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Comparative Evaluation of Efficacy of Three Different Storage Media in Maintaining the Viability of Periodontal Ligament Cells: An In Vitro Study

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ABSTRACT

Introduction: An ideal storage medium should be one that is capable of preserving the viability, mitogenicity and clonogenic capacity of the damaged Periodontal ligament cells to facilitate proliferation of these cells over the denuded root surface, thereby preventing further root resorption.

Aim: The aim of this study is to evaluate the effectiveness of Casein phosphopeptide amorphous calcium phosphate as a storage media for avulsed tooth in maintaining periodontal ligament cell viability in comparison with Hank's Balanced Salt Solution and Oral Rehydration Solution.

Materials and Methods: Forty freshly extracted human premolar teeth with normal periodontium and closed apices were taken. Forty teeth were randomly assigned into five experimental groups. It was then incubated for 30 minutes in falcon tubes with 2.5 ml solution of 0.2 mg/ml of collagenase II and 2.4 mg/ml solution of dispase grade II in phosphate buffered saline. After incubation, 50 µl of fetal bovine serum was added to each tube with the help of micropipette. Cells were labelled with 0.4% trypan blue for determination of viability. The number of viable cells in a grid of Neubauer's chamber were counted under a light microscope at 40X magnification.

Results: Results were analysed using Kruskul-Wallis test and Mann-Whitney U test.

Conclusion: GC Tooth Mousse, Hanks balanced salt solution and Oral rehydration solution can be used as storage medium. GC Tooth Mousse is better than Hanks balanced salt solution and Oral rehydration solution as a storage medium.

Keywords: Casein phosphopeptide amorphous calcium phosphate, Hank's balanced salt solution, Oral rehydration solution, Storage medium.

INTRODUCTION

Dental trauma is the most common injury which occurs in oro-facial region. Traumatic dental injuries are often seen among injuries to the face. Among them, tooth avulsion (0.5%-16%) is a complex traumatic injury characterized by the rupture of the neurovascular bundle and periodontal ligament (PDL) exposing the tooth to the outer environment. It occurs most often in the age group of 7-10 years, when the alveolar bone is resilient and offers minimal resistance to extrusive forces.[1]

Avulsion is a potential threat to the vitality of Periodontal ligament cells which are essential for the healing of replanted avulsed teeth. Hence management protocols should include management of the pulp and the periodontal ligament cells in the long-term survival and prognosis of avulsed teeth.[2] The types of healing that takes place after the avulsion injury are as follows: 1.Favorable healing: a. Healing with a normal periodontal ligament (without root resorption) b. Healing with surface resorption (repair-related resorption) 2.Unfavorable healing: a. Healing with ankylosis (replacement). b. Healing with inflammatory resorption (infection related resorption).[3] Two of the most critical factors affecting the prognosis of an avulsed tooth after replantation are extra oral dry time and the storage medium in which the tooth is placed.[4]

As replantation of avulsed teeth occurs more frequently between 1 and 4 hours after avulsion, degeneration of cemental periodontal ligament fibers is a common event and the presence of necrotic Periodontal ligament remnants on root surface stimulates the occurrence of inflammatory root resorption, which is the major cause of loss of replanted teeth.[5]

Secondly, storage or transport medium to support cell viability is more important than the extra oral

time to prevent ankylosis and replacement resorption.[4] A storage medium may be defined as a physiological solution that closely replicates the oral environment to help preserve the viability of Periodontal ligament cells following avulsion.[6]

An ideal storage medium should be one that is capable of preserving the viability, mitogenicity and clonogenic capacity of the damaged Periodontal ligament cells to facilitate proliferation of these cells over the denuded root surface, thereby preventing further root resorption. The storage medium should have a physiological osmolality, pH and temperature to allow for optimal cell growth or survival. The most important factor is that it should be readily available or be easily accessible for use in emergency situations.[7]

Most of the studies revealed HBSS as the most suitable transport medium since it helps in reducing replacement resorption by maintenance of a normal Periodontal ligament. Unfortunately HBSS is not widely used because it is not readily available to the public. Eagle's medium and Viaspan® have the capacity to maintain cell viability for a longer period, but their use is limited because they are expensive products and are not readily available in many locations (e.g. schools) where tooth avulsions are likely to occur.[8,9]

Milk is better than saliva, tap water or dry storage, but it is not so efficient as Hank's balanced salt solution as a storage medium for avulsed teeth for the maintenance of vitality and proliferative capacities of periodontal ligament fibroblasts, because milk does not re-establish and/or rebuild the vitality of damaged periodontal ligament cells. Egg white is a promising storage medium for avulsed teeth due to high nutrient value as well as availability at the trauma site. Though, none of these storage media could be adopted as ideal because of their mixed efficacy and other limitations, the search for a more suitable transport medium is still on.[9,10]

Despite their excellent properties, all storage media cannot be considered as practical for use due to limited availability. Hence readily available sources can be considered as alternatives. The present study is therefore undertaken to assess the effectiveness of casein phosphopeptide amorphous calcium phosphate as a storage media for avulsed tooth in maintaining periodontal ligament cell viability in comparison with Hank's balanced salt solution and oral rehydration solution.

MATERIALS AND METHODS

Inclusion criteria:
The permanent teeth either maxillary or mandibular first or second premolar teeth that were freshly extracted for orthodontic purposes were collected.

Exclusion criteria:
Teeth with moderate to severe periodontal disease, Teeth with fractured crown or root, Teeth with dental caries.

Armamentarium

Materials:
- Collagenase II
- Dispase II
- Phosphate buffer solution
- Fetal bovine serum
- Casein phosphopeptide amphorphous calcium phosphate
- Hank's balanced salt solution
- Oral rehydration solution
- Trypan blue
- 15 ml Falcon tubes
- Micropipette
- Neubauer's chamber
- Light microscope with hemocytometer
- Centrifugation machine

Method:
The study protocol was approved by Institutional Ethical Committee. Forty freshly extracted human premolar teeth with normal periodontium and closed apices were taken. Coronal 3mm of periodontal ligament was scraped with the curette to remove the cells that have been damaged. Forty teeth were randomly assigned into five experimental groups. The teeth in experimental groups were dried for 30 minutes, followed by 45 minutes immersion in one of the following experimental storage media like:

Group 1- Casein Phosphopeptide amorphous calcium phosphate- 10 freshly extracted teeth were stored in Casein Phosphopeptide

amorphous calcium phosphate.
Group 2- Hank's balanced salt solution -10 freshly extracted teeth were stored in 10ml of Hank's balanced salt solution.
Group 3- Oral rehydration solution-10 freshly extracted teeth were stored in 10ml of oral rehydration solution.
Group 4- Positive control- 5 freshly extracted teeth were immediately treated with collagenase and dispase.
Group 5- Negative control- 5 freshly extracted teeth were bench dried for 8 hours with no follow up storage time and then placed in collagenase and dispase.

It was then incubated for 30 minutes in falcon tubes with 2.5 ml solution of 0.2mg/ml of collagenase II and 2.4 mg/ml solution of dispase grade II in phosphate buffered saline. After incubation, 50 µl of fetal bovine serum was added to each tube with the help of micropipette. Cells were labelled with 0.4% trypan blue for determination of viability. The number of viable cells in a grid of Neubauer's chamber were counted under a light microscope at 40X magnification.

Calculations
1. Viability % = Viable cells/ Total cells X 100

2. Cells per ml = Average count per square X Dilution factor X 10^4 (count 10 squares)

3. Total cell number = Cells per ml X Original volume of fluid from which cell sample was removed

Statistical Analysis
Results were analysed using Kruskal-Wallis test and Mann-Whitney U test.

RESULTS
The mean values for the number of viable cells obtained following 45 minutes of storage in casein phosphopeptide amorphous calcium phosphate were 792, Hank's balanced salt solution were 150, Oral rehydration solution were 82.

While comparing Hank's balanced salt solution with positive control group in terms of viability, the difference was found to be statistically highly significant (P=0.002).

While comparing Oral rehydration solution with positive control group there is no statistically significant difference in viability (P=0.111).

While comparing Casein phosphopeptide amorophous calcium phosphate with positive control group there is statistically high significant difference in viability (P=0.002).

While comparing Hank's balanced salt solution with positive control group in terms of number of live cells, the difference was found to be statistically highly significant (P=0.003).

While comparing Oral rehydration solution with positive control group in terms of number of live cells, the difference was found to be statistically highly significant (P=0.002).

While comparing Casein phosphopeptide amorophous calcium phosphate with positive control group in terms of number of live cells, the difference was found to be statistically highly significant (P=0.002).

While comparing Hank's balanced salt solution with positive control group in terms of number of dead cells, the difference was found to be statistically highly significant (P=0.002).

While comparing Oral rehydration solution with positive control group in terms of dead cells, the difference was found to be statistically highly significant (P=0.002).

While comparing Casein phosphopeptide amorophous calcium phosphate with positive control group in terms of dead cells, the difference was found to be statistically highly significant (P=0.002).

DISCUSSION
A storage medium may be defined as a physiological solution that closely replicates the oral environment to help preserve the viability of PDL cells following avulsion.[2] The ideal storage medium should be capable of preserving the feasibility of cellular periodontal ligament, so that the cells could go through mitosis and form clones of the damaged fibroblast of the periodontal ligament and its generating cells and it should be

readily available or easily accessible at the site of accident.[7]

Epidemiological studies reveal that the prevalence of dental avulsion is three times more in boys than girls, most probably accredited to their active participation in hostile games and sports of a more aggressive nature. It occurs most commonly in the permanent dentition of 8 to 12-year-old children, according to Andreasen et al. (1995) it is the loosely structured periodontal ligament surrounding the erupting tooth, which often exhibits short, incompletely formed roots and favours the avulsion of these teeth, along with this an added confounding factor being the pronounced elasticity of alveolar bone in these children when compared to adults.[11]

Though the complete loss of a permanent tooth can be managed by various treatment modalities as the majority of these injuries usually takes place during the active stages of growth and development, the prosthetic rehabilitation has been severely questioned and or been contradicted, thus immediate re-implantation is the only answer. This not only prevents the negative psychological influences to the child as well as to the parent, but also prevents incurrence of a heavy economic burden on the parents owing to complex prosthetic rehabilitation with suboptimal treatment results in this age group.[12]

Whenever tooth avulsion occurs, immediate replantation at the trauma site is the ideal procedure for maintaining the viability of periodontal ligament cells. In contrast, excessive drying results in loss of vitality of periodontal ligament cells, which elicit a severe inflammatory response on the diffuse area over the root surface, upon re-implantation, which is meant for repair by new tissue. When extraoral dry time is more the cementoblasts move slowly and osteoblasts dominate the migration of cementoblasts leading to direct contact of osseous tissue to the root surface this, in turn, causes physiological bone re-contouring thus the entire root will be slowly replaced by bone and this process is termed as 'Osseous Replacement' or 'Replacement resorption'.[13]

To avoid above mentioned complications, it is advisable to re-implant the avulsed tooth immediately or it should be stored under moist condition in a suitable storage medium until re-implantation.

HBSS is essentially a pH-balanced salt solution contains ingredients, such as glucose, calcium, and magnesium ions, which can sustain and reconstitute the depleted cellular components of the PDL cells. It can preserve cells and tissues for 24 hours and both the pH (7.8) and the osmolality (270mOsmol Kg-1) are ideal.[9]

In the present study while comparing the HBSS with the positive control group it was seen that the difference was found to be highly significant (P=0.002).

While comparing the HBSS with the positive control the number of live cells were found highly significant (P=0.003)

HBSS was the most effective medium for preserving viability, mitogenicity, and clonogenic capacities of PDL cells for up to 24 hours at 4°C when compared with other solutions.[14]

The number of viable periodontal ligament (PDL) cells were compared between different storage media (HBSS, milk, saline and water) using a collagenase assay. The number of viable and non-viable PDL cells were counted with a haemocytometer and analyzed. They observed approximately 90% cell viability with HBSS and no statistically significant differences in the viability of PDL cells among saline, HBSS and milk.[15]

In the present study while comparing the oral rehydration solution with the positive control group no statistically significant difference (P=0.111) was seen in the viability of the cells.

The number of viable periodontal ligament cells in oral rehydration solution is significantly less than the HBSS although the mean osmolality (270 mOsmol kg-1) and mean pH (7.8) was similar in this study.

A study done by Subramaniam et al showed a similar results with HBSS, and found that significant difference in the mean number of viable cells was observed, following an extra oral dry time of 60 min. ORS-L was comparable to that of HBSS with regard to cell viability.[16]

While comparing the Oral rehydration solution and CPP-ACP group with the positive control the number of live cells was found highly significant (P=0.002).

The effectiveness of Oral rehydration solution at various concentrations as a storage media for avulsed teeth was compared, the viability of the cells was determined by MTT assay and found that although different concentrations of ORS have the possibility of preserving PDL cell viability, the 25% and 50% concentrations are more effective and could on average preserve 79.98% and 68.34% of the PDL cells respectively.[17]

Casein phosphopeptides (CPP) are derived from casein, which account for 80% of the total protein in bovine milk. Furthermore, they have been shown to exert cytomodulatory effects. The pH of CPP-ACP was found to be 7.37, which is similar to the pH of the oral cavity thereby promoting a favorable environment to maintain the vitality of the cells.[17]

In the present study a highly statistically significant difference was found between the CPPACP group and the positive control group (P=0.002) which is in accordance with the study done by Hegde K et al. who evaluated GC Tooth Mousse Plus: A potential storage media for avulsed teeth and concluded that although milk is a better storage media as compared to saline and GC Tooth Mousse Plus. GC Tooth Mousse Plus can be considered as storage media to prevent desiccation of PDL cells up to the duration of 60 minutes.[18]

In the present study, the mean viability percentage by CPP-ACP was (99.17%), HBSS (95.52%), ORS (88.21%), and there was a statistically significant difference (p<0.001) in viability percentage between the three groups. The probable reason for this may be the pH of CPP-ACP was found to be 7.37, which is similar to the pH of the oral cavity thereby promoting a favorable environment to maintain the vitality of the cells.

In the present study to minimize the exposure of cells to active trypsin and to preserve maximum cell viability, the root surface was treated with collagenase and dispase grade II as was performed in the work by Pileggi et al.[19] This procedure allowed rapid cell retrieval and maintained maximum cellular integrity, as was demonstrated by the positive control samples. Collagenase and dispase assay provides a combination of collagenolytic and proteolytic enzymes required for tissue disaggregation.[4,20]

Also, this study used fetal bovine serum as a growth supplement for cell culture media because of its high content of embryonic growth promoting factors. When used at appropriate concentrations, it supplies many defined and undefined components that have been shown to satisfy specific metabolic requirements for the culture of cells in vitro.[21]

In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. In the protocol presented here, a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm.[22-23]

The results of the present study indicated that CPP-ACP group demonstrated a significantly higher number of viable PDL cells than HBSS, and Oral rehydration solution. There was a significant difference between all the groups which is in accordance with the study done by Hegde K et al.[18]

However, more long term studies and further research are required with a larger sample size to validate the benefits of CPP-ACP as an appropriate storage media.

CONCLUSION

GC Tooth Mousse , Hanks balanced salt solution and Oral rehydration solution can be used as storage medium. GC Tooth Mousse is better than Hanks balanced salt solution and Oral rehydration solution as a storage medium. However, further research is required with a large sample size to validate the benefits of CPP-ACP as an appropriate storage media.

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LEGENDS

Comparisons	Z	p
Positive control vs Hanks balanced salt solution	3.062	0.002 hs
Negative control vs Hanks balanced salt solution	3.062	0.002 hs
Positive control vs Casein phosphopeptide amorphous calcium phosphate[a]	3.062	0.002 hs
Negative control vs Casein phosphopeptide amorphous calcium phosphate[a]	3.062	0.002 hs
Positive control vs Oral rehydration solution	1.592	0.111 ns
Negative control vs Oral rehydration solution	3.062	0.002 hs

Table 1. Comparison of viability of different storage media

	N	Mean	Std. Deviation	Minimum	Maximum
Hanks balanced salt solution	10	95.5250	95.5250	2.69465	100.00
Casein Phosphopeptide Amorphous	10	99.1720	99.1720	0.27067	99.51
Oral Rehydration Solution	10	88.2150	88.2150	2.56946	92.63
Positive control	5	90.3000	90.3000	1.20233	91.32
Negative control	5	24.7080	24.7080	9.51289	40.44

Table 2. Means and standard deviations of different storage media
a. H=34.297 $p<0.001$ very highly significant (vhs)

Comparisons	Z	p
Positive control vs Hanks balanced salt solution	2.092	0.003 hs
Negative control vs Hanks balanced salt solution	0.736	0.462 ns
Positive control vs Casein phosphopeptide amorphous calcium phosphate[a]	3.062	0.002 hs
Negative control vs Casein phosphopeptide amorphous calcium phosphate[a]	3.065	0.002 hs
Positive control vs Oral rehydration solution	3.07	0.002 hs
Negative control vs Oral rehydration solution	3.073	0.002 hs

Table 3. Comparison of number of live cells between storage media

	N	Mean	Std. Deviation	Minimum	Maximum
Hanks balanced salt solution	10	150.4000	48.02361	80.00	234.00
Casein phosphopeptide amorphous	10	792.5000	39.45814	707.00	834.00
Oral Rehydration Solution	10	82.3000	2.79086	78.00	88.00
Positive control	5	244.6000	15.91540	225.00	264.00
Negative control	5	160.8000	19.33132	136.00	185.00

Table 4. Means and standard deviations of live cells in different storage media

a. H= 34.311 p<0.001 very highly significant (vhs)

Comparisons	Z	P
Positive control vs Hanks balanced salt solution	3.067	0.002 hs
Negative control vs Hanks balanced salt solution	3.067	0.002 hs
Positive control vs Casein phosphopeptide amorphous calcium phosphate[a]	3.078	0.002 hs
Negative control vs Casein phosphopeptide amorphous calcium phosphate[a]	3.078	0.002 hs
Positive control vs Oral rehydration solution	3.07	0.002 hs
Negative control vs Oral rehydration solution	3.07	0.002 hs

Table 5. Comparison of dead cells between different storage media

	N	Mean	Std. Deviation	Minimum	Maximum
Hanks balanced salt solution	10	6.3000	3.267669	.00	11.00
Casein phosphopeptide amorphous	10	6.3000	1.88856	4.00	10.00
Oral Rehydration Solution	10	11.0000	2.44949	7.00	15.00
Positive control	5	26.2000	2.86356	23.00	30.00
Negative control	5	38.6000	10.78427	26.00	55.00

Table 6. Means and standard deviations of dead cells between different storage media a. H=29.973 p<0.001 very highly significant (vhs)

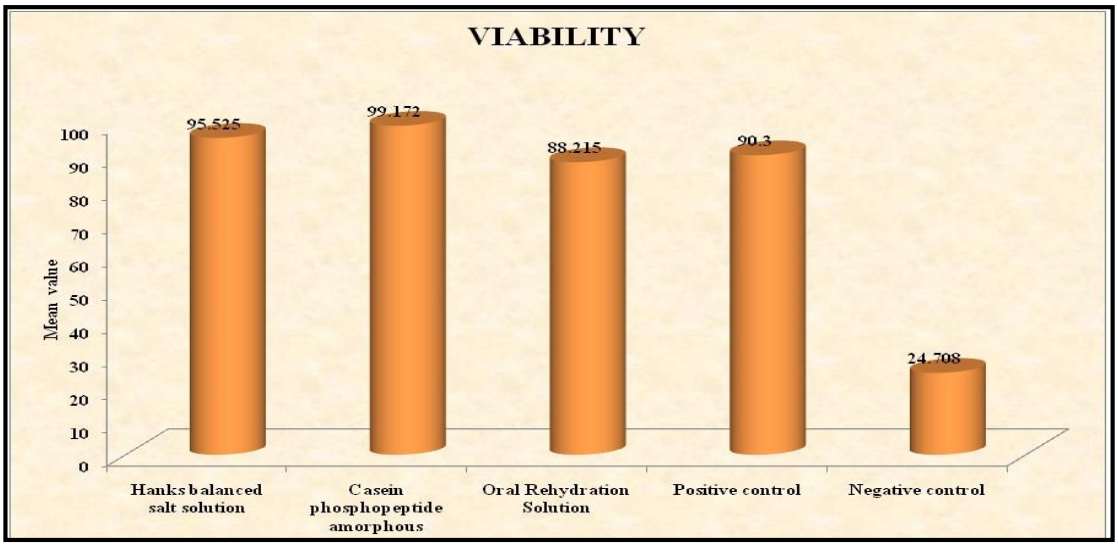

Figure 1. Comparison of viability of different storage media

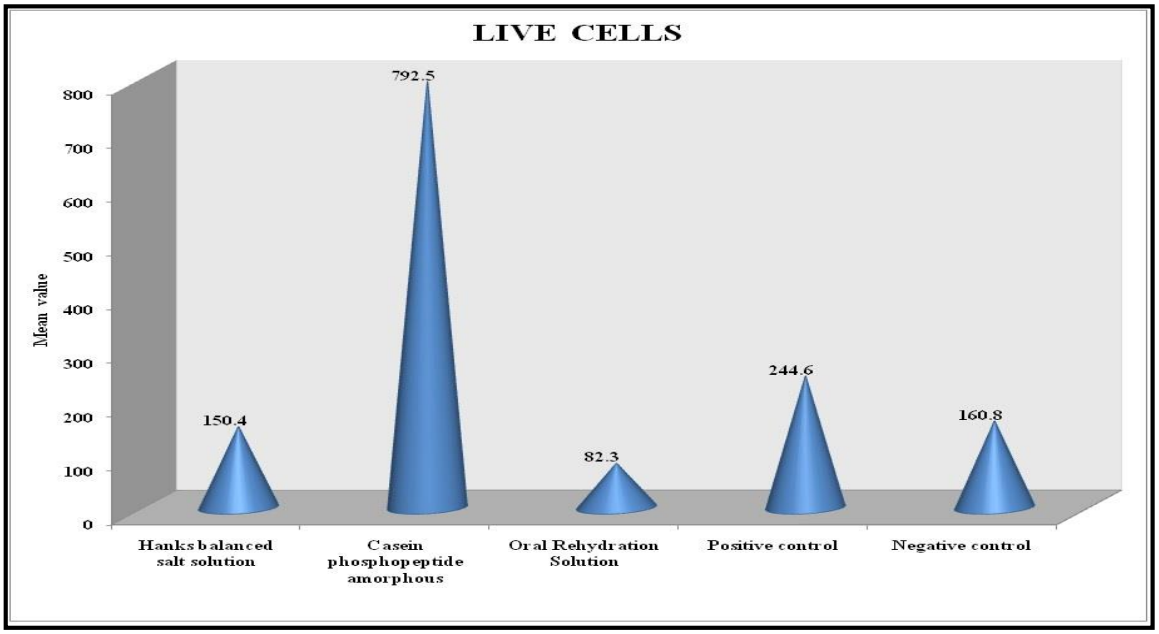

Figure 2. Comparison of viability of live cells between different storage media

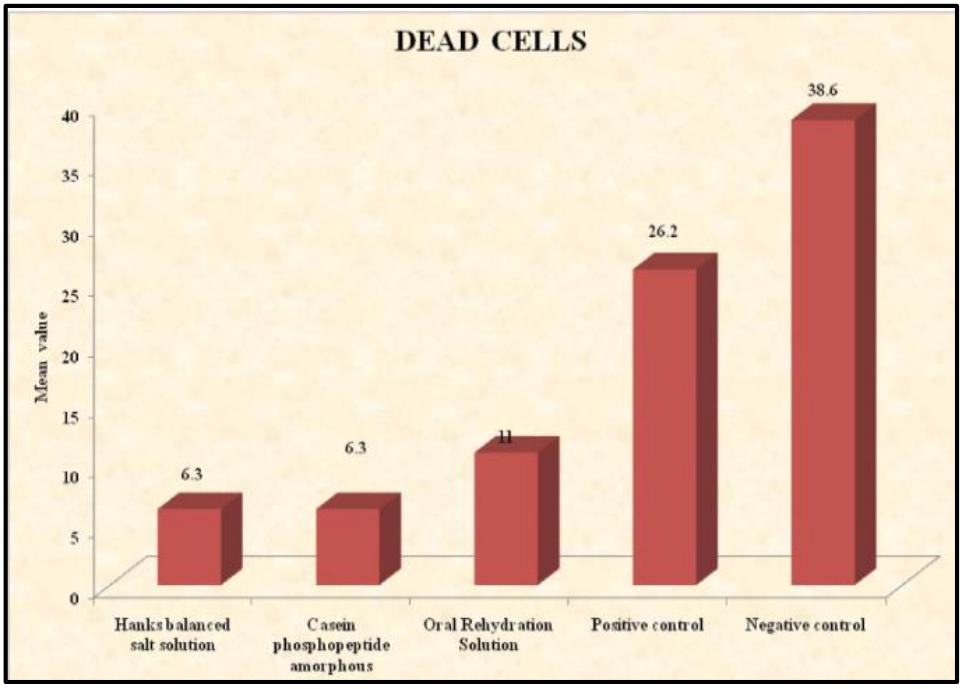

Figure 3. Comparison of dead cells between different storage media