CHAPTER 3

MATERIALS AND METHODS
3. MATERIALS AND METHODS

3.1. Materials used in the study:

Two varieties of composites resins, different in composition were selected for the study. Silorane based composite - Filtek P90 from now on will be abbreviated as ‘SBC’ and Methacrylate based composite- Z100 from now will be abbreviated as ‘MBC’. The details of these two compositions are presented in Table 3.1

3.1.1. Sample preparation of silorane based composite and methacrylate based composite:

The specimens for the present study were prepared using Brass molds (10 mm diameter x 2 mm thickness). The mold were sandwiched between transparent matrix strip and glass slide. The uncured composites were inserted into the mold and intentionally overfilled. Light pressure was applied to expel excess material from the mold. Each specimen was light cured through the top and bottom glass slide for the duration recommended by the manufacturers. The intensity of the light-curing unit was checked before each sample run using a Hilux curing light meter. The set cylindrical specimens were separated from the mold. The specimens were stored at 100% relative humidity at 37°C for 24hours. All specimen preparation was done by a single operator, in order to reduce variability.
3.2. Methodology: General plan of the study

![Diagram showing biocompatibility and biomechanics evaluations for Silorane based composite (SBC) and Methacrylate based composite (MBC).]

Silorane based composite- SBC (Filtek P90)  
Methacrylate based composite- MBC (Z100)

**Biocompatibility (Biological evaluation)**
- Anti microbial activity
  - **Antibacterial:** *Streptococcus mutans, Lactobacilli acidophilus* (MIC & Disc diffusion Method)
  - **Antifungal:** *Candida albicans* (MIC & Disc diffusion Method)
  - Adhesion and penetration assay

**Biomechanics (Physical evaluation)**
- Fluoride release & recharge (Fluoride sensitive ion electrode)
- Color stability (Reflectance Spectrophotometer)
- Surface roughness (Surface analyzer profilometer)
- Surface Hardness (Vickers Hardness tester)
- Degradation resistance (Water sorption & solubility-weight loss method)
- Elevated temperature changes (Electric furnace)

3.3. Evaluation of biocompatibility:

3.3.1. Measurement of cytotoxic effects:

3.3.1.1. Preparation of stock solution:

The two composites used in the study (Table 3.1) were dissolved in dimethysulfoxide (DMSO). From the stock solution (10mg/ml), seven different concentration of composite (20, 10, 5, 2.5, 1.25, 0.625 and 0.3125 μg/ml) were used to treat cells at the final concentration of DMSO less than 0.2%.

3.3.1.2. Isolation and cell culture method for human gingival fibroblasts:

The fresh biopsy specimens of human gingival tissue of patients undergoing orthodontic or periodontal treatment were collected with all sterile precautions in Hank's
Balanced Salt Solution (HBSS) with 10% antibiotics after obtaining informed consent according to institutional ethical committee regulations. Within 12 hours after sample collection, samples were washed thrice with HBSS+10% antibiotics (penicillin+streptomycin), tissues were cut into small pieces using sharp, sterile surgical blade. The cells were cultured in Dulbecco’s modified Eagle medium (DMEM) (Sigma, UK) supplemented with 10% fetal calf serum/fetal bovine serum (FBS) (Biowest Ltd., UK), 2% l-glutamine (Sigma), 100 U/ml penicillin, (Sigma), and 100μg/ml streptomycin (Sigma). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Cells were passaged and freeze-dried to use when required.

3.3.1.3. Isolation and cell culture method for dental pulp cells:

Dental pulp tissue from non-caries permanent teeth of patients undergoing orthodontic treatment was extracted after obtaining informed consent according to institutional ethical committee regulations was harvested. Following extirpation, dental pulp tissue was cultured using Dulbecco’s modified Eagle medium (Sigma, UK) supplemented with 10% fetal calf serum (Biowest Ltd., UK), 2% l-glutamine (Sigma), 100 U/ml penicillin, (Sigma), and 100μg/ml streptomycin (Sigma) at 37 degree centigrade, in 5% CO₂ incubator. After 24 hours of incubation, the supernatant media was removed and fresh media containing α-modified Eagle’s medium (α-MEM, Sigma) supplemented with 10% FBS, glutamine and 1% polysialic acid (PSA) was placed in each well, and the plates were incubated at 37°C in a humid atmosphere of 5% CO₂. Once these cells reach confluence of 80%, the cells were trypsinized and sub-cultured. After 2-3 passage, once sufficient number of cells is available, these cells were subjected to MTT assay.
3.3.1.4. Passaging and freezing of cells:

Once cells become 80% confluence, the cells were split with a mixture of 0.02% trypsin (Sigma) and 0.02% Ethylene diamine tetra acetic acid (EDTA) (Sigma), and incubated for 2–5 min at 37°C. Culture plates were flushed with DMEM containing 10% FBS. Sample pallet was made into cell suspension by vertexing and/or pippeting the digested tissue several times. The resulted cell suspension was replated into fresh culture plate. Excess cell were frozen in liquid nitrogen with appropriate media containing 10 % DMSO.

3.3.1.5. Treatment:

Cells derived from cell lines, after attaining required confluence, cells were treated with different doses of SBC and MBC for different time point. Then these cells were subjected to (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) MTT assay to assess the proliferation and viability.

3.3.1.6. Methyl Thiazolyl Tetrazolium (MTT) assay:

The cells were seeded into a 96 well plate at a density of $10^4$ cells / well and incubated for 48 hours to allow the cells to attach. The attached cells were treated with various doses of both the composites. DMSO treated cells and untreated cells served as control. Culture was incubated for 48 and 72 hours. At the end of the incubation period, 20 µl of MTT stock solution were added to each well and were incubated for 4 hours at 37°C. During this incubation, the MTT enters the cells and mitochondrion of the cells reduces it to insoluble formazan crystals. Then, the formazan crystals were dissolved in lysis solution (1:24 ratio of Hydrochloric acid (HCl): isopropanol) for 1hr at room temperature. The absorbance of the colored solution was measured using ELISA plate.
Reader at wavelength 570nm. Since reduction of MTT is done only in the metabolically active cells, the level formazan crystal formation is considered as a measure of the viability of the cells. Readings were analyzed using Microsoft Excel program. Each experiment was done in triplicates.

3.3.1.7. Statistical Analysis:

The data were presented as mean and SD. The effects of the two composites on the HGF and/or DPC was compared by ‘t’ test and to compare the toxicity within the various concentration of composite used and time period one way ANOVA was used followed by Tukey’s multiple comparison test at significance level of $p < 0.05$.

3.3.2. Evaluation of antimicrobial activity:

*Streptococcus mutans* (*S. mutans*) (gram positive facultative anaerobic cocci shaped bacteria), *Lactobacillus acidophilus* (*L. acidophilus*) (gram positive facultative anaerobic or microaerophilic rod shaped bacteria) and *Candida albicans* (*C.albicans*) (diploid fungus that grows both as yeast and filamentous cells) were selected as test organisms and Agar disc diffusion method and Broth macro dilution method (MIC) was used to evaluate the antimicrobial potential against these organisms on the test specimens, SBC and MBC, in the present study.

3.3.2.1. From strains of *Candida albicans*:

The readily available strain of *Candida albicans* (ATCC 2091) was used for experimental purpose. Before the experiment 100ml stationary phase culture of Candida were prepared in SDB. Cells were harvested by centrifugation (750g, 20min), washed 3
times in sterile phosphate buffered saline (PBS), pH 7.2 and resuspended in PBS to a 2 McFarland which corresponds to a cell concentration of $1.5 \pm 0.3 \times 10^{-7}$ cells/ml.

3.3.2.2. From Strains of *Streptococcus mutans* and *Lactobacillus acidophilus*

The readily available strains of *S. mutans* (ATCC 25175) and *L. acidophilus* (ATCC 4356) were chosen as test strain for the study. After frozen (-60°C) precultures were established, the bacteria was exposed on Brain Heart Infusion agar Media (BHI) for *S. mutans* and Rogosa agar for *L. acidophilus* and incubated at 37°C for 48hours. Single colonies were cultivated in sterile trypti case soy broth supplemented with yeast extract for 16hr at 37°C. The day before the experiment 1 ml of each bacterial suspension were inoculated with 250ml of the sterile trypti case soy broth and incubated for 12hours at 37°C. The bacterial suspension were centrifuged for 5 min at 2000 rpm at 18°C and washed twice with PBS. The optical density of the suspensions will be adjusted to 0.3 at 540nm with a spectrophotometer. The cells of *L. acidophilus* were suspended in thioglycolate broth. *L. acidophilus* was incubated anaerobically for a period of 2days.

3.3.2.3. Antimicrobial activity by agar disc diffusion method:

The lawn culture of the organisms was grown on the agar plates. Uniform perforations 10 mm diameter was made at equidistant points in agar (SDB for *C. albicans*, tryptic soy broth for *S. mutans* and rogosa agar for *L. acidophilus*) by means of sterile loop, to receive the testing materials. The agar plates were inoculated at 37°C for 24- 48 hours to allow the microorganisms to grow. After incubation, the mean diameter of the inhibition zones formed around the wells was measured. All the assays were done
in triplicates, under aseptic conditions and the results were average of three records. Data was statistically analyzed using students t test at a significance level of 5% using SPSS 15.0.

3.3.2.4. Antimicrobial activity by broth macro dilution method (determination of MIC):

The procedure for the carry out of the study was adopted as explained by Andrews 2001⁹¹.

*Preparation of bacterial inoculums:* Three to four isolated colonies on blood agar was picked using straight wire and inoculated into BHI broth and incubated at 37°C for 4-6 hours. The turbidity was adjusted to Mc Farlands 0.5 standard. The turbidity matched broth culture was diluted 1 in 10 in BHI broth to get bacterial inoculums having bacterial concentration approximately 1.5x 10⁸ cfu/ml.

*Broth Macro dilution test:* Ten test tubes each containing 2ml sterile BHI broth was arranged in a rack. Two ml of SBC / MBC (test solution) (in broth) were added to the first tube and thus mixed and transferred into the next tube in 2 ml quantities till 9th tube to get doubling dilutions (1 in 4 to 1 in 1024). Tenth tube served as growth control. Standardized bacterial inoculums were added (0.02ml) to each tube. The tubes were incubated at 35°C for 18 hours and examined. The minimum concentration (higher dilution) of the SBC / MBC that inhibits bacterial growth was considered the minimum inhibitory concentration (MIC).
3.3.3. Adhesion and penetration assay:

3.3.3.1. Adherence test:

SBC and MBC were pre-sterilized and separately placed in the sterile bottles with a magnetic stirrer. Microbial suspensions around 600µl and 1ml of BHI broth in case of *C. albicans* and *S. mutans* and thioglycolate broth in case of *L. acidophilus* was poured into the bottles were sealed tightly and the suspension was stirred at a constant speed for two hours at 37°C. (It was preliminarily determined that the bacterial adherence to the restoratives saturated within two hours). Then the restorative plates were removed, washed with a large amount of distilled water, fixed with 2.5% glutaraldehyde at 4°C for 30 min, and stained with 1% acridine orange for 30 min. The numbers of adhered bacteria were counted directly under a fluorescence microscope (40 x magnification) (Olympus CX41, Olympus corporation Ltd). The numbers of bacteria in each of 20 fields selected at random on each plate of the restorative were counted and divided by the area of the view. From the values thus obtained, the average number and standard deviation were calculated. Data was statistically analyzed using chi-square test (fishers exact) at a significance level of 5% using SPSS 15.0.

3.3.3.2. Penetration test:

The dried test specimens was held horizontally with a clamp and sectioned. Sections were immersed in 0.03% acridine orange for 1 min, rinsed in distilled water, then allowed to dry lying horizontally with the cut section kept uppermost. Sections were viewed via fluorescence microscopy x 1000 magnification and oil immersion. The
objective was focused on one end of the section and moved down so that the entire length of the sample was examined.

The procedure was repeated 3 times:

Level one - once with the objective focused on the outer layer of the sample (upper surface of the material),

Level two- once in the middle of the material and

Level three- once adjacent to the lowermost layer of the material.

The penetration of microorganism into SBC/ MBC was measured by counting the number of cells visible within each microscopic field. The number of cells counted was then totaled for each level and the mean calculated for each level. Data was statistically analyzed using chi-square test (fishers exact) at a significance level of 5% using SPSS 15.0.

3.4. Evaluation of biomechanics:

3.4.1. Effect of temperature, time interval and storage condition on fluoride release and recharge from silorane based and methacrylate based restorative material

This study compared the amounts of fluoride release and recharge capacities, in distilled water and artificial saliva at 4°C, 37°C, and 55°C of SBC and MBC in comparison with Glass ionomer cement (GIC) as control over time periods of 1day, 7day, 14day and 28day. Table-3.2 lists the details of the glass ionomer cement used as a gold standard which is known to release fluoride. Preparation of test specimen was done as described in section 3.1.1.
**Experimental design:**

<table>
<thead>
<tr>
<th>Preparation of SBC / MBC/ GIC (60 samples each)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immersion in Artificial Saliva</td>
</tr>
<tr>
<td>Temperature</td>
</tr>
<tr>
<td>Time interval</td>
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<td></td>
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<td></td>
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</tr>
</tbody>
</table>

**Fluoride release:** Sixty samples of each restorative material were fabricated as described in section 3.1.1. Thirty samples each were immersed in one of the two groups: 20ml of distilled water or artificial Saliva in 30ml plastic containers. Composition of artificial saliva: NaCl 0.4g, KCl 1.21g, Na₂S.9H₂O 0.0005g, NaH₂PO₄.2H₂O 0.7g, CO(NH₂) 1g, all the ingredients were dissolved in 1000ml distilled water with pH adjusted to 6.75±0.75 with 0.1N NaOH. Sterile plastic containers were used throughout, as glass vessels are known to absorb and leach fluoride. Samples were further subdivided into three groups (n=10) to be stored at 4°C (in refrigerator), 37°C (in an incubator), 55°C (in a water bath) for one day. At the end of 24hours the samples were removed, washed with distilled water and dried with blotting paper, before being transferred to a fresh jar containing 20ml of distilled water or artificial saliva. This procedure was continued for 7day, 14day and 28day time interval. Solution at the end of one day, 7 day, 14day, and 28day time interval was collected and fluoride release was measured using a fluoride sensitive electrode (Orion Ion Analyzer -Orion™ 2109XP Fluoride Analyzer) by adding...
1:1 quantity of Total Ionic Strength Adjustment Buffer (TISAB) solution. For each sample, analysis was done three times and mean was taken to obtain accurate results.

**Statistical Analysis:** Comparison between immersion media was done using student ‘t’ test, and comparison between materials, and temperatures was done using One way ANOVA and tukeys posthoc. Time period were assessed using repeated measures ANOVA and bonferoni post hoc. The data was expressed as mean ± SD. p values < 0.05 was considered as significant.

**Fluoride Recharge:** Each specimen was recharged in 5000ppm neutral sodium fluoride (NaF) solution for 5 minutes. Specimen recharge was repeated for 3 weeks. The fluoride re-release was measured at 1, 3 and 7 days after each recharge episode.

**Statistical Analysis:** Comparison between immersion media, between materials and time interval (weeks and days) was done using repeated measures ANOVA and tukeys posthoc. The data was expressed as mean ± SD. p values < 0.05 was considered as significant.

**3.4.2. Effect of staining solutions and immersion periods on color stability of silorane based and methacrylate based restorative material**

In this study, six commonly consumed beverages were used as staining agents and distilled water as control, to evaluate the effects of color stability of SBC in comparison with its methacrylate counterpart MBC.

Table 3.3 lists the details of the resins and staining agents used in the present study. It is known that obtaining conditions of oral environment *in vitro* is not easy. To simulate *in vivo* conditions in this study, six different staining solutions and distilled water (control)
were used at a constant temperature of 37±1°C. The quantities of staining ingredients to make the test solutions were prepared closer to the amount consumed per person at a time. This study is an indicator of cumulative effect of repeated short immersions of composite resins used for restorations, during prolonged service.

*Preparation of test solutions:* 30g of tea, coffee and cocoa and one gram of turmeric were separately taken in one liter of distilled water and solution was prepared by boiling, followed by simmering for five min and filtered through a filter paper. In case of lime, 100ml of lime juice concentrate was dissolved in one liter of distilled water.

*Preparation of test specimens:* Preparation of test specimen was done as described in section 3.1.1. A total of 150 samples (70 samples for each group of SBC and MBC, [n=10]) were fabricated which were to be stained for 1, 7, 14 and 28 days’ time interval. All the specimens were coded to avoid manual bias while evaluating through spectrophotometer. The samples were polished with different grades of silicon carbide paper (80, 100, and 120) and buff polished with pumice slurry.

*Experimental Design:*

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparing of SBC / MBC specimens (150 samples)</td>
<td>Baseline color measurements</td>
<td>Preparation of test solutions (Turmeric [T], Tea [E], Coffee [C], Cocoa [O], Lime [L], Yoghurt [Y]) and Staining</td>
</tr>
</tbody>
</table>

Measurement of color
Method: The resin samples were stored in distilled water for 24 hours before immersing into the test solutions. After the baseline measurement, the samples were immersed in staining agents: Turmeric, Yoghurt, Coffee, Tea, Cocoa and Lime for 8 hours of immersion period daily (simulating overnight soaking period) for 1 day, 7 days, 14 days and 28 days at 37°C. The solutions were stirred twice a day to reduce precipitation. The solutions were refreshed each day. The samples were then rinsed in distilled water for 8-10 dips and blot dried with a tissue paper. They were stored in a desiccator for the remaining period until they were placed in a viewing port for color measurement. The control samples were stored in distilled water at 37°C, the water being changed every day.

Measurement of color: Color changes (ΔE) were measured objectively by a reflectance spectrophotometer (Spectrolino, Gretag-Macbeth AC, Germany), to potentially eliminate the subjective errors of color assessment. To measure chromatic differences Standard Commission International de L’ Eclairage (CIELab) color system was used. It quantifies color in terms of three coordinate values L*, a* and b*. L* represents brightness or lightness (value), a* and b* serve as numeric, correlates both for hue and chroma on green- red axis and blue- yellow axis respectively. The magnitude of color difference perceived between two objects is calculated by formula $\Delta E = (\Delta L^2 + \Delta b^2 + \Delta a^2)^{1/2}$.

Before each measurement session, the instrument was calibrated according to manufacturer instruction by using the supplied white calibration standard. Color was then measured according to CIE Lab color scale. D50 standard illuminant from a tungsten lamp was used with a viewing angle of 2°. UV filter was positioned to 100% UV. Color
measurements were made in three randomly selected areas for each specimen and average of three reading was recorded. The mean and standard deviation was calculated. 

Statistical analysis: Data were statistically analyzed by repeated measures of ANOVA and sidak post hoc test (for immersion period); ‘t’ test (for each material) and one way ANOVA (for staining agents). The data was expressed as mean ± SD. p values < 0.05 was considered as significant.

3.4.3. Effect of finishing / polishing techniques and time on surface roughness and surface hardness of silorane based and methacrylate based restorative material

Experimental design:

Forty specimens of each restorative material were fabricated (n=10) as described in section 3.1.1. The matrix strip formed surface was used as a baseline for all tests. Forty specimens were finished and polished immediately using four finishing and polishing procedures and the remaining forty specimens were finished and polished after a week.
Specimens were examined for obvious voids, labeled on the bottom and randomly separated into four treatment groups.

**Finishing and Polishing Procedures:**

Method I: Extra fine finishing diamond bur followed by Soflex discs (Al₂O₃-coated, abrasive disc system, fine grit, and extra-fine grit) was employed with a high-speed turbine with water-spray-coolant, and an air-dried slow hand-piece, respectively as per the work of Ozgunaltay and his co-workers.¹⁷⁵

Method II: Extra fine finishing diamond bur followed by the Astropol and Astrobrush polishing system (silicon-based abrasive polisher point and polisher brush) was employed with a high-speed turbine with water-spray coolant, and a low-speed hand-piece with water spray, respectively.

Method III: Thirty-fluted tungsten carbide bur followed by the Soflex discs (Al₂O₃-coated, abrasive disc system, fine grit and extra fine grit) was employed with a high-speed turbine with water-spray coolant, and an air-dried slow hand-piece, respectively.

Method IV: Thirty-fluted tungsten carbide bur followed by the Astropol and Astrobrush polishing system was employed with a high-speed turbine with water-spray coolant, and a low-speed hand-piece with water spray, respectively.

Each step of the finishing–polishing was applied for 30s. Each bur was applied using light pressure in multiple directions. The Soflex discs was changed after the polishing of each sample and each silicon-based polisher point was discarded after use, while the diamond burs and carbide burs was changed every three samples.

**Measurement of surface roughness:** The surface roughness was evaluated using Taylor/Hobson Precision, Surtronic 3+ (Taylor Hobson Limited, England) coupled to a
computer with Talyprofile software surface analyzer with a cut off length of 0.80mm and a crosshead speed of 0.25mm per second to obtain average surface roughness (Ra, μm) and a surface profile tracing. Each sample was rotated 120°, relative to the center, for each of three readings and averaged to generate average roughness value (Ra).

**Measurement of surface hardness:** The surface hardness was evaluated using Matzusawa Micro Vickers hardness Tester (Model MMT X 7A, Matsuzawa Co Ltd, Japan). Prior to testing, all specimens were sonically cleaned for 10 min to remove any influence of precipitate on surface hardness. Specimens were placed on platform with the surface under testing facing the diamond indenter. Load applied was 300g for 15s. Three indentations were made no closer than 1 mm to the adjacent indentations on the surface of each specimen. Average of three readings was taken as mean value. The hardness was measured by using formula:

\[
Vickers\text{ Hardness Number (VHN)} = \frac{0.1854 \times P}{d^2} \text{ (N/mm}^2) \quad \text{where } P = \text{ load in N},
\]

\[d = \text{average length of diagonal of indentation in mm.}\]

**Statistical analysis:** The data was expressed as mean ± SD. p values < 0.05 was considered as significant. Two way repeated measures ANOVA followed by tukey test was used.

### 3.4.4. Degradation resistance of silorane based and methacrylate based restorative material: Evaluation of water sorption and solubility

Ten disc shaped specimens of each composite SBC and MBC as described in section 3.1.1. The degradation protocol for sorption and solubility test was based on ISO
Water sorption and solubility were assessed by mass gain or loss after storage in water or artificial saliva after 1 day, 15 days and 30 days. (Composition of artificial saliva: NaCl 0.4g, KCl 1.21g, Na₂S.9H₂O 0.0005g, NaH₂PO₄.2H₂O 0.7g, CO(NH₂) 1g, all the ingredients were dissolved in 1000ml distilled water with pH adjusted to 6.75±0.75 with 0.1N NaOH).

The diameter of each specimen was measured at two points at right angles to one another, and the mean diameter was calculated with a digital caliper (Digimatic Caliper, Mitutoyo, Tokyo, Japan; accuracy = 0.002 mm). The thickness of each specimen was measured at the center of the specimen and at four equally spaced points on the circumference, and the mean thickness was calculated. The volume (V) of each specimen was calculated in mm³ using the formula \( V = \pi \times r \times h \), where \( r \) is the mean sample radius (diameter/2) and \( h \) is the mean sample thickness.

Specimens were weighed using an analytical scale accurate up to 0.0001mg. For each material, 10 specimens were placed in a glass vial containing 10mL of artificial saliva, 10 in a glass vial containing 10mL of distilled water.

The specimens were stored in desiccators at 37°C for 24 hours until constant mass was achieved (m1). Specimens were then stored for one day, day 15 and one month in water and artificial saliva at 37°C, and the water-saturated mass was measured (m2). Finally, the specimens were dried again in the desiccator until constant mass was obtained, and their masses were once again determined (m3). The difference in mass between the initial dry and final dry mass represented the amount of solubility (SO) (m1-m3/volume of specimen) in micrograms per cubic millimeter. The difference in mass
between the saturated and final dry specimens provided water sorption (WS) values (m² - m³/ volume of specimen) in micrograms per cubic millimeter.

Statistical Analysis: Independent t test was done to compare water sorption and solubility in different solutions and test materials. Repeated measure ANOVA was used to compare water sorption and solubility between time interval and materials. P value <0.05 was considered significant.

3.4.5. Effect of elevated temperature on silorane based and methacrylate based restorative material

In this study, 54 extracted teeth were collected from the Department of Oral and Maxillofacial Surgery, in the Manipal College of Dental Sciences, Mangalore. Teeth with decay, abrasion, erosion, hypoplasia, fracture and / or restorations were excluded from the study. After the extraction, every tooth were washed with deionized water to eliminate blood residues and disinfected in a 5% sodium hypochlorite solution for one hour and stored in a sodium chloride 0.9% solution at room temperature for up to one month, according to the ISO/ TS 11405/2003.

The 54 teeth were divided into three groups of 18 teeth each

<table>
<thead>
<tr>
<th>Group 1 - Unrestored teeth,</th>
<th>Plate 1 (n=6)</th>
<th>Plate 2 (n=6)</th>
<th>Plate 3 (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Group 1</td>
<td>Group 1</td>
<td>Group 1</td>
</tr>
<tr>
<td>Group 2</td>
<td>Group 2</td>
<td>Group 2</td>
<td>Group 2</td>
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<tr>
<td>Group 3</td>
<td>Group 3</td>
<td>Group 3</td>
<td>Group 3</td>
</tr>
</tbody>
</table>

All the restored cavities were class I. Once fillings was performed, teeth were set in three individual custom made trays made of dental investment material of approximately equal dimension to facilitate their manipulation and were numbered as —
Plate I, Plate 2 and Plate 3: the roots of the unrestored and restored teeth were kept totally immersed in the investment material, with the aim of simulating the real oral cavity. Each plate contained teeth arranged in three columns, with each column made up of six teeth so that each plate contained 18 teeth. Three plates containing teeth of different groups were prepared by an investment material.

All plates contained a first column of unrestored teeth, a second column of teeth restored with SBC, a third column of teeth restored with MBC. They were exposed to direct heat inside an oven with a muffler chamber (Thermolyne®) previously calibrated to three different temperature degrees with the specified time period as below:

- Plate I was incinerated at 500°C for 15 minutes,
- Plate II was incinerated at 500°C for 30 minutes and
- Plate III was incinerated at 1000°C for 30 minutes.

As soon as each target temperature was reached, the samples were removed from the oven and allowed to cool to room temperature. The samples were then examined both macroscopically and with a stereo-microscope (Reichert, Stereostar Zoom) and data recorded.

Analysis: Damage to the teeth and restorative materials to variable temperature and time can be categorized as intact (no damage) scorched (superficially parched and discolored), charred (reduced to carbon by incomplete combustion) and incinerated (burned to ashes).
Table 3.1 Composites used in the study

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Manufacturer</th>
<th>Shade</th>
<th>Organic matrix</th>
<th>Inorganic fillers</th>
<th>Filler content [vol.%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtek P90</td>
<td>Silorane based micro hybrid composite</td>
<td>3M/ESPE, St. Paul, MN, USA</td>
<td>A2</td>
<td>3,4-Epoxycyclohexylethyl cyclopolsiloxane, bis-3,4-poxycyclohexylethylphenylmethylsilane</td>
<td>Silanized quartz, yttrium fluoride</td>
<td>55</td>
</tr>
<tr>
<td>Z100</td>
<td>Methacrylate based hybrid composite</td>
<td>3M/ESPE, St. Paul, MN, USA</td>
<td>A2</td>
<td>Bis-GMA and TEGDMA</td>
<td>Zirconium, silica</td>
<td>66</td>
</tr>
</tbody>
</table>
Table 3.2 Glass Ionomer Cement

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Manufacturer</th>
<th>Shade</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC Gold Label Universal Restorative (Conventional)</td>
<td><strong>Glass Ionomer Cement -GIC</strong></td>
<td>GC Corporation, Tokyo, Japan</td>
<td>A2</td>
</tr>
</tbody>
</table>
Table 3.3 Staining agents used in the study

<table>
<thead>
<tr>
<th>Staining Agents</th>
<th>Code</th>
<th>Manufacturer</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turmeric</td>
<td>T</td>
<td>MTR Food Ltd, Bangalore, India</td>
<td>1g/ltr boiling water, simmer for 5 min and filtered</td>
</tr>
<tr>
<td>Coffee</td>
<td>C</td>
<td>Nescafe Sunrise Premium, Nestle India Ltd, New Delhi, India</td>
<td>30g/ltr boiling water, simmer for 5 min and filtered</td>
</tr>
<tr>
<td>Tea</td>
<td>E</td>
<td>Brooke Bond Red Label Hindustan Unilever Ltd Mumbai, India</td>
<td>30g/ltr boiling water, simmer for 5 min and filtered</td>
</tr>
<tr>
<td>Cocoa</td>
<td>O</td>
<td>Cadbury Cocoa, Cadbury India Ltd, Mumbai, India.</td>
<td>30g/ltr boiling water, simmer for 5 min and filtered</td>
</tr>
<tr>
<td>Lime</td>
<td>L</td>
<td>Home made</td>
<td>100ml/ltr of distilled water</td>
</tr>
<tr>
<td>Yoghurt</td>
<td>Y</td>
<td>Home made</td>
<td></td>
</tr>
</tbody>
</table>