4.1 Introduction

Dental caries are characterized by dissolution of tooth enamel and are cause of public health concern [Nakano et al. 2007; Falsetta et al. 2014]. Major factor influencing the dental decay is an assault of tooth surface by oral microbial biofilms [Selwitz et al. 2007; Nance et al. 2013]. \textit{S. mutans} is considered to be one of the main etiological agents of dental caries and is best known biofilm forming oral bacterium [Loesche 1989; Hasan et al. 2015]. Acid production by fermentation of dietary carbohydrate (acidogenesis), formation of exopolysaccharide, biofilm formation along with its ability to survive in an acidic environment (aciduracity) are some of the prominent characteristics which help \textit{Streptococcus mutans} in their cariogenic process [Koo et al. 2003; Krol et al. 2014]. Eradication of dental biofilm is very difficult and only mechanical cleaning like brushing or flossing the teeth is not sufficient. Thus, to improve the oral health it is important to formulate approaches that can inhibit or delay the biofilm formation.

Fluorides and its various preparations are of great importance in dentistry [Marquis et al. 2003]. In its ionic form fluoride prevents the demineralization and helps in remineralization of tooth enamel [Featherstone 1999]. Fluoride also exerts effects on biological activity of caries causing bacteria. They reduce the ability of plaque forming bacteria to produce acid and can impair glycolysis by inhibition of enolase activity [Hamilton 1977]. Furthermore, they work on membrane associated proton pump (H+\text{-ATPase}) by inhibiting it and in turn reducing the cellular level of ATP [Sutton et al. 1987; Eshed et al. 2013].

It is believed that topical application of fluoride on tooth surface leads to the formation of calcium fluoride like material which act as the reservoir of fluoride ions and during caries challenge it releases fluoride at low pH in plaque and protects the tooth's surface from caries [Rošin-Grgetić & Lincir 2001; Rølla & Saxegaard 1990]. Nevertheless, the limited concentration of calcium ion in mouth results in the formation of only limited amount of calcium fluoride like deposits after topical application of conventional fluoride formulations [Saxegaard & Rolla 1989].

Nanoscale based approaches are being widely used and have been proven to be more effective in the elimination of biofilms and in inhibition of dental caries [Eshed et al. 2013; Kulshrestha \textit{et al.} 2014; Hernández-Sierra \textit{et al.} 2008]. Their high surface to
volume ratio provides them with unique properties which can be exploited for the
development of new therapies and drugs [Raghupati et al. 2011]. Sun and Chow have
demonstrated that calcium fluoride nanoparticle (CaF$_2$-NPs) rinse can increase the level
of fluoride ions in the oral fluid and in another study the strength and the fluoride release
capacity of dental composite having CaF$_2$-NPs have been shown [Sun and Chow 2008;
Xu et al. 2008]. However, there are no studies focusing on the direct effect of CaF$_2$-NPs
on caries causing virulence factors like exopolysaccharide production, biofilm formation,
aciduracity and acidogenesis of $S$. mutans as well as its effect on demineralization of
dental enamel.

The main objective of this study was to formulate calcium fluoride nanoparticles (CaF$_2$-
NPs) and to evaluate its effect on some of the major virulence factors of $S$. mutans.
Furthermore, we have investigated CaF$_2$-NPs effect on caries development in in vivo
model to evaluate its use as a topical applicant for prevention of dental caries.

3.2 Experimental Overview

Calcium fluoride nanoparticles (CaF$_2$-NPs) were synthesized using methodology
described in section 2.2.3.3. Characterization of nanoparticle was performed by UV-
visible spectroscopy (2.2.4.1), TEM (2.2.4.2), SEM (2.2.4.3), XRD (2.2.4.6) and FTIR
(2.2.4.4). The minimum inhibitory concentration and minimum bactericidal concentration
of CaF$_2$-NPs were evaluated method outlined in section 2.2.5. The effect of sub inhibitory
concentrations of CaF$_2$-NPs on virulence traits of $S$. mutans viz. adherence, biofilm
formation, exopolysaccharide production, water soluble and insoluble glucans and
glycolytic pH drop was investigated by methodology described in sections 2.2.5, 2.2.7,
2.2.8.1, 2.2.12 and 2.2.14 respectively. The effect of sub inhibitory concentrations of
CaF$_2$-NPs on the dispersion of preformed biofilm was assessed using method given in
section 2.2.9. The growth pattern of $S$. mutans in presence of sub inhibitory
concentrations of CaF$_2$-NPs was evaluated by method provided in section 2.2.11. TEM
and CLSM analysis were performed to investigate the effect of CaF$_2$-NPs on the cells and
biofilm architecture of $S$. mutans respectively, methodology is described in section 2.2.17.
Moreover, the effect of CaF$_2$-NPs on expression of gene involved in $S$. mutans virulence
pathway was also studied by quantitative RT-PCR as outline in section 2.2.18. The oral
toxicity of these nanoparticles and their anti-cariogenic effect in vivo was evaluated by
method given in section 2.2.20. Cytotoxicity assay was also performed on HEK-293 cell line (section 2.2.21).

4.3 Results

4.3.1 Characterization of calcium fluoride nanoparticles

TEM analysis of CaF$_2$-NPs was performed to determine its morphology and size (Figure 4.1a). The particles were found to be in the nanometre range with average particle size of 15-25 nm (Figure 4.1b). SEM image (Figure 4.1d) of nanoparticle revealed the morphology of synthesized nanoparticle. UV-visible spectroscopy of the nanoparticle is represented in Figure 4.1c. FTIR spectrum of CaF$_2$-NPs showed a strong band at ~3400 cm$^{-1}$, 1678 cm$^{-1}$, 430 cm$^{-1}$ (Figure 4.1e). Furthermore, Figure 4.1f is a typical XRD pattern of CaF$_2$-NPs.

![Figure 4.1 Characterization of CaF$_2$-NPs: (a) Transmission electron microscopy image of CaF$_2$-NPs, (b) Particle size ~ 15-25 nm, (c) UV visible spectrum of CaF$_2$-NPs, (d) Scanning electron microscopy image, (e) FTIR spectrum, and (e) XRD pattern of CaF$_2$-NPs.](image-url)
4.3.2 Effect on bacterial viability

The minimum inhibitory concentration of CaF$_2$-NPs on *S. mutans* was found to be > 64 mg/ml.

4.3.3 Significant reduction in biofilm

Biofilm formation ability of *S. mutans* in presence of different concentration (4, 2, 1 mg ml$^{-1}$) of CaF$_2$-NPs was evaluated using crystal violet assay (Figure 4.2a). There was almost 89%, 71% and 62% reduction in biofilm forming ability of *S. mutans* as compared to control when treated with 4 mg ml$^{-1}$, 2mg ml$^{-1}$ and 1mg ml$^{-1}$ concentration of nanoparticles respectively. With the decrease in nanoparticles concentrations there is gradual increase in its biofilm formation ability. This suggests a concentration dependent reduction in biofilm formation.

4.3.4 Effect on EPS production

A considerable decrease in EPS production in *S. mutans* in the presence of CaF$_2$-NPs was observed and the reduction is in a concentration dependent manner (Figure 4.2b). A 90%, 65% and 64% decrease in EPS production by *S. mutans* was observed when treated with 4 mg ml$^{-1}$, 2mg ml$^{-1}$ and 1mg ml$^{-1}$ of CaF$_2$-NPs respectively, as compared to untreated sample. The highest EPS production was seen in control when no nanoparticles were present. When nanoparticle concentration increased, the EPS production decreased.

![Inhibitory effect of sub-MIC concentration of CaF$_2$-NPs](image)

**Figure 4.2** Inhibitory effect of sub-MIC concentration of CaF$_2$-NPs (a) Biofilm formation (b) EPS production. Data are mean ± S.D. (n=3), statistical significance as compared with the untreated control (p< 0.05) denoted by an asterisk (*).
4.3.5 *Growth curve pattern*

Growth curve was used to investigate the effect of CaF$_2$-NPs on *S. mutans* growth. The results displayed a typical sigmoidal pattern and there was no significant variation between control and treated samples (Figure 4.3). The results clearly indicated that bacterial growth is not hindered at concentration of nanoparticle used in the study.

![Growth curve](image)

**Figure 4.3** Growth curves of CaF$_2$-NPs treated and untreated *S. mutans* (O.D values, 1:10 times diluted). The data represent mean ± S.D.

4.3.6 *Effect on adherence*

The inhibitory effect of different concentration of CaF$_2$-NPs on initial adherence of *S. mutans* is shown in Figure 4.4 (a). There was 70%, 57% and 44% inhibition of attachment of *S. mutans* to the glass surface in presence of 4mg ml$^{-1}$, 2mg ml$^{-1}$ and 1 mg ml$^{-1}$ concentrations of CaF$_2$-NPs respectively.

4.3.7 *Dispersion of preformed biofilm*

Different concentrations of CaF$_2$-NPs were used to evaluate their effect on treatment of preformed biofilm of *S. mutans* (Figure 4.4b). There was 11% , 7% and 5% reduction of preformed biofilm on treatment with 4mg ml$^{-1}$, 2mg ml$^{-1}$ and 1 mg ml$^{-1}$ concentrations of CaF$_2$-NPs respectively.
4.3.8 Reduction in glucan production

A significant reduction in both insoluble glucan and soluble glucan production was observed in *S. mutans* when treated with CaF$_2$-NPs (Figure 4.5). Almost 90% reduction was observed in treated sample as compared to control. Soluble and insoluble glucans both were reduced to the same extent in the present experiment.
4.3.9 Decrease in rate of acid production and stress tolerance

As shown in Figure 4.6, the acid tolerance ability of S. mutans was inhibited appreciably in the presence of CaF$_2$-NPs. The onset pH of 7.2 dropped to 4.5 in control while in treated samples (4 mg ml$^{-1}$) final pH was 5.2. Furthermore, the rate of initial pH drop in 10 min was calculated to be 0.14 min$^{-1}$ to 0.09 min$^{-1}$ in case of control and treated samples respectively, demonstrating pronounced reduction in the acid production ability of S. mutans.

![Figure 4.6](image.png)

**Figure 4.6** Effect on sub-MIC levels of CaF$_2$-NPs on glycolytic pH-drop (the values enclosed in box corresponds to the initial rate of pH drop). Data are mean ± S.D. (n=3), statistical significance as compared with the untreated control (p< 0.05) denoted by an asterisk (*).

4.3.10 Expression profile of virulence gene

Quantitative RT-PCR was performed to gain insight into the effect of CaF$_2$-NPs treatment on the expression of virulence genes (gtfC, vicR, ftf, comDE, spaP) in S. mutans. An entire set of genes was down regulated after treatment with nanoparticles (Figure 4.7). The expression spaP decreased by 80% and that of vicR and comDE genes by > 50%. The decrease in expression of gtfC gene was 32% and a suppression of 14% was observed in the expression of ftf gene.
Figure 4.7 Expression profile of various genes of *S. mutans* in response to treatment of sub-MIC concentration of CaF$_2$-NPs. The data presented were generated from at least three independent sets of experiments (Data is mean ± Standard deviation) Statistical significance as compared with the untreated control (p < 0.05) denoted by an asterisk (*).

**4.3.11 Impairment of biofilm architecture visualized through confocal microscopy**

Confocal laser scanning microscopy (CLSM) images of *S. mutans* illustrate an apparent obliteration of biofilm architecture in the presence of CaF$_2$-NPs without affecting its growth (Figure 4.8). The upper panel shows the control sample images while lower three panel represent images of biofilms when treated with various concentrations of CaF$_2$-NPs. In control images (Figure 4.8a-d) majority of cells shows green fluorescence with a mat of *S. mutans* cells showing rich biofilm architecture while in treated samples (Figure 4.8e-h) cells were highly dispersed and alive, which depicted inhibition of biofilm formation and not the viability.
**Figure 4.8** Effect of CaF$_2$-NPs on biofilm architecture: Confocal laser scanning micrographs of control biofilm (a, b, c, d), micrographs of treated biofilm 4mg/ml (e, f, g, h), 2mg/ml (i, j, k, l), 1 mg/ml (m, n, o, p).

### 4.3.12 Insignificant effect on cell wall

TEM analysis was performed to visualize the effect of nanoparticle on the cell wall of *S. mutans* (Figure 4.9). In control, the cell wall is intact with the healthy intracellular content of bacterium (Figure 4.9a) on the contrary in image of treated samples (Figure 4.9b) little damage was observed (indicated by red arrows) but the damage was not prominent and majority of cells were having intact membrane. The results suggest that there was insignificant damage to cell walls in the presence of CaF$_2$-NPs.
Figure 4.9 Transmission electron microscopy images of *Streptococcus mutans*: (a) control (b) treated with sub-MIC concentration of CaF₂-NPs (Magnification of 4000X).

4.3.13 Cytotoxicity

Relative cell viability of HEK-293 cell line in presence of CaF₂-NPs is shown in Figure 4.10. Almost 100% viability was observed at all concentration (4 mg ml⁻¹, 2 mg ml⁻¹ and 1 mg ml⁻¹). Thus test concentration of CaF₂-NPs are nontoxic to HEK-293 cell line.

Figure 4.10 *In vitro* cytotoxicity assay (MTT) on HEK-293 cell line.
4.3.14 Oral toxicity profile

No mortality was observed after oral toxicity assay and animals did not exhibit any behavioural or weight changes. Thus, indicating that nanoparticles were absolutely non-toxic to the animals used in this study.

4.3.15 In vivo Caries reduction

The weekly recovery of *S. mutans* cells over 5 weeks post treatment is presented in Table 4.1. There was a substantial decrease in the recovery of *S. mutans* cells from rats treated with nanoparticles as compared to the untreated group (control). It was also found that there was significant reduction of caries score in rats treated with CaF$_2$-NPs as compared to the control. Figure 4.11 shows the reduction in smooth as well as sulcal surface caries after treatment. The overall reduction in smooth surface caries was comparable to sulcal surface caries post treatment. The severity of smooth surface caries was reduced to 52.6% (slight), 72.1% (moderate) and 70.7% (extensive) as compared to sulcal surface caries where they were reduced by 50.8% (slight), 65.6% (moderate) and 69.5% (extensive). However, the reduction in the extensive caries was found to be pronounced over slight and moderate caries.

**Table 4.1 Recovery of *S. mutans* on the following weeks after inoculation (×10$^4$ CFU)**

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>110.77± 5.06</td>
<td>127.56 ± 4.44</td>
</tr>
<tr>
<td>3</td>
<td>127.67 ± 2.12</td>
<td>110.83 ± 6.01</td>
</tr>
<tr>
<td>6</td>
<td>148.06 ± 6.73</td>
<td>95.77 ± 2.82</td>
</tr>
<tr>
<td>8</td>
<td>166.35 ± 7.04</td>
<td>88.89 ± 4.56</td>
</tr>
<tr>
<td>10</td>
<td>188.08 ± 7.02</td>
<td>74.08 ± 3.87</td>
</tr>
</tbody>
</table>
Figure 4.11 Effect of sub- MIC level of CaF$_2$-NPs on dental caries development in rats; Data represents mean± S.D. of Keyes’ score.

4.3.16 Scanning electron micrograph of untreated and treated rats teeth

The SEM analysis of the rats teeth clearly depicted the demineralization of the dental margins in untreated group Figure 4.12a while the groups treated with CaF$_2$-NPs showed smooth dental margins as clearly shown in Figure 4.12a'. Furthermore, the Figure 4.12b, c and Figure 4.12b', c' show the dental surface of untreated and treated tooth respectively. It was observed that in untreated samples the surface of the tooth has an evident biofilm embedded in the glucan pool, whereas in the treated groups the dental surface was found clear from any such exopolysaccharide projections as previously detectible in control.
**Figure 4.12** SEM analysis of rats’ teeth to evaluate the effect of CaF$_2$-NPs on caries development and extent of demineralization in treated (lower panel) and untreated groups (upper panel): (a) Untreated rat tooth showing caries (magnification 200X), (b, c) magnified view of marked region showing biofilm of *S. mutans* on untreated tooth (magnification 10X), (a’) CaF$_2$-NPs treated tooth (magnification 200X), (b’, c’) magnified view of marked region of a treated tooth (magnification 10X).

**4.4 Discussion**

*S. mutans* is the key organism of dental caries and its cariogenic potentials are well documented [Islam *et al.* 2008; Dmitriev *et al.* 2011]. Control of its virulence factors like exopolysaccharide production, biofilm formation, aciduracity and acidogenesis and enhancement of remineralization of tooth enamel are major approaches which can be used for combating dental caries. In the present study, we have reported CaF$_2$-NPs to be very effective in suppressing *S. mutans* biofilm and other virulence factor (exopolysaccharide formation, acidogenesis, aciduracity).

CaF$_2$-NPs were prepared using a co-precipitation method a type of liquid- phase methods. The main advantages of using liquid phase methods are simple methodology and high surface activity of produced nano-materials [Omolfajr 2011]. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) results revealed the shape and size of nanoparticles. The particles are not fully dispersed but are agglomerated. The larger particles in SEM image exhibit several spherical perturbances on their surface.
implying that these larger particles are formed by fusion of smaller particle during their preparation process [Pandurangappa & Lakshminarasappa 2011]. CaF₂-NPs show characteristic absorption peaks in UV range. The origin of these bands is due to the nanosize of particle. It has been suggested that large surface to volume ratio of nanoparticle results in the development of voids on the surface and inside the agglomerated nanoparticles. These voids lead to fundamental adsorption in UV range [Kumar et al. 2007]. Furthermore, it is well established that surfaces of nanoparticle comprise of numerous defects like Schottky or Frenkel resulting in absorption of light by nanocrystal [Zang et al. 2008]. Thus, absorption band at 202 nm in the present study confirms formation of CaF₂-NPs. FTIR spectrum was measured to analyze the structural properties and bonds of CaF₂-NPs. Two strong peaks at ~3400 cm⁻¹ and ~1678 cm⁻¹ in FTIR spectrum are due to H-O-H bending of water molecule. Band at ~ 430 cm⁻¹ arise due to hindered rotation of hydroxyl ion [Khan et al. 2013]. The XRD pattern of CaF₂-NPs matched well with the standard JCPDS card NO 87-0971 which reveals a cubic phase fluorite type structure [Fujihara et al. 2002]. The broad peaks in XRD pattern suggest small crystalline size [Pandurangappa & Lakshminarasappa 2011]. The crystalline size was calculated to be ~ 6.8 nm by using scherer`s formula. Moreover, from XRD spectra it can be estimated that the particles are in single phase and pure sample has been synthesised as there is no extra peaks found.

Formation of biofilm is the crucial virulence factor of S. mutans by virtue of which it causes dental caries [Hamada & Slade 1980; Nance et al. 2013]. Biofilm are adherent bacterial communities embedded in the hydrated matrix of exopolysaccharide and exhibiting a complex three dimensional structure [Costerton et al. 1999; Selwitz 2007]. The cells in biofilms behave differently in their functionalities as compared to their planktonic counterparts [Fux et al. 2003]. Biofilm architecture imparts bacteria with the ability to resist antibiotic and lead to persistent bacterial infection [Mah & O’Toole 2001]. CaF₂-NPs were found to substantially decrease the biofilm formation after 24 hours of incubation. EPS (Exopolysaccharide) is essential for the formation, maintenance and spread of biofilm and it is one of the key virulence factors of S. mutans [Flemming & Wingender 2010]. A considerable reduction in EPS production in the presence of CaF₂-NPs may be associated with the diminution of biofilm forming ability of S. mutans.
Moreover, the similarity in the growth curve of treated and control samples indicated that CaF₂-NPs reduced biofilm formation without affecting bacterial viability.

Attachment of *S. mutans* cells to the adhering surface is significant steps in the process of caries formation and its deterrence could be a prophylaxis against its virulence [Islam *et al.* 2008]. Adherence occurs mainly by virtue of the hydrophobic interactions between the cells and the adhering surface. The marked inhibition in adherence after short term exposure of sub inhibitory concentrations of CaF₂-NPs shows that nanoparticles are modifying the physical properties of cell surface which intern reducing the hydrophobic interactions between *S. mutans* and adhering surface. Moreover, very less reduction in preformed biofilm on treatment with CaF₂-NPs suggest that these nanoparticle are best suited in prophylactic treatment of dental caries. Glucan is the main exopolysaccharide produced by *S. mutans* and are integral components in the sucrose dependent colonization of *S. mutans* biofilm on the tooth surface. It is elicited from the data that there is a phenomenal reduction in glucan synthesis. Almost equal reduction was observed in both water soluble and water insoluble glucans. This indicates that CaF₂-NPs are acting on the GTFs and impairing their enzymatic activity, thus the reduction in EPS production was due to malfunctioning of GTFs.

Acid production and acid tolerance are cardinal virulence factors which attributes to the cariogenic ability of *S. mutans* [Kuramitsu 1993]. Pursuing these abilities *S. mutans* easily survives in stress condition and impose stress on other species of cariogenic plaque eventually evolves out as dominant species. Furthermore, the sustained pH values below pH 5.4 aids in the demineralization of enamel and development of dental caries [Banas 2004].

The rate of pH drop reflects acidogenic capacities of the cells, while final pH values of the suspensions represent acid tolerance [Gregoire *et al.* 2010]. In the present study the results show a significant drop in the final pH of the suspension in the presence of CaF₂-NPs suggesting deterioration in the acid tolerance capacity of *S. mutans*. Along with this the rate of pH drop was decreased in the presence of CaF₂-NPs as compared to control which implies the impairment of acid production capacity.

It is evident that CaF₂-NPs are acting against some of the major virulence factors of *S. mutans*. One of the reasons behind this anti biofilm property of CaF₂-NPs may be the
release of fluoride ions. Fluoride ions have been reported to act directly or in the form of metal complexes to inhibit many enzymes [Li 2003]. In \textit{S. mutans}, fluoride ions combine with H ions forming HF molecule, which can eventually inhibit the glycolytic enzymes like Enolases [Sutton \textit{et al.} 1987; Eshed \textit{et al.} 2013]. In addition, a fluoride ion hinders the proton extrusion by F-ATPases through lending a proton back into the cell [Li 2003; Svensäter \textit{et al.} 2000]. Thus, it is possible that suppression of the acid and glucan production ability in the presence of CaF$_2$-NPs is due to release of fluoride ions from the nanoparticles.

Gene expression profile of selected genes of \textit{S. mutans} revealed a considerable reduction in gene expression in the presence of CaF$_2$-NPs. \textit{spaP} (Ag I/II or P1) is a protein of the antigen I/II family is crucial in \textit{S. mutans} for initial adhesion to tooth surface [Khan \textit{et al.} 2010]. Down regulation of this gene in \textit{S. mutans} probably results in poor adhesion and reduced ability to form biofilm on smooth surfaces. Gene \textit{vic R} is a two component regulatory system and is known to regulate a set of gene encoding for important surface proteins which are critical for sucrose dependent adherence to a smooth tooth surface [Hasan \textit{et al.} 2012]. Thus suppression of these two genes may further lead to inhibition of adhesion and may be a cause of anticariogenic action. In addition, \textit{gtf C} and \textit{ftf} which encode GTFC and FTF enzyme that catalyse the cleavage of sucrose to synthesize extracellular glucan and fructan polysaccharides [He \textit{et al.} 2012], were also down-regulated. The reduction in aforesaid genes will thereby suppress the exopolysaccharide synthesis pathway eventually inhibiting the biofilm formation. Furthermore, \textit{com DE} which is a part of the quorum sensing cascade of \textit{S. mutans} was also suppressed considerably. It has been shown to regulate genetic competence, acid tolerance, and biofilm formation [Yung-Hua \textit{et al.} 2002]. Hence, down regulation of this gene will not only attenuate internal communication system, but also adversely affect the acid tolerance potential of \textit{S. mutans}. As the gene examined are only selected set of genes of \textit{S. mutans} genome, additional assessment of other virulence gene is further required to get a broader spectrum of effect of CaF$_2$-NPs on cariogenic potentials of \textit{S. mutans}.

The findings of the present study indicated that anti biofilm effect of CaF$_2$-NPs against \textit{S. mutans} is a combination of both the suppression in enzymatic activity associated with glucan synthesis and of gene involved in adhesion, acid production, acid tolerance and quorum sensing. Interaction of CaF$_2$-NPs with enzymes and suppression of genes are
interlinked to each other at different steps of regulatory network. This may lead to impairment of the whole metabolic network, eventually forbidding bacterial pathogenesis.

Confocal laser scanning microscopy results were in consistence with the above discussed results. A disruption of biofilm architecture was observed by CLSM in the presence of sub-MIC concentrations of CaF$_2$-NPs. In control sample a green mat is clearly visible which shows that the cells are interacting with each other and forming a healthy biofilm while in treated samples there were more live cells as compared to dead cells, but less biofilm was formed suggesting that at tested concentrations there was a tremendous decrease in biofilm formation ability of *S. mutans*. Moreover, TEM images of *S. mutans* are exhibiting insignificant destruction of peptidoglycan layer and there is no damage to the cells which validate that CaF$_2$-NPs is not affecting bacterial viability.

Further, the use of animal models to study the *S. mutans*-host interactions under controlled conditions demonstrated that the daily topical exposure CaF$_2$-NPs dramatically affected the ability of *S. mutans* to colonize the tooth surfaces, consequently inhibiting the development of smooth surface caries and sulcal surface carious lesions.

These nanoparticles were able to lodge themselves deep in the cavity and could release calcium fluoride in a sustained manner to mineralize the cavity. However, the depth of slight caries was not deep enough to lodge the nanoparticles within them. This is the probable reason for the reduction of extensive caries over the slight and moderate caries. The *in vivo* effect of CaF$_2$-NPs was also confirmed by the scanning electron micrographs demonstrating a reduced demineralization and biofilm formation on tooth surfaces treated with CaF$_2$-NPs. It has been reported that topical application of fluoride on tooth surface results in the formation of calcium fluoride like material which act as the reservoir of fluoride ions that when released, protect the tooth's surface and help in remineralisation [Rošin-Grget & Linčir 2001; Rølla & Saxegaard 1990]. Hence, the reduction in caries may be due to attachment of nanoparticle on the tooth surface and sustained release of fluoride ions [Sun & Chow 2008; Xu et al. 2008] from CaF$_2$-NPs, which not only helps in the suppression of virulence traits of *S. mutans* but also promote remineralisation.

Despite the potential benefits of using nanoparticle it is necessary to be concerned about their probable harmful effects to human health. In present study the most likely harmful effect may be the entry of nanoparticles in the human gastrointestinal tract. CaF$_2$-NPs
were found to be non-cytotoxic to human normal cell line (HEK-293) and there was no oral toxicity. Thus, substantiating no detrimental effect to human normal cells. Apart from that there is diverse microbial ecosystem in human intestine and metabolic activities of these microbes directly actuate human health [Rajilić-Stojanović et al. 2007]. Hence, it is important to address the interaction of gut microbes with CaF$_2$-NPs and whether these interactions are deleterious, positive, or insignificant. Consequently, further research is required in this aspect before using CaF$_2$-NPs in therapeutics.

In conclusion, the present study validates the anti-cariogenic potential of CaF$_2$-NPs against *S. mutans*. These nanoparticles appears to be ideal for prevention of dental caries with no oral toxicity. Moreover, they are non-cytotoxic to normal human cell line (HEK-293). Thus, CaF$_2$-NPs could possibly be used as topical applicant on tooth surface and as a potential therapeutic agent against *S. mutans* to inhibit caries related problems.