professor and Head, Pedodontics and Preventive Dentistry, Bapuji Dental College and Hospital, Davanagere, Karnataka
** Ameet J Kurthukoti MDS, Reader, Pedodontics and Preventive Dentistry, Bapuji Dental College and Hospital, Davanagere, Karnataka
*** Pranjali Gupta (MDS), Post graduate student, Pedodontics and Preventive Dentistry, Bapuji Dental College and Hospital, Davanagere, Karnataka

Send all correspondence to: Dr. Prabhakar A.R, Professor and Head, Dept of Pedodontics and Preventive Dentistry, Bapuji Dental College and Hospital, Davanagere - 577004, Karnataka, India

Phone No: 91-8192-220575
Fax No: 91-8192-220578

E-mail:attiguppeprabhakar@yahoo.com

dogenic and cariogenic potential of human breast milk and plain and sweetened bovine milk. The results of this study should shed light on the question of whether milk is cariogenic or not.

MATERIALS AND METHOD

The study included human breast milk and plain and sweetened packaged bovine milk as the experimental groups and distilled water and 10% sucrose solution as the control groups.

The specimens were grouped as follows:

Control Groups: which comprised of Group I (Negative Control): Distilled water; Group II (Positive Control): 10% Sucrose solution.

Experimental Groups; which comprised of Group III: Human Breast Milk; Group IV: Plain Packaged Bovine Milk.**;** Group V: Sweetened Packaged Bovine Milk.

Preparation of experimental groups

Human Breast Milk specimens: Mature HBM donations were collected from nursing mothers with infants of ages 12 weeks to 2 years. The milk specimens were collected in sterile plastic containers after gentle manual expression of the breast tissue.[6]

Plain Packaged Bovine Milk specimens: Packaged BM (Mfg at Shimoga co-operative milk producer's society union, limited) was collected from a retail dairy outlet. It was pasteurized at a temperature of 72^{O}C for 16 seconds and allowed to cool to room temperature.

Sweetened Packaged Bovine Milk specimens: Ten grams of sugar was added to 100 ml of pasteurized cool milk, obtained via the method described above and the resulting mixture was stirred for ten seconds.

All of the experimental and control specimens were kept in sterile plastic beakers.

Part I: Bacterial fermentation and growth

Collection of unstimulated saliva:

Ten children between 3 and 5 years of age were selected for saliva collection using a simple random sampling technique from the Outpatients Department. The inclusion criteria were: presence of early childhood caries, absence of systemic diseases, no history of any fluoride intake/ use of topical fluorides, and no history of antibiotic use in the previous month.

Saliva was collected over a period of 5 minutes by repeatedly spitting every 60 sec.[9] The process was repeated for all 10 children. The saliva was then pooled and inoculated onto an MSB agar plate (Hi Media laboratories Pvt Ltd, Mumbai) and incubated at 37ºC for 2 days to obtain a culture of *Streptococcus mutans*.[10]

In each group (Group I-Group V), six specimens were inoculated with *S. mutans*. Todd Hewitt Broth (Hi Media laboratories Pvt Ltd, Mumbai) was used as a control culture media. All of the specimens and the control culture media were incubated at 37ºC for 3 hours.[6] After optimal growth in the Todd Hewitt Broth was ensured, 0.01 ml samples were taken from each sample (Groups I-V), spread onto individual MSB agar plates,[6] and incubated at 37ºC for 2 days.[10] The individual plates were subsequently assayed for the number of colony forming units (cfus) of *SM* using a digital colony counter.

The pH changes associated with bacterial fermentation were also assessed by comparing the pH of each culture plate before and after 48 hours of culture using a pH meter.[6]

Part II: Buffer Capacity

In order to ensure standardization, six specimens from each of the experimental and control groups from part I of the study were evaluated for their buffering capacity (as suggested by the statistician). The initial pH of each individual sample in the various groups was assessed using a pH meter prior to the beginning of the experiment using two separate electrodes standardized with standard buffer solutions of pH 7. Then, 4.5 ml of each individual sample was drawn out using a sterile glass pipette and placed in a sterile glass beaker and 0.01 M hydrochloric acid was added from a microburette to each sample while the sample was stirred continuously to obtain a homogenous solution and stable pH value according to the pH meter. The acid was added until a drop in pH of 2 units was attained.[6]

The number of moles of HCl required to reduce the pH by 2 units was calculated, and the buffer capacity of each sample was determined using the buffering intensity formula given by Van Slyke.[11]

$$\text{B value} = \frac{dB}{dpH} = \frac{(\text{ml acid added})\,(\text{normality of acid})}{(\text{volume of milk})\,(\text{pH change})}$$

Part III: *In Vitro* Caries Progression

Seventy-five primary maxillary incisors extracted for therapeutic reasons were included in this study. All of the teeth were evaluated under a stereomicroscope to ensure the absence of caries, developmental defects, enamel fractures and micro-fractures, discoloration, and internal / external pathologic resorption. Pulp treatment was not attempted for any of the teeth.

Preparation of test specimen (tooth)

All soft tissue and calculi were removed from the teeth with a periodontal scaler. The teeth were cleaned using slurry of pumice, steam autoclaved, and stored in distilled water at room temperature until further use. An enamel window was then prepared as follows.

A circular piece of plaster adhesive tape measuring 2.5 mm in diameter was fixed to the labial cervical third surface of each of the 75 primary maxillary incisors, and the remaining portion of the teeth was painted with nail varnish. After the varnish had dried, the masking tape was removed to leave an exposed enamel surface of 0.049 cm^2 (enamel window)

on each tooth.[6] The teeth were then randomly divided into five groups of 15 each and mounted onto carving wax blocks. Mounted teeth were suspended in a *Streptococcus mutans* suspension and incubated at 37ºC for 18 hours to achieve *in vitro* enamel colonization.[6]

In vitro caries progression was then checked: Fifteen sets of mounted teeth were immersed in individual glass bottles containing 2.5 ml samples of the various groups for 12 weeks. All of the test and control solutions were freshly replenished daily for a period of 12 weeks. Positive bacterial culture in all specimens was verified at weekly intervals.

The development of caries was assessed by visual and histological evaluation of the teeth, and the progression of dental caries was assessed radiographically as follows:

VISUAL EXAMINATION

The extent of caries progression was numerically scored on a 0-4 scale using the following visual scoring criteria.[12]

Score	Criteria
0	No detectable change indicative of caries
1	Opacity or discoloration hardly visible on the wet surface, but distinctly visible after air drying
2	Opacity or discoloration distinctly visible without air drying
3	Localized enamel breakdown in opaque or discolored enamel
4	Cavitation in opacity or discolored enamel exposing the dentin

HISTOLOGIC EXAMINATION

The individual teeth were unmounted from the wax blocks and sectioned through the enamel window, in the bucco-palatal direction, using a diamond disc. Both sectioned surfaces were examined under a stereomicroscope (x 10) and the section with more extensive changes was chosen for scoring. The extent of caries progression was numerically scored on a 0-4 scale using the histological scoring criteria.[12]

Score	Criteria
0	No enamel demineralization or a narrow surface zone of opacity (edge phenomenon)
1	Enamel demineralization limited to the outer 50 % of the enamel layer
2	Demineralization involving the inner 50% of the enamel, up to the enamel-dentin junction
3	Demineralization involving the outer 50% of the dentin
4	Demineralization involving the inner 50% of the dentin

RADIOGRAPHIC EXAMINATION

Each of the histologically scored specimens was subsequently individually radiographed using Ultraspeed film[6] and a standardized exposure (8 mA, 70 KVP, 0.08 sec, 15 cm cone-film distance). A magnifying lens was used to examine the radiographs.

The caries progression was evaluated using a 0-4 scale according to the radiographic scoring criteria.[13]

Score	Criteria
0	No radiolucency visible
1	Radiolucency visible in the enamel
2	Radiolucency visible in the dentine, but restricted to the outer third of the dentin
3	Radiolucency extending to the middle third of the dentin
4	Radiolucency in the pulpal third of the dentin

During all of the scoring procedures i.e.; visual, histologic, and radiographic, the examiner's diagnostic reproducibility was monitored by randomly selecting 10 % of the samples/radiographs interpreted on one day and placing them at random amongst the samples/radiographs to be examined on the next working day. All of the samples/radiographs were scored independently by two blinded investigators and their results correlated with the original scores.

Results were expressed as mean ± standard deviation (SD) for continuous data and numbers for scoring pattern. Since the number of specimens was small and measurements were in scores, non-parametric analyses were performed. Kruskal-Wallis ANOVA was used for multiple group comparison followed by the Mann-Whitney test for pair-wise comparisons. Categorical data were analyzed by Chi square test.

RESULTS

Bacterial fermentation and growth

In this study, the mean bacterial growth in the presence of human breast milk was 101.5 cfus. The mean drop in culture pH was 0.81 (7.00 to 6.19). The mean bacterial growth in the presence of plain packaged bovine milk was 145.2. The mean drop in culture pH was 1.00 (7.00 to 6.00). The mean bacterial growth in the presence of sweetened packaged bovine milk was 257.8. The mean drop in culture pH was 1.93 (7.02 to 5.09). The differences in pH levels and cfu counts were significantly different between all combinations of the groups ($p<0.01$). (Table 1).

Buffer Capacity of milk groups

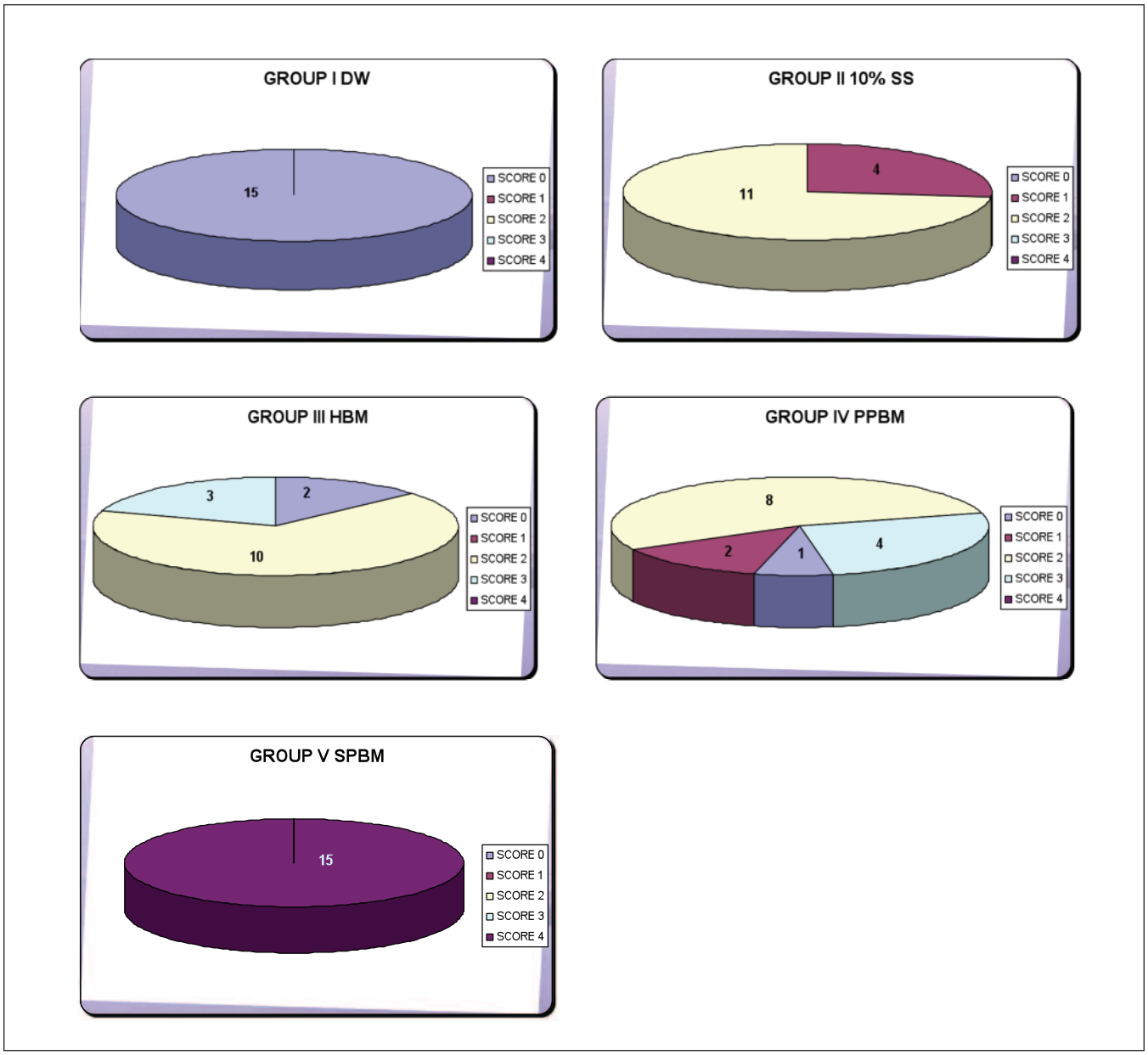
The mean initial pH of the human breast milk was 7.03 and the B value was 0.022. The mean initial pH of the plain packaged bovine milk was 6.71 and the B value was 0.035. The mean initial pH of the sweetened packaged bovine milk was 6.72 and the B value was 0.032. The B values were significantly different between all combinations of the groups ($p<0.01$) except for Group I and Group II ($p= 1.00$) and Group IV and Group V ($p= 0.052$). (Table 1).

In vitro caries progression

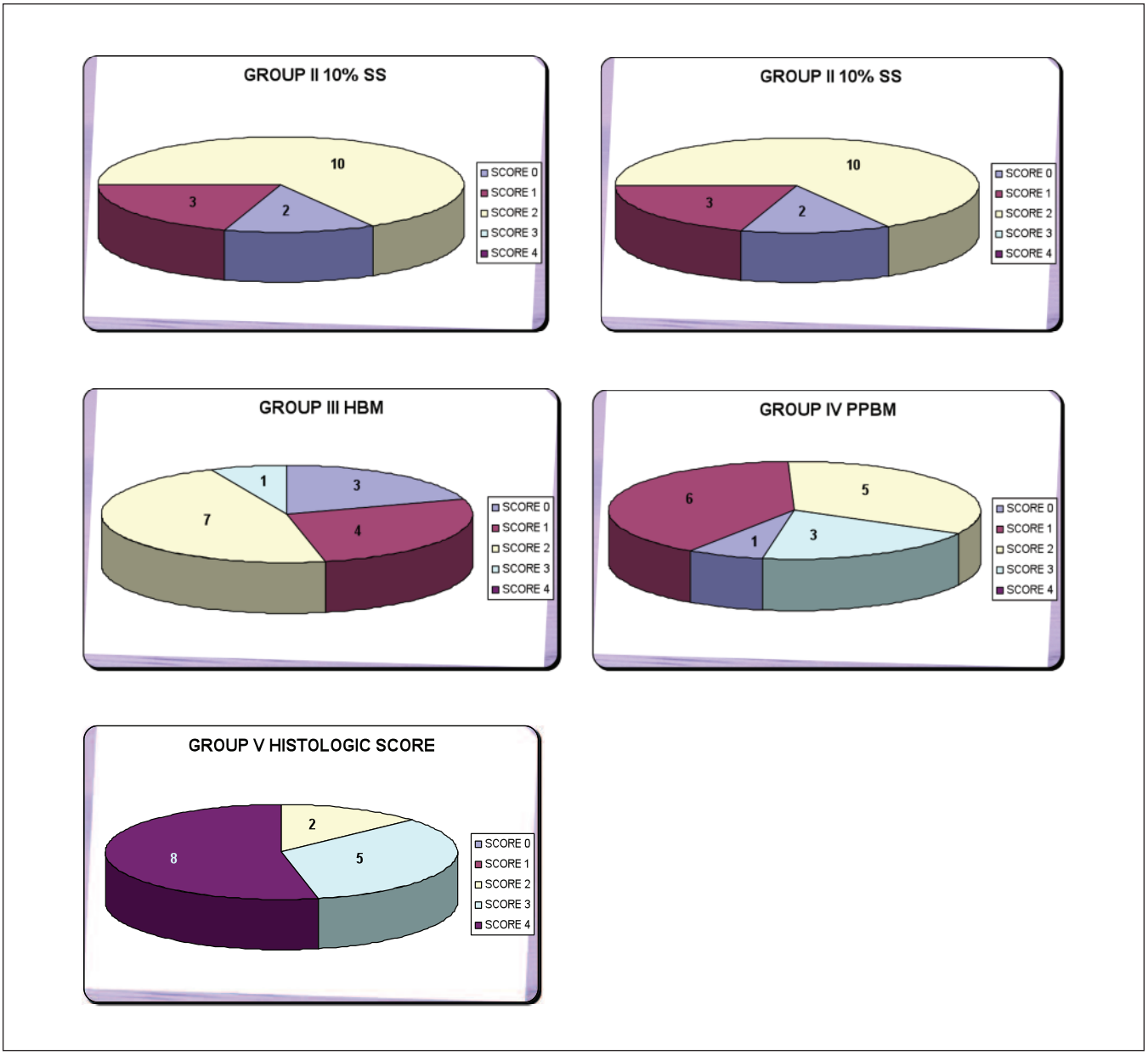
On visual examination (Graph 1), it was found that in the human breast milk group, two teeth scored 0, ten teeth scored 2, and the remaining three teeth scored 3. In the plain packaged bovine milk group, one tooth scored 0, two teeth

Table 1. Caries-related variables of the control and experimental groups.

Variable	Distilled water	Sucrose solution	Human breast milk	Plain packaged bovine milk	Sweetened packaged bovine milk
Bacterial fermentation and growth -Colony forming units (cfus)	49.3±6.1	70.7±5.5	101.5±8.3	145.2±7.1	257.8±18.0
-Difference of initial and final pH	0.23±0.07	1.72±0.08	0.81±0.09	1.00±0.04	1.93±0.13
Buffer capacity -Initial pH	7.08±0.02	6.93±0.05	7.03±0.11	6.71±0.03	6.72±0.03
-B value	0.003±0.001	0.003±0.001	0.022±0.002	0.035±0.003	0.032±0.003



Graph I: Comparison of the visual scores of caries progression for the various experimental and control groups.



Graph 2: Comparison of the histologic scores of caries progression for the various experimental and control groups.

scored 1, eight teeth scored 2, and the remaining four teeth scored 3. In the sweetened packaged bovine milk group, all fifteen of the teeth scored 4.

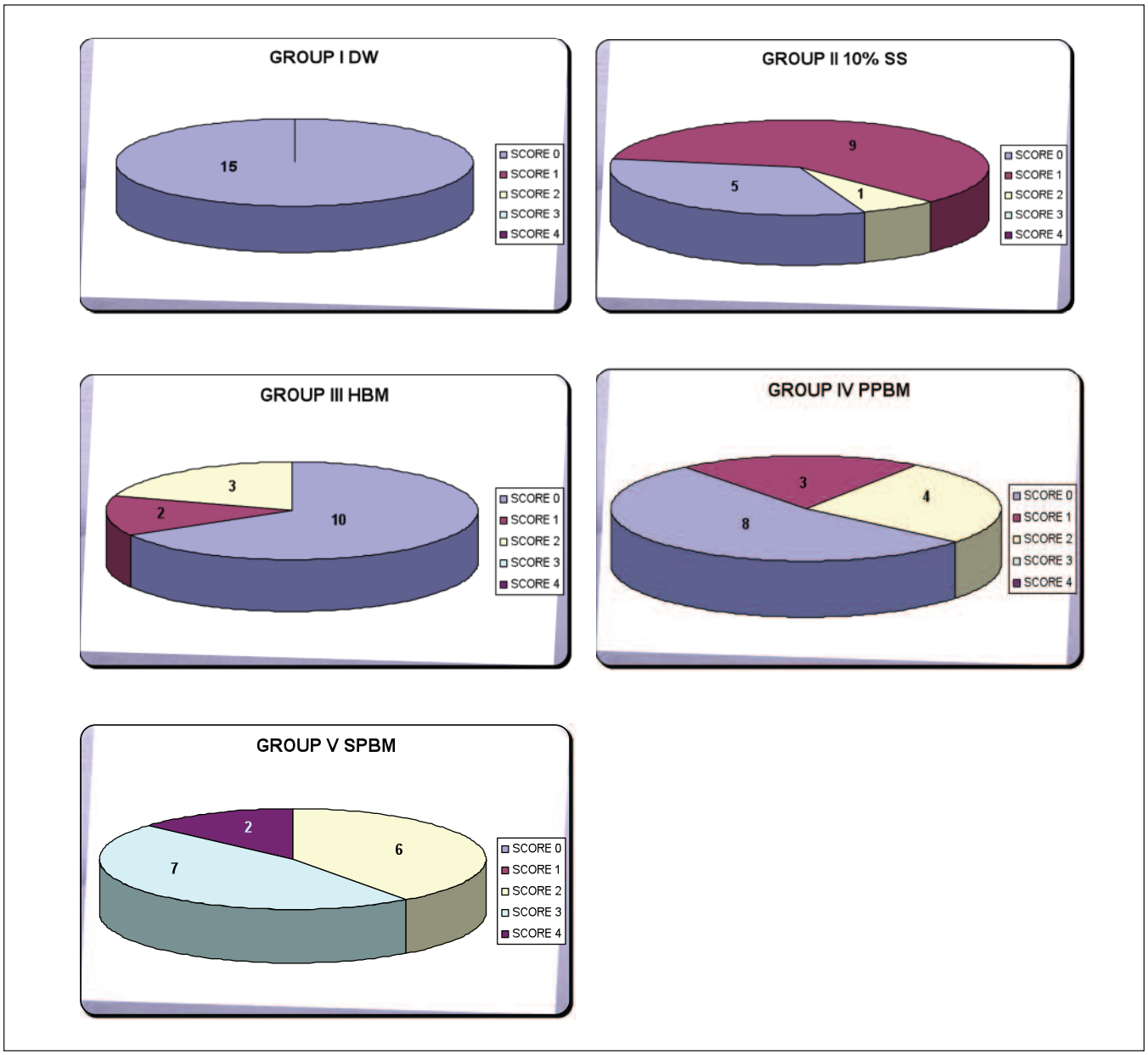
On histologic examination (Graph 2), it was found that in the human breast milk group, three teeth scored 0, four teeth scored 1, seven teeth scored 2, and the remaining one tooth scored 3. In the plain packaged bovine milk group, one tooth scored 0, six teeth scored 1, five teeth scored 2, and the remaining three teeth scored 3. In the sweetened packaged bovine milk group, two teeth scored 2, five teeth scored 3, and the remaining eight teeth scored 4.

On radiographic examination (Graph 3), it was found that in the human breast milk group, ten teeth scored 0, two teeth scored 1, and the remaining three teeth scored 2. In the plain packaged bovine milk group, eight teeth scored 0, three teeth scored 1, and the remaining four teeth scored 2. In the sweetened packaged bovine milk group, six teeth scored 2, seven teeth scored 3, and the remaining two teeth scored 4.

DISCUSSION

Traditionally in research, ecosystems that cannot be studied intact are separated into their component parts to be analyzed in isolation under controlled conditions. Hence, in the present study, the cariogenicity of the various milk groups were evaluated by:

1. Bacterial fermentation and growth potential of milk groups.
2. Buffer capacity of milk groups.
3. *In vitro* caries progression



Graph 3: Comparison of the radiologic scores of caries progression for the various experimental and control groups.

In the first part of the study, the bacterial fermentation and growth potential of the various milk groups were examined in order to ascertain the extent to which they supported the growth of *SM*.

It has been noted that bovine milk supports maximal bacterial growth, while human breast milk supports moderate bacterial growth.[6] The results of the present study are analogous to these previous reports, revealing that sweetened packaged bovine milk supported maximal bacterial growth, followed by plain packaged bovine milk, while human breast milk only supported moderate bacterial growth.

The final pH of the culture media was directly influenced by the total number of cfus of *SM* present.[6] In the present study, high numbers of colony forming units were observed in all of the experimental groups.

In previous studies, it was found that the addition of as little as 2% sucrose to milk enhanced its acidogenicity."[1]

The non sucrose containing milk groups also demonstrated a considerable drop in the final culture pH, but the drop was significantly smaller compared to that of the sweetened packaged bovine milk group. This observation could be attributed to the presence of lactose, which is the predominant sugar in natural milk. Lactose does not lower pH values as drastically as sucrose.[14] Sucrose, which is a fermentable carbohydrate, was absent in the human breast milk and plain packaged bovine milk groups. This may have been an additional factor responsible for the smaller pH drop.

The evidence from the present study indicates that the

presence of a fermentable carbohydrate in milk definitely influenced its bacterial fermentation and growth potential. The pH drop associated with sucrose fermentation is more drastic than that associated with lactose.[15] Thus, sucrose is personified as the arch criminal in dental caries.[16]

Part II: Buffer capacity:

Whenever acids are produced in the oral milieu, the buffers present in saliva and milk play a role in neutralizing the acid produced by these bacteria In general, the constituents that contribute to the pH buffering capacity of milk and milk products can be broadly divided into two categories: 1. Proteins and 2. Weak acids, bases, and their complexes with metal cations. Proteins and free amino acids are major contributors to pH buffering in any biological system. The pH buffering capacity of proteins is due to amino acids with basic and acidic side chains and interactions of cations with functional groups in these side chains.[17]

The B values derived for bovine milk in our study were comparable to the values reported in another study.[11]

The control groups in the present study attained the final pH with very low volumes of acid addition, suggesting that they had very poor or no buffering capacity. However, due to their milk protein content, the experimental groups showed significantly greater buffering capacity compared to the controls. This buffering capacity of natural milk could be attributed to its protein content, predominantly casein.[18]

It was found that the maximum buffering in natural milk occurs at an approximate pH of 5.5. The buffering intensity curve of casein determined by difference indicates that the buffer action of casein is exerted principally between pH 4.5 and pH 5.7, with a maximum at approximately pH 5.2. Thus, casein is evidently one of the chief factors in the buffer action of milk in this range.[19]

Bovine milk (plain and sweetened) exhibited greater buffering capacity than human breast milk. An assessment of the composition of human breast milk is important to understand the reduced buffer capacity in comparison to bovine milk. Human breast milk has significantly less phosphate (5 mg/dL) than bovine milk (75 mg/dL inorganic phosphate). Human breast milk also has lower protein levels, with approximately one-fifth the amount of amino acids contained in bovine milk. Of particular importance is the concentration of histidine, which has a highly buffering imidazole ring. The concentration of histidine in human breast milk (23 mg/dL) is significantly less than that present in bovine milk (110 mg/dL). In contrast, the concentration of organic acids is two to three times higher in human breast milk than bovine milk. It is likely that the phosphates and proteins present within human breast milk are capable of buffering the free hydrogen ions associated with these organic acids and thereby maintaining the pH near neutral when unchallenged by other acid sources. However, when additional acid is present, the buffering capacity is exceeded.[6]

The buffering values of milk obtained in the present study need to be carefully interpreted, because salivary buffers, which are important in controlling the pH of the oral fluids bathing the teeth, were not evaluated.

Bacterial growth and fermentation and buffer capacity, though interrelated, are separate facets that do not provide sufficient information to draw conclusions about the cariogenic potential of milk. After having tested the bacterial fermentation and buffer capacity of the experimental groups, it was imperative that their true cariogenic potential be tested in an in-vitro caries model.

Part III: *In vitro* caries progression

In vitro caries models act as a laboratory "microcosm" by attempting to model many of the physical aspects of the oral cavity.[20] These models completely expose the true cariogenic potential of a substrate, since they include all three features needed for caries formation and progression i.e. the host (tooth), substrate (milk), and flora (*Streptococcus mutans*). The presence of all three entities in the present study in an in-vitro model for a period of 12 weeks enabled substantial conclusions to be drawn.

When the visual scoring criteria were employed, it was evident that the positive control and all of the experimental groups showed caries progression. Comparison of the caries scores using visual criteria across the experimental groups showed that the addition of sucrose to milk directly influenced its cariogenicity. As such, maximum caries lesions were evident in the sweetened packaged bovine milk group.

When the histologic and radiographic scoring criteria were employed, the findings of the visual scoring were reconfirmed. It was again evident that the greatest extent of caries progression into dentin was found in the sweetened packaged bovine milk group. The results obtained from the present study are comparable to those from a previous study. The authors reported that caries progression into the dentin was evident at the end of 4 weeks in the positive control group (10% sucrose).[6] They also found that the caries progression into dentin was observed in a time period as short as 3.2 weeks when human breast milk was supplemented with sucrose. The authors employed only visual and radiographic criteria to evaluate caries progression.[6] In the present study, however, visual, histologic, and radiographic scoring criteria were employed to evaluate caries progression. Though the time needed for caries progression was not evaluated, it was clearly evident that supplementation with an external carbohydrate source (sucrose) enhanced the cariogenic potential of milk.

The results of the present study are also comparable to those of an animal study, in which the authors concluded that the addition of sucrose to milk enhanced the severity of caries scores. They reported that milk did not possess cariostatic properties when the dietary challenge and milk were administered separately. They also suggested that milk containing sucrose was more cariogenic than milk alone.[1]

A similar study comparing the cariogenic potential of infant formulae, milk, and a sugar solution concluded that

milk was reasonably cariogenic.[21] These previous reports are comparable to those of the present study in which natural milk (human and bovine) was found to be relatively cariogenic and caused precavitated lesions in an in vitro caries model.

Some authors have cautioned that "to relate the process of dental caries to milk alone may be an inappropriate condemnation of a product that is nutritionally essential to the growth and welfare of the infant."[22] The reduced caries scores associated with human breast milk and plain packaged bovine milk could be attributed not only to the absence of sucrose, but also to the protective effects of milk.

In the *in situ* model using bovine enamel, it was evident that phosphoproteins in milk which strongly adsorbed onto enamel apparently prevented its dissolution.[1] It was also observed that milk could remineralize enamel in vitro.[23] A range of antibacterial substances, including lysozyme, peroxidase, and lactoferrin, that are present in milk can affect the microbial flora of the mouth.[1]

The caries progression scores obtained for the human breast milk and plain packaged bovine milk groups showed that all of the lesions had not yet cavitated and hence were reversible. In a clinical scenario, the presence of saliva would have a synergistic effect on the cariostatic property of milk. The roles that saliva plays in protecting against caries include: diluting and eliminating sugars and other substances, buffering, balancing demineralization/ remineralization, and its antimicrobial action.[24]

It has been suggested that non-cavitated lesions can be reversed/arrested by preventive/non-operative treatment, irrespective of whether they are in the enamel only or in the enamel and dentin. Hence, in an *in vivo* situation, in the presence of saliva and preventive procedures, milk may have no cariogenic effects.

In the present study, it was evident that the imbalance between demineralization and remineralization determines the severity of caries. In a mild to moderate bacterial acid attack, the presence of buffers in milk plays an important role and limits the extent of caries to only the outer layers of enamel in the form of a non-cavitated lesion. This non-cavitated lesion can eventually be mineralized by the fluoride or calcium and phosphate ions present in saliva. However, when the acid attack surpasses the neutralizing capability of milk, cavitation occurs. The presence of cavitated lesions associated with sweetened milk signifies the utilization of fermentable carbohydrates in addition to milk sugar by the bacteria to produce caries.

The results of the present study are intended to serve as a guide; whether the cariogenic potential of any substrate depends upon the manner and pattern of use. Ideally, an infant should not be allowed to sleep with a bottle of sweetened milk or even breastfed at will. In the event that this occurs, the data presented here aids in forecasting the dire consequences. In any case, it is evident that the addition of sucrose to milk is likely to induce high levels of dental diseases.

CONCLUSIONS

The following conclusions were drawn from the present study:

1. Bovine milk (sweetened and plain) supported greater bacterial growth and fermentation than human breast milk.
2. Bovine milk (sweetened and plain) showed greater buffer capacity than human breast milk.
3. In an *in situ* model, in the absence of saliva, human breast milk and plain bovine milk were also found to be relatively cariogenic. However, it was evident that the addition of an external source of carbohydrate to milk increased its cariogenic potential and the extent of caries progression into dentine.

In the present study, though human breast milk and plain bovine milk were found to be only relatively cariogenic, the experiments were conducted in the absence of saliva. In an in vivo situation, the beneficial remineralizing effects of saliva cannot be ignored. Hence, it can be concluded that milk is unlikely to be cariogenic when supplemented with "liquid enamel" in the form of saliva. However, further investigations are recommended with a larger sample size and an in vitro caries model utilizing saliva to more accurately determine the cariogenic potential of human breast milk.

This study was a modest attempt to investigate the acidogenic and cariogenic properties of milk. The results of our study demonstrate the need to educate parents about the harmful effects of adding an external carbohydrate source to all forms of milk. It is prudent that parents weigh the benefits against the risk before sweetening milk just to make it more palatable to children.

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