Indian Council of Medical Research, New Delhi
FINAL REPORT

1. **Title of the Project** : Evaluation of molecular toxicity of newly developed materials intended from biomedical applications **(**ICMR NO. 5/20/1 (Bio)/07-NCD-I)
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3. **Implementing Institution :** Sree Chitra Tirunal Institute for Medical Sciences and Technology, Biomedical Technology Wing, Satelmond Palace Campus, Poojappura, Thiruvananthapuram, Kerala - 695 012.
4. **Date of commencement :** 15.10.2009
5. **Duration :** 3 years
6. **Date of completion :** 31.10.2011
7. **Objectives as approved :**

The objective of the project is to evaluate the molecular level toxicity effects of six newly developed materials and their chemical leachants on mitochondrial DNA, antioxidant enzymes, lipid peroxidation and cytogenetic effects.

Methodology

- **Experimental animals**

  Studies were carried out using animals, procured from Division of Laboratory Animal Sciences (DLAS), of Sree Chitra Tirunal Institute for Medical Sciences and Technology of this Institute. Both sexes were used in the experiment for comparative studies. The list of animals used for the present study is stated in table 1. They were housed in polypropylene cages over husk beddings in a controlled environment (temperature: 22±3°C; humidity: 50 to 70%), fed with standard feed and free access to water and a 12 h light and dark cycle was maintained. This study conformed to the guiding principles of Institutional Animal Ethical Committee (IAEC), Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Guide for the care and use of laboratory animals.

- **Biomaterials for experiment**

  The biomaterials used for this study were procured from the Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. The list of experimental material used is stated in table 2.

- **Preparation of material extract**

  HA-BG, HA-EVA, fibrin glue, dental material and latex were weighed and then extracted with physiological saline and artificial saliva. 2g of the material was added to 10ml of the physiological saline and artificial saliva and incubated at 37 ºC /70ºC for 24/72h with shaking at 50 rpm. The extract of these materials were used for the analysis of lipid peroxidation, protein, reduced glutathione, glutathione reductase, glutathione peroxidase, genomic DNA, mitochondrial DNA extraction etc.

- ***In vitro* studies.**
  - **Preparation of liver homogenate for antioxidant assay**

    Mice/rat liver was dissected out aseptically and 10% liver homogenate was prepared with 0.1M Tris-HCl (pH 7.4). The samples were incubated with different concentrations of physiological saline/artificial saliva extract of the biomaterials and were used for following studies. The suspended mixtures were centrifuged at 3500 rpm for 10 min at 4°C in a refrigerated centrifuge (Eppendorf, 5810R). The resultant supernatants were maintained in an ice bath until used for the estimation of total protein and different antioxidant parameters using standard protocols with slight modifications. Following parameters were done with material extracts.
    - Lipid Peroxidation (LPO)
    - Estimation of protein
    - Reduced glutathione (GSH)
    - Glutathione reductase (GR)
    - Glutathione peroxidase (GP_x)

- Genomic DNA extraction
- Mitochondrial DNA extraction
- Analysis of 8OHdG

Methodologies of the above parameters were explained in the in vivo experimental areas (page No 5.)

- **Chromosomal aberration study**

Human peripheral blood samples were collected from healthy donors (non-smokers and with no recent history of exposure to mutagens) in heparinized tubes for initiation. Whole blood cultures in duplicate were also set up. In vitro assay was performed in the presence and absence of a rat liver exogenous metabolic activation system (S9 mix). Lymphocytes prepared in karyotype medium were stimulated to divide using phytohemagglutinin an atmosphere of 5% CO_2. After 48 h of culture, cells were treated with physiological saline extract of latex material. CP at a dose of 100 µg/culture (Sowjanya et al 2009) and MC at a dose of 1 µg/culture (Nowell 1964) were used as positive controls for with and without metabolic activator experiments, respectively. The treatment lasted for 24h in the absence of S9 mix and 3 h in the presence of S9. The S9 fraction was used at a final concentration of 5% in RPMI medium. After 3 h of treatment with material extracts and CP in the presence of S9, the cells were replaced with fresh karyotyping medium. Prior to cell harvest, the cultures were treated with spindle poison colchicine (final concentration of 1µg/culture) for 2h. Finally cells were harvested at 72h, metaphase slides were stained with 10% Geimsa solution and then screened for chromosomal aberrations using automated metaphase finder (Carl Zeiss, Germany).Respective lymphocyte culture slides with metaphase chromosomes were photographed.

- ***In vivo* studies**
  - **Implantation of HA-BG and PMMA-HAP materials in rabbit femur bone**

The animals were grouped into two; one set as control and other as the test. All the animals were anaesthetized with ketamine (2ml/kg) and xylazine (0.25ml/kg) following atropine sulphate (0.25ml/kg) subcutaneously and diazepam (0.4ml/kg) pre- medication, by intra-muscular administration and place the animal on the surgical table. Swab the clipped skin lightly but thoroughly with 70% alcohol followed by betadine solution. Expose the cortex region of each femur and 2.0 mm size holes were made by drilling using low speed drill with profuse irrigation with saline. Both the test and control materials were made into cylindrical shape of 2.0mm in diameter and 6.0mm in length. The implants were inserted into the holes and the wounds were closed using sutures.

  - **Implantation of HAP-EVA and fibrin glue in rat brain**

The animals were randomly divided into different groups with eight numbers of animals in each group. All animals were anesthetized by means of intramuscular injection of ketamine hydrochloride 100mg/kg (Levent, Istanbul, Turkey) and xylazine 5 mg/kg IM (Bayer, Istanbul, Turkey). They were then fixed on the table and their scalps were shaved and cleaned with 10% Povidone iodine. A calvarial defect was performed with a high-speed drill on the right parietal bone of all rats. The dura mater was kept intact during this procedure. About 100µL of fibrin glue was then applied on the defect using a special applicator having double chamber syringes.

  - **Implantation of dental composite in rat gluteal muscle**

The animals were randomly divided into different groups with eight numbers of animals in each group. Xylaxin (5mg/kg) and ketamine (100mg/kg) were given intramuscularly as anesthesia. The animals were clipped tightly and swabbed thoroughly with 70% alcohol. On the dorsal surface 2cm incisions were made using klema bone marrow puncture needle with stylet. Implants with dimensions of 1-3mm width and 10mm length were pushed into the holes. Close the incision with sterile nylon suture and apply antiseptic cream.

  - **Implantation of latex material in rat subcutaneous tissue**

Latex material, Size of 10 mm diameter and 0.5mm thickness was used in the experiment and sterilized in an autoclave at 121ºC for 1 hour. The animals were anaesthetized with 100 mg/kg ketamine and 5mg/kg xylazine. Skin at the site of surgery was shaved and disinfected and implantation was done by making a subcutaneous tunnel pocket on the dorsal surface, on one

side of the spinal column by blunt dissection, such that the base of the pocket is 10 mm away from the line of incision. The test sample was then pushed into the tunnel. Four test materials were implanted on either side of the animal (2/side). The skin wounds were closed with 5/0 PDS II (Polydioxanone) monofilament absorbable sutures (Ethicon Ltd., UK).

All the implanted animals were observed for an observation period of 1, 4, 12, 26 and 52 weeks. The details of the implantation in animals were listed in table 3. After the experimental periods the animals were sacrificed bone, muscle, skin, brain and liver were rapidly excised, washed in normal ice-cold physiological saline, and immediately placed in an ice bath.

- **Measurement of enzymatic activities**

For antioxidant assays 10% liver homogenates were made in Tris-HCl (0.1M, pH 7.4) using an ice-chilled glass homogenizing vessel in a rotor stator homogenizer at 900 rpm (Polytron, PT 3100).

For *in vitro* studies mice liver was dissected out and 10% liver homogenate was prepared with 0.1M Tris-HCl (pH 7.4). The samples were incubated with different concentrations of physiological saline extract of the biomaterials and were used for the following antioxidant assays. The suspended mixtures were centrifuged at 3500 rpm for 10 min at 4°C in a refrigerated centrifuge (Eppendorf, 5810R). The resultant supernatants were maintained in an ice bath until used for the estimation of total protein and different antioxidant parameters using standard protocols with slight modifications. Enzymatic antioxidant status alters due to oxidative stress mediated material leachants. Alterations in the levels reflect the severity of the damage.

- **Estimation of protein in liver homogenate**

Determination of protein concentration was performed in liver homogenate by the method of Lowry *et al*. (1951) with bovine serum albumin as standard and expressed as mg/ml.

- **Assay for Lipid Peroxidation (LPO)**

The extent of LPO was determined as the concentration of malondialdehyde (MDA) generated by the thiobarbituric acid reactive substances (TBARS), as described by *Ohkawa et al.1979.* The amount of malondialdehyde (MDA) formed was measured spectrophotometrically at 532 nm.

- **Assay for Reduced glutathione (GSH)**

GSH level in the liver homogenate was determined by the method of *Moron et al* with slight modifications in which Ellman's reagent or DTNB (5, 5'- dithiobis-(2-nitrobenzoic acid), reacts with GSH to form a spectrophotometrically detectable product at 412 nm. The change in absorbance at 412 nm is a linear function of the GSH concentration in the reaction mixture and is based on the reaction of GSH with DTNB to give a compound that absorbs at 412 nm. The amount of GSH was expressed as n mole / mg protein.

- **Assay for glutathione reductase (GR)**

Determination of glutathione reductase activity was performed as previously described by *Mize $ Langdon 1962*. Briefly, this assay measures the rate of NADPH oxidation to $NADP^+$, which is accompanied by a decrease in absorbance at 340nm, so GR activity can be monitored spectrophotometrically. Thus, one GR unit is defined as the reduction of one µM of GSSG per minute at 25°C and pH 7.6.

- **Assay for glutathione peroxidase (GP_x)**

Activity of GPX was assayed by the method described by *Rotruck et al*. The remaining GSH after the enzyme catalyzed reaction was complexes with 5, 5'dithiobis 2-nitrobenzoic acid (DTNB) that absorbs at maximum wavelength of 412 nm. Enzyme activity was expressed as µg of GSH consumed /min/mg protein.

- **Genomic DNA extraction**

Genomic DNA was extracted from rat brain tissues, bone, subcutaneous tissues, using the DNeasy blood and tissue Mini Kit (Qiagen, Hilden, Germany) with minor modifications in manufacture's protocol. Each brain tissue was incubated overnight at 56°C with 180µl Buffer ATL and 50µl Proteinase K (20mg/ml). 10µl RNase (1mg/ml) was added and incubated at room temperature for 5min. Incubated samples were treated with 230µl of AL buffer at 70 °C

for 10min. Ethanol (230µl of 99%) was added to the suspension, mixed thoroughly by vortexing. The mixture was applied to the DNeasy spin column sitting in a 2ml centrifuge tube and centrifuged at 8000rpm for 2min. DNA binds to the silica spin filter while contaminants pass through. Remaining contaminants and enzyme inhibitors are removed by a wash step. Pure DNA is then eluted in a low salt Tris buffer to allow for pH stabilization of the DNA in storage. To maximize DNA yield, two successive elution steps were performed each with 50µl elution buffer.

- **Mitochondrial DNA extraction**

The frozen brain(100 mg), bone ,skin and muscle tissue(250 mg) was homogenized with 1 mL ice cold buffer supplied along with mtDNA extractor kit (Wako, Japan) followed by centrifuge at 1000 g for 1 minute. The supernatant was discarded and the pellet was resuspended in 50 µL of solution I and vortexed thoroughly. Further, 100µL solution II equal volume of solution II A and II B was added in the suspension and vortexed again and was kept on ice for 5 min. To this suspension 75µl solution III was added and briefly vortex the tube and again it was kept on ice for 5 minute again. The mixture was centrifuged at 12,000 g for 10 minute at 4ºC, after centrifugation 250µl supernatant was transferred to another centrifuge tube, 300µl sodium iodide and 500 µl isopropanol was added and again centrifuged at 12,000g for 10 minute. After centrifugation the pellet was washed with 1mL washing solution .and centrifuged at 12000g for 5 minute(2 times) at room temperature. The final mtdna pellet was dried carefully at vacuum and further suspended in TE buffer.

- **Conformation of genomic DNA by PCR amplification**

The genomic DNA isolated from blood, liver and brain using the DNeasy blood and tissue Mini Kit (Qiagen, Hilden, Germany) was confirmed by PCR using specific primer of genomic gene β-actin. The sequences of primers are 5'GCCAACCGTGAAAAGATGAC3' (Forward Primer) and 5'AGCCACCAATCCACACACAGA 3' (Reverse Primer). The reaction mixture contained in a total volume of 25µl containing 1 µL of forward and reverse primers (each at 0.5mM), 1µL of 10 mM dNTPs (dATP, dCTP, dGTP, and dTTP), 1.52µ L of $MgCl_2$ (24mM) , 0.1µ l of *Taq* DNA polymerase, (Fermentas), 2µL of Taq buffer , 2 µL of isolated mt DNA and finally 16.4 µL of sterile distilled water. . All of the samples were subjected to 30 cycles of amplification, consist of denaturation at 94ºc for 30 seconds, annealing at 60º c for 30 seconds, and extension at 72ºc for 1 minute.

- **Confirmation of Mitochondrial DNA by Polymerase chain reaction**

The mitochondrial DNA was isolated from skin, bone, brain and muscle by mtDNA isolation kit (WAKO, Japan) as described above, and it was confirmed by PCR using specific primer of mitochondrial gene cytochrome C. The sequences of primers are 5'GCCA ACCGTGAAAAGATGAC' and antisense strand 5'AGCCACCAATCCACACAGAGTA3'. The reaction mixture contained in a total volume of 25µl containing 1µL of forward and reverse primers (each at 0.5mM), 1µL of 10mM dNTPs (dATP, dCTP, dGTP, and dTTP), 1.52µL of $MgCl_2$ (24mM), 0.1µ l of *Taq* DNA polymerase, (Fermentas), 2µL of Taq buffer, 2 µL of isolated mtDNA and finally 16.4µL of sterile distilled water. . All of the samples were subjected to 30 cycles of amplification, consist of denaturation at 94ºc for 30 seconds, annealing at 60º c for 30 seconds, and extension at 72º c for 1 minute.

- **Determination of DNA concentration**

Purity check: After ensuring complete solubility of DNA, the purity factor (260/280nm) was determined spectrophotometrically (UV-1601, Shimadzu, Japan). The concentration of 50µg/ml was taken as one unit of optical absorption for double stranded – helical DNA at 260nm with optical path of 1cm.

- **Agarose gel electrophoresis** : The integrity of nuclear DNA and mitochondrial DNA was checked on 0.7% agarose gel stained with ethidium bromide (10mg/mL) by loading 12µl of DNA preparation (2µl extracted DNA, 2µl gel loading buffer containing 25% bromophenol blue and 30% glycerol, 8µl sterilized deionized water). The electrophoresis was carried out at 100V using 1xTAE buffer for 30min. After electrophoresis, the gel was observed under an UV lamp in a gel documentation system (Alpha Innotech).

- **Analysis of 8OHdG**

8-OHdG in rat brain, subcutaneous tissue, muscle, and bone nuclear DNA& mitochondrial DNA digests was determined by a competitive Immunosorbent assay using High sensitive 8-OHdG check (Japan Institute for the Control of Aging, Fukuroi, Japan or Genox Corp.,

Baltimore, MD). Samples pretreatment includes DNA were dissolved in water at a concentration of 50µg and made into single stranded by incubating at 95ºC for 5min and rapidly chilling on ice. Samples were then digested into nucleosides, by incubating the denatured DNA with 10µl 0.5M sodium acetate, 1.25µl magnesium chloride and 1µl DNease I for 10min at room temperature. The reaction mixtures were then centrifuged for 1min at 2000rpm and the supernatants were collected and stored for further study. 50µl of pretreated samples along with standards were placed in the 96-well plate (Nunc). Assays were carried out as per the manufacture's instruction. At the end of experiment the color developed is proportional to the amount of antibody in the plate, which in turn is inversely related to the amount of 8-OHdG in the serum sample. Lower color means higher amounts of 8-OHdG. Results are expressed in nanograms per milliliter. The absorbance of the reaction mixture was read at 450 nm with a micro-plate reader (Asys Expert plus, Austria). Curve fitting was done with Digi Swift software. The unknown concentration of 8-OHdG in each sample was determined by generating standard curves for each lot of assay reagents from standardized samples contained in each ELISA kit. The mean of each subject's three samples was computed and then interpret by statistical analyses.

- **Chromosomal aberrations Analysis**

Animals were administered intraperitonealy with a single injection of test, positive and negative control. Colichicine (20µg/kg) was administered 90 minutes before sacrificing the animals to arrest mitosis. All the animals were sacrificed at the end of 24h and 48h by cervical dislocation. Both femora were removed through the pelvic bone just below the knee and cleaned of any adhering muscle. Bone marrow cells were collected from both tibias by flushing in 5mL of physiological saline and centrifuged at 2000 rpm for 10min. The pellet was resuspended in KCl (0.075 M, at 37ºC, 5 mL) and incubated at 37ºC for 10min. Material was centrifuged at 2000 rpm for 10 min, fixed in methanol: acetic acid (Carnoy's fixative, 3:1 v/v). Centrifugation and fixation (in the cold) were repeated three times at least at intervals of 10 min. The material was resuspended in a little volume of fixative, dropped onto chilled slides, flame-dried and stained in 1:6 Sorenson buffered Giemsa (pH: 7.2). 100 metaphases containing 40 chromosomes were examined per animal to score different types of aberrations. To avoid the bias, slides were coded as blind (Chaubey RC, Mohanan PV, Sobti RC)

- **Micronucleus Analysis**

Animals were administered intraperitonealy with a single injection of test, positive and negative control and were sacrificed by cervical dislocation 24 h and 48 h after treatment. The frequency of micronucleated erythrocytes in femur bone marrow was evaluated. The bone marrow was flushed out from bones into 2mL fetal bovine serum and centrifuged at 2000 rpm for 10 min. Resuspend the pellet with a small volume of calf serum and smear were prepared. The slides were fixed by incubation in methanol for 3 h. Air dry, numbered, and stained with diluted May-Grunwald (1:1 with buffer) for 15 minutes and counter stain with diluted Giemsa stain (1:6 with buffer) for 30 minutes. After each step wash with buffer, air dry and subjected for evaluation. Two thousand polychromatic erythrocytes (PCE) were analyzed microscopically in each preparation to assess the incidence of micronuclei. The ratio of polychromatic to normal chromatic erythrocytes was also recorded (Schmid W, 1973).

- **Statistical analysis**

All the samples were run in duplicate; differences were statistically assessed using student t-test. The results obtained were expressed as Mean ± Standard deviation (SD). For all comparisons, a p value >0.05 was considered for statistically significant analysis.

RESULTS

- ***In vitro* studies**
- **Antioxidant assay of biomaterials in physiological saline extract**
- **Lipid peroxidation**

HAP-EVA and HA-BG

Incubation of liver homogenate with leachants of HA-EVA did not show any significant deviation from the negative control levels. Figure 1 shows that the leachants of HAP EVA and HABGA did not have any adverse effects on the reduced glutathione level.

Dental material

Different concentrations of physiological saline extract of the material were incubated with mice liver homogenate and assayed for the presence of malondialdehyde for *in vitro* studies. Figure 2 shows that LPO level was not significantly decreased in different concentration such as 1mL (1.83nmol/mg protein ,) 2mL (2.22 nmol/mg protein) than control(4.85 nmol/mg protein) .The results didn't show up any significant deviation from the negative control groups.

Latex

Different concentrations of physiological saline extract of the material were incubated with mice liver homogenate and assayed for the presence of malondialdehyde for *in vitro* studies. Figure 3 shows that LPO level was non significantly decreased indifferent concentration such as 1mL (3.78 nmol/mg protein, 2mL (4.35nmol/mg protein) than control(6.94nmol/mg protein) .The results didn't show up any significant deviation from the negative control groups.

Fibrin glue

The *in vitro* concentration of LPO on mice liver incubated with physiological saline and physiological saline extract of fibrin glue is represented in figure 4. The amount of LPO in control at different incubation periods were 0.52nmol/mg protein, 0.405nmol/mg protein, 0.307nmol/mg protein respectively and at all the three different concentrations and incubation periods were found to be increasing with an increase in the incubation period. Even though there was an increase in concentration of LPO, the increase was insignificant in comparison with the control.

- **Reduced Glutathione**

HAP-EVA and HA-BG

Incubation of liver homogenate with leachants of HA-EVA and HA BG did not show any significant deviation from the negative control levels. These results are shown in Figure 5 and was confirmed that the leachants of HA-EVA and HA-BG did not have any adverse effects on the reduced glutathione level.

Dental material

Incubation of liver homogenate with leachants of dental material did not show any significant deviation from the negative control levels. These results are shown in Figure 6 and was confirmed that the leachants of dental material did not have any adverse effects on the reduced glutathione level.

Latex

Physiological saline extracts of latex material were incubated with liver homogenate and these homogenate were used for *in vitro* assay. Figure 7 depicts the levels of GSH was increased in 1ml physiological saline extract (0.63nmol/mg protein) than control and 2ml physiological saline extract.(0.4nmol/mg protein).

Fibrin glue

Figure 8 exhibits the *in vitro* concentration of GSH in the mice liver treated with normal physiological saline and physiological saline extract of fibrin glue. The level of GSH was observed to be decreasing at all the concentrations and was also found to be insignificant with that of the control at all incubation periods.

- **Glutathione reductase**

HAP-EVA and HA-BG

Incubation of liver homogenate with leachants of HA-EVA did not show any significant deviation from the negative control levels. These results are shown in Figure 9 and was confirmed that the leachants of HA-EVA and HA-BG did not have any adverse effects on the Glutathione reductase.

Dental material

The level of GR on mice liver homogenate incubated with normal physiological saline and physiological saline extract of dental material respectively. All the enzyme concentration was found to be decreasing with increasing concentration of the material extract and also with an increase in incubation period as shown in figure 10, and the levels of expression of the enzymes were of only slight significance when compared with their respective controls.

Latex

The physiological saline extract of latex material has not shown any significant changes as compared with control as depicted in figure 11. The results indicated that the glutathione reductase levels were non significant in both studies as compared with control.

Fibrin glue

The level of GR on mice liver homogenate incubated with normal physiological saline and physiological saline extract of fibrin glue was shown in figure 12. All the enzyme concentration was found to be decreasing with increasing concentration of the material extract and also with an increase in incubation period. The levels of expression of the enzymes were of only slight significance when compared with their respective controls.

- **Glutathione peroxidase**

Latex

The liver homogenate incubated with different concentrations of physiological saline extract of the material was assessed for *in vitro* studies. There was no statistically significant alterations in the GPX level as compared with the control as shown in figure 13.

Fibrin glue

The level of GPx on mice liver homogenate incubated with normal physiological saline and physiological saline extract of fibrin glue respectively. Figure 14 depicts that all the enzyme concentration was found to be decreasing with increasing concentration of the material extract and also with an increase in incubation period, and the levels of expression of the enzymes were of only slight significance when compared with their respective controls

Antioxidant assay of biomaterials in artificial saliva

- **Lipid peroxidation**

HAP EVA and HABGA

Incubation of liver homogenate with leachants of HA-EVA did not show any significant deviation from the negative control levels. Figure 15 & 16 was confirmed that the leachants of HAP EVA and HABGA did not have any adverse effects on Lipid peroxidation level.

Dental material

Incubation of liver homogenate with leachants of Dental material did not show any significant deviation from the negative control levels. These results are shown in Figure17 and was confirmed that the leachants of Dental material did not have any adverse effects on the peroxidation of lipids.

Latex

Different concentrations of artificial saliva extract of the material were incubated with mice liver homogenate and assayed for the presence of malondialdehyde for *in vitro* studies. Figure 18 shows that LPO level was non significantly decreased indifferent concentration. The results didn't show up any significant deviation from the negative control groups

- **Reduced glutathione**

HAP EVA and HABGA

Incubation of liver homogenate with leachants of HA-EVA and HA BG did not show any significant deviation from the negative control levels. These results are shown in Figure 19 and 20 and was confirmed that the leachants of HA-EVA and HA-BG did not have any adverse effects on the reduced glutathione level.

Dental material

The level of reduced glutathione on mice liver homogenate incubated with normal artificial saliva and artificial saliva extract of Dental material respectively. All the enzyme concentration was found to be increasing with increasing concentration of the material extract as shown in figure 21, and the levels of expression of the enzymes were of only slight significance when compared with their respective controls.

Latex

Artificial salival extracts of latex material were incubated with liver homogenate and these homogenate were used for *in vitro* assay. Figure 22 depicts the levels of GSH was increased in increasing the artificial salival extract.

- **Glutathione reductase**

HAP-EVA and HA-BG

Incubation of liver homogenate with leachants of HA-EVA did not show any significant deviation from the negative control levels. These results are shown in Figure 23 and figure 24 that was confirmed that the leachants of HA-EVA and HA-BG did not have any adverse effects on the Glutathione reductase.

Dental material

The level of GR in mice liver incubated with artificial saline extracts dental material has also not shown any significant changes as compared with control as depicted in figure 25.

Latex

The physiological saline extract of latex material has also not shown any significant changes as compared with control as depicted in figure 26. The results indicated that the glutathione reductase levels were non significant as compared with control.

- **Quantitation of nuclear DNA damage by 8-Hydroxy-2-Deoxyguanosine ELISA**

Latex

Different concentrations of physiological saline extract of latex, were treated with subcutaneous tissue of mice showed non significant changes in the concentration of 8-OHdG in all the concentration of the test 8-OHdG level as shown in figure 27.

Fibrin glue

The in vitro level of 8-OHdG in rat tissues treated with physiological saline extract of fibrin glue is shown in figure 28. Even though there was a slight difference in the expression of 8-OHdG at all the four different concentrations of the test, the results indicated any significant difference with the respective controls.

- **Quantitation of mitochondrial DNA damage by 8-Hydroxy-2-Deoxyguanosine ELISA**

HA-EVA and Fibrin glue.

8OHdG content in mice brain tissues treated with physiological saline extract of fibrin glue and HAP EVA was shown in figure 29 and 30. HAP- EVA and fibrin glue leachents showed that that the level of 8-OHdG was slightly increase at the increasing concentration of extract, than control, but they are statically nonsignificant.

HA-BG

In *in vitro* study physiological saline leachants with different concentration showed that at 2ml of leachents (3.258-OHdG/10^5dG). 8-OHdG content was slightly increase than control (2.025 8-ohDG/10^5 dG) and other concentration as shown in figure 31.

Dental material

Different concentrations of physiological saline extract of Dental material were treated with muscle tissue of mice showed non significant increase in increasing the concentration of extract, as shown in figure 32.

Latex

In vitro study physiological saline leachants with different concentration showed that level of 8-OHdG content was slightly decrease than control as shown in figure 33. But they were statically nonsignificant than control $p \geq 0.01$.

- ***In vitro* chromosomal aberration studies.**

Results of chromosomal aberration studies with and without the metabolic activator are depicted in table 4. The positive controls CP (100µg/5ml of culture) and MC (1µg/5ml of culture) induced significant increase in the number of cells with chromosomal aberrations in their respective experiments. Among 100 cells scored CP induced chromosomal aberrations in 93.00±5.67 cells and chromatid aberrations in 15.00±1.41 cells. HA-EVA induced chromosomal and chromatid aberrations with S9 was in 5.50±0.71 , and 5.00±2.83 cells and with out was in 9.50 ±0.70 and 6 ±2.83 Dental material induced chromosomal and Chromatid aberrations in with S9 is in 4.34 ±1.15and without S9 is in 3.34.±1.527 cells 2.00±1.00 and 2.34 ± 0.57 and Latex induced chromosomal and chromatid aberrations with metabolic activator in 4± 0.82 and 4.5±0.58 cells and without S9 is in 3.00±1.00 and 3.34±0.56 respectively. Chromosomal aberration like chromatid gap, chromosome break and other chromosomal aberration has occurred in Cyclophosphamide and Mitomycin as shown in (figure 34 a, and 34b).When compared to the HA-EV, HA-BG , Latex extract treated, and Dental material, both in the absence and presence of metabolic activator (Fig. 34 c,34 d,34e,34f,34g,34h, and 34,i). The chromosomal architecture of lymphocytes treated with HA-

BG and HA-EV showed a more or less normal pattern with mild degree of chromosomal aberrations almost comparable to negative control treated groups. The positive controls CP (100µg/5ml of culture) and MC (1µg/5ml of culture) induced significant increase in the number of cells with chromosomal aberrations in their respective experiments.

- ***In vivo* study**
- **Lipid peroxidation**

HAP-EVA

The LPO level in liver homogenates of control and four different observation period of rabbits implanted with BGA are demonstrated in figure 35. Similar to that of EVA the amount of malondialdehyde in 4th week (22.76nmol/mg protein) was seemed to be increased when compared with the respective control. The concentration of LPO in the later periods was decreasing and observed to reach the control values (15.99nmol/mg protein). All the periods were found to be slightly significant $p \leq 0.01$.

HA-BG

The amount of malondialdehyde in control and four different observation period of rats implanted with the biomaterial EVA is demonstrated in figure 36. The level of LPO present in the 4th week (44.7 nmol/mg protein) is observed to be higher than the control value (35.12nmol/mg protein). The amount found to be decreasing in the subsequent period. Except 26th week all other periods were found to be slightly significant ($p \leq 0.01$).

Dental composite

The results of these LPO level was measured at the end of each observation period from the respective group of animals are depicted in figure 37. It is evident from the results that the experimental animals implanted with DC pins did not endure any significant oxidative stress even for longer observation periods.

Latex

In the *in vivo* study, MDA products were insignificantly increased in 4th week (5.90nmol/mg protein)&12th week (6.90 nmol/mg protein) than control(5.96 nmol/mg protein). But MDA level was back to the control value at the 52nd week (4.76 nmol/mg protein) shown in figure 38.

Fibrin glue

Figure 39 depicts the level of LPO in the control and fibrin glue treated rats of four different observation period. The level of LPO in the 4th week (27.54nmol/mg protein) was lower than the control (28.86nmol/mg protein) and it was found to be increasing till 26th week (32.51nmol/mg protein). Interestingly, impaired hepatic antioxidant enzymes activities were brought back to control at the end of the observation period. Results at all the time periods were found to be insignificant while relating with the control values.

- **Reduced Glutathione**

HAP-EVA

Figure 40 depicts the concentration of GSH in control and four different observation period of rats implanted with the biomaterial EVA. The amount of GSH in the 4th week was 4.04860nmol/mg protein and seemed to be increasing in the succeeding periods. The concentration of GSH in the 52nd week (4.183nmol/mg protein) is found to be similar to that of the control value. All the period were found to be non significant $p \geq 0.05$.

HA-BG

The GSH concentration in control and four different observation periods of rabbits implanted with BGA is represented in figure 41. GSH concentration in liver homogenate was observed to be increasing in all the observation period when compared to that of the control value 5.971nmol/mg protein. All the periods were found to be slightly significant $p \geq 0.001$.

Dental material

The results of GSH measured at the end of each observation period from the respective group of animals are depicted in figure 42. It is evident from the results that the experimental animals implanted with DC pins did not endure any significant oxidative stress even for longer observation periods

Latex

The reduced glutathione activity in latex implanted rats exhibited statistically non significant increase in 12th week (8.89 nmol/mg protein) as compared with control (8.89nmol/mg

protein) as shown in figure 43. It is evident from the results that experimental rats did not endure any significant stress even for longer observation period.

Fibrin glue

The amount of GSH in rats subjected to fibrin glue implantation and the control values is shown in figure 44. The concentration of GSH in all the observation periods were found to be similar to that of the control values except the deduction observed in the levels of GSH in the 4^{th} (3.57nmol/mg protein) and 26^{th} week (3.28nmol/mg protein) which might be due to a stress created as a result of implantation. In the 52^{nd} (3.7nmol/mg) protein week of observation the amount of intracellular GSH seemed to increase and reached up to the control. The p-value of all the observation periods was shown to be insignificant with that of the control.

- **Glutathione reductase**

HAP-EVA

The concentration of GR in rats liver homogenate implanted with EVA and control are shown in figure 45. The amount of GR in the control (0.61514 units/mg protein) and 26^{th} week were observed to be similar. The level of GR in 4^{th} week was decreased to 0.5567 units/mg protein and increased to 0.625 units/mg protein when compared with the control. All the period were found to be nonsignificant $p \geq 0.05$.

HA-BG

Figure 46 shows the level of GR in control and four different observation period of rabbit implanted with BGA. GR concentration found to be increasing in all the observation period with that of the respective control value (0.699 units/mg protein). The concentration of GR in 12^{th} week was found to be slightly significant $p \geq 0.01$ and all other periods were non significant with the control value $p \geq 0.05$.

Dental composite

Glutathione activity was measured at the end of each observation period from the respective group of animals are depicted in figure 47. It is evident from the results that the experimental animals implanted with DC pins did not endure any significant oxidative stress even for longer observation periods.

Latex

The *in vivo* studies of the latex material were carried out using implanted material in different batches of wistar rats as described above. Due to the oxidative stress, GR activity was non significantly decreased in 12^{th} week (0.563 U/mg protein) than control (0.58 U/mg protein) and other observation period, shown in figure 48. But all the periods were observed to be statistically non significant ($p \geq 0.05$).

Fibrin glue

Figure 49 illustrates the level of GR activity in un implanted and fibrin glue implanted rats. During the 4^{th} week (1.83 U/ mg protein), the concentration of GR was enhanced due to the stress generated by implantation. But it seemed to be decreasing in the 12^{th} week (0.069 U/ mg protein). At the end of the observation period the GR concentration increases and reaches to the control values. All the periods were observed to be significant ($p \geq 0.05$).

- **Glutathione peroxidase**

HAP-EVA

GPx expression in the liver homogenate of control and rats implanted with EVA are represented in figure 50. The level of GPx in the 4^{th} week (0.443 units/mg protein) is found to be increased when compared with that of the respective control (0.437 units/mg protein). But the level observed to be decreased in the 12^{th} week (0.415 units/mg protein) and increasing in the following period. All the observation period were found to be nonsignificant $p \geq 0.05$.

HA-BG

Figure 51 demonstrates the level of GPx concentration in the liver homogenate of control and the four different observation periods of rabbits implanted with BGA. The expression of GPx in the 4^{th} week (0.638 units/mg protein) was increased when compared with that of the control value (0.218 units/mg protein) and seemed to be decreasing in the subsequent weeks. The 12^{th} and 26^{th} weeks were observed to be slightly significant with the control $p \geq 0.01$ and the 4^{th} and 52^{nd} week were found to be non significant $p \geq 0.05$.

Latex

Figure 52 illustrates the level of GPx concentration in the liver homogenate of control and the five different observation periods of rabbits implanted with latex. The liver homogenate of the

implanted rats were used for *in vivo* study. When compared with control, GPx activity in liver was slightly increased (0.29U/mg protein .0.43 mg/protein, 0.52 U/mg protein, 0.59 U/mg protein, 0.52 U/ mg protein , 0.56U/ mg protein and ,0.59U/mg protein respectively, but they are statically not significant.($p \geq 0.05$.)

Fibrin glue

The amount of GPx expressed in all the four observation status of rats treated with fibrin glue was compared with control values and is represented in figure 53. The levels were found to be increasing in all the periods of observation as 0.241U/ mg protein, 0.249U/ mg protein, 0.32 U/ mg protein and 0.504U/ mg protein. But the increase in values showed only slight significance ($P \geq 0.05$) with the control.

- **Agarose gel electrophoresis of nuclear and mitochondrial DNA**

Clear bands were obtained on agarose gel corresponding to nuclear DNA and mitochondrial DNA was shown in figure 54.

- **Confirmation of nuclear DNA by Polymerase chain reaction.**

Total mtDNA was isolated by nuclear DNA isolation kit (QIAGEN,) was analyzed by PCR, A substantial 691bp amplification product corresponding to the mitochondria genome detected was shown in figure 55.

- **Confirmation of mitochondrial DNA by Polymerase chain reaction**

Total mtDNA was isolated by mtDNA isolation kit (WAKO) was analyzed by PCR, A substantial 630bp amplification product corresponding to the mitochondria genome detected was shown in figure 56.

- **Quantitation of nuclear DNA damage by 8- Hydroxy-2-Deoxyguanosine ELISA.**

HAP-EVA

Figure 57 depicts the amount of 8-OHdG in the genomic DNA isolated from the control and four different observation period of rats brain implanted with EVA. The concentration of 8-OHdG was found to be increasing in the 4^{th} week (2.226 8-OHdG/ 10^5dG) and decreasing in the 12^{th} week (2.209 8-OHdG/ 10^5dG) when compared to that of the control (2.2185 8-OHdG/ 10^5 dG). The level found to be increasing in the succeeding periods. Except 12^{th} week all other periods were found to be non significant $p \geq 0.05$.

HA-BG

The concentration of 8-OHdG in the genomic DNA isolated from the control and four different observation period of rabbits brain implanted with BGA is presented in figure 58. The expression of 8-OHdG was found to be increasing in all the observation period. The level observed to be decreased in the 4^{th} week (1.7205 8-OHdG/ 10^5 dG) and increasing in the following weeks when compared with the control value (1.8358-OHdG/ 10^5 dG). All the weeks observed to be non significant ($p \leq 0.05$).

Dental composite

The oxidative DNA damage was analyzed in muscle tissue at the implantation site of both control animals and test animals. Figure 59 depicts that there was no significant change in the concentration of DNA adduct formed in test animals when compared to negative control animal. Hence our results suggests that both short and long term implanted polymerized DC pins did not elicit any ill effects to the animal.

Latex

Figure 60 depicts that ,8-OHdG level was slightly increase in of 12^{th} week (27.42 8-OHdg/10^5 dG) than control (un implanted) (18.35 8-OHdg/10^5dG) From *in vivo* study, 8-OHdG levels was found to any significant changes has not occurred in any period as compared with control ($p \geq 0.05$).

Fibrin glue

Figure 61 demonstrates the expression of 8-OHdG at different observation periods together with the control. It was observed that the concentration of 8-OHdG in the brain tissues of rats (160.66 8-OHdG/ 10^5 dG, 166.75 8-OHdG/ 10^5dG, 172 8-OHdG/ 10^5 dG, 182.5 8-OHdG/ 10^5 dG respectively) at different observation periods showed a slight significance when compared with the control values (220 8-OHdG/ 10^5dG) at all the four observation periods.

- **Quantitation of mitochondrial DNA damage by 8- Hydroxy-2-Deoxyguanosine ELISA.**

HAP-EVA.

Total mtDNA was isolated from HA-EVA implanted brain tissue of different period such as 1st week,4th week,12th week,26th week and 52nd week, that was subjected to 8-OHdG ELISA for the measurement of mitochondrial damage due to oxidative stress. The amount of 8-OHdG in DNA was non significantly increased at 1st week.(3.175 8-OHdG/ 10^5 dG) 4th week 3.3 8-OHdG/ 10^5 dG) 12th week (3.39 8-OHdG/ 10^5 dG), 26th week(3.738-OHdG/ 10^5 dG) and 52nd week respectively than control (3.12 8-OHdG/ 10^5 dG) as shown in figure 62.

HA-BG

Figure 63 shows the level of 8-OHdG content of implanted HA-BG bone tissue of 12th week (2.98 8-OHdG/ 10^5 dG), 26th week,(3.05 8-OHdG/ 10^5 dG)and 52nd week (3.3 8-OHdG/ 10^5 dG) was more than control (2.83 8-OHdG/ 10^5 dG) and beginning period (2.828-OHdG/ 10^5 dG).).But it was statically nonsignificant than control. ($p \geq 0.05$).

Dental material

Total mtDNA was isolated from Dental material implanted muscle tissue of different period such as 1st week,4th week,12th week,26th week and 52nd week, that was subjected to 8-OHdG ELISA for the measurement of mitochondrial DNA damage due to oxidative stress. Figure 64 shows the mean level of 8-OHdG content of different periods (3.8 8-OHdG/ 10^5 dG, 3.818-OHdG/ 10^5 dG ,3.8 8-OHdG/ 10^5 dG , and 3.698-OHdG/ 10^5dG was lower than that control value 3.88 8-OHdG/10 ^{5}dG.

Latex

As shown in Figure 65, 8-OHdG content was increase , at 1st week(2.658-OHdG/ 10^5 dG), 4th week (2.728-OHdG/ 10^5dG) and 12th week(2.88 8-OHdG/ 10^5 dG) than control (2.632 8-OHdG/ 10^5 dG) which progressively decreased at the end of the observation period (2.618-OHdG/ 10^5 dG).

Fibrin glue

Figure 66 depicts that the level of 8-OH dG was slightly decreased than control at 1st week(2.718-OHdG/ 10^5 dG),4th week(2.96 8-OHdG/ 10^5 dG), and 12th week (3.125 8-OHdG/ 10^5 dG), than control (3.358-OHdG/ 10^5 dG) But at the end of the observation period ,the level was progressively increased at the end of observation period at 52nd week (3.4 8-OHdG/ 10^5 dG),than any other period, because due to the stress.

- **Micronucleus**

MNPCE frequencies and the PCE/NCE ratios at 24 and 48 hours in single dose of physiological saline extract of five different biomaterials, physiological saline alone and cyclophosphamide are depicted in figure 67 and 68. Table 5 and 6 demonstrates the results of micronucleus test at 24hr and 48hr. Positive control animals exposed to cyclophosphamide showed a significant increase in MN frequency in both 24hr (2.592 % MN/2000 PCE) and 48 hr (2.333 % MN/2000 PCE) after treatment when compared with negative control animals treated with physiological saline ($p \geq 0.05$). In a similar manner, the MN frequencies at physiological saline extract of all the five different biomaterials were comparable ($p \leq 0.001$) to the negative control in both 24hr and 48hr after treatment (0.083 % MN/2000 PCE).

- **Chromosomal Aberrations**

No significant change in the mitotic indices were detected in different groups treated with physiological saline extract of biomaterials (figure 69 and 70) when compared with their control group. The results obtained from the chromosomal aberration study of positive and negative control and the test materials are represented in table 6 and 7 (24hr) and 6 (48hr). The percentage of chromosomal break in the latex material shows slight significance ($p \leq 0.01$) in both 24hr (0.096% of chromosomal break in 100 metaphase/ animal) and 48hr (0.025 % of chromosomal break in 100 metaphase/ animal). The percentages of chromatid gap formed in 24h and 48h are shown in table 7 and 8. All the other four biomaterials were found to be statistically insignificant when compared with the negative control animals ($p \geq 0.05$). Similarly positive control cyclophosphamide induced a statistically significant percentage of breaks and gaps.

Conclusions

The biocompatibility or safety evaluation of materials/medical devices have largely been reported in terms of its biological response induced by the physical presence of materials or chemical leachants from the materials. Recently, there is an emergent anxiety that the long term use of medical devices/implants can contribute to changes in DNA structure. The chemical leachants may have an influence on oxidative stress, antioxidant enzymes and lipid

peroxidation. The results on the toxicity of materials at the molecular level indicated that all the biomaterials used for study (HA-BG, HA-EVA, Dental material, Latex and fibrin glue) did not induce any lipid peroxidation both in *in vitro* or *in vivo* methods. There was no alternation observed in the levels of (both in vitro and in vivo) GR, GSH and GPx. The treatment of material extracts or implantation of material (HA-BG, HA-EVA, Dental material, latex material and fibrin glue) did not show any damaging effects on nDNA or mtDNA. The effect of genotoxicity by *in vitro* using cultured human peripheral blood lymphocytes with and without metabolic activation demonstrated that the physiological saline extract of HA-EVA, HA-BG, Dental material and Latex was comparable with negative control values. It was also observed that the physiological saline extract of all the materials did not induced the any chromosomal anomalies or micronuclear potential in mice. The PCE and NCE ratio indicated that the material extract showed no influence on the cell proliferation. The results of the study emphasis that the physical presence of the material or chemical leachants of the material did not induce any toxic effects at the molecular level and established that all the material used were truly safe, thus fulfilling the promise of utmost safety in health care. Hence it can be concluded that the evaluation of toxicity at the molecular level is a cardinal change in approach to biocompatibility evaluations leading to a paradigm shift in bringing in newer regulations for development of safer medical devices, implants and tissue engineered organs for life time application.

FIGURES

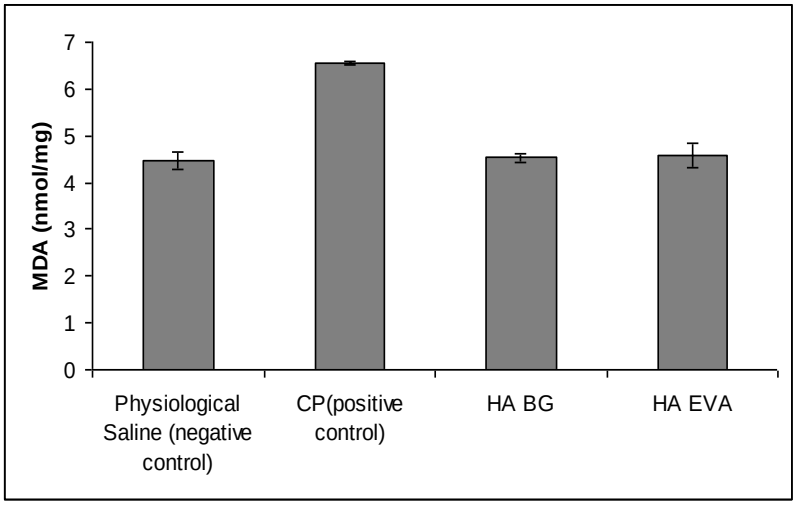


Figure 1: Concentration of malondialdehyde in liver homogenate incubated with Cyclophosphamide and physiological saline extracts of HA BG and HA EVA .Values are expressed as mean±S.D, $n = 3$. $p \leq 0.05$

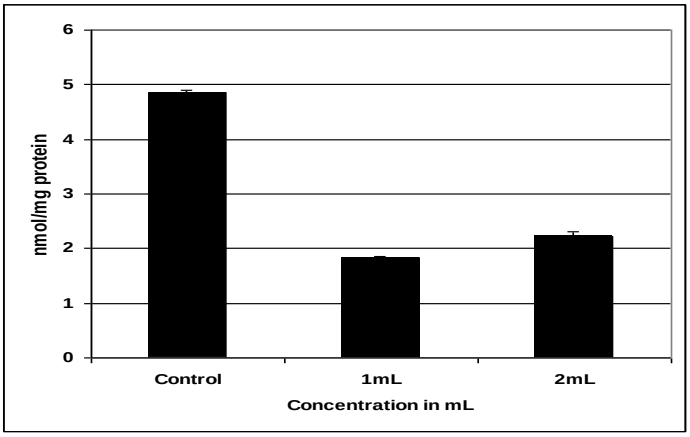


Figure 2: Concentration of malondialdehyde in liver homogenate incubated with different concentration of physiological saline extracts of Dental material .Values are expressed as mean±S.D, $n = 3$. $p \leq 0.05$

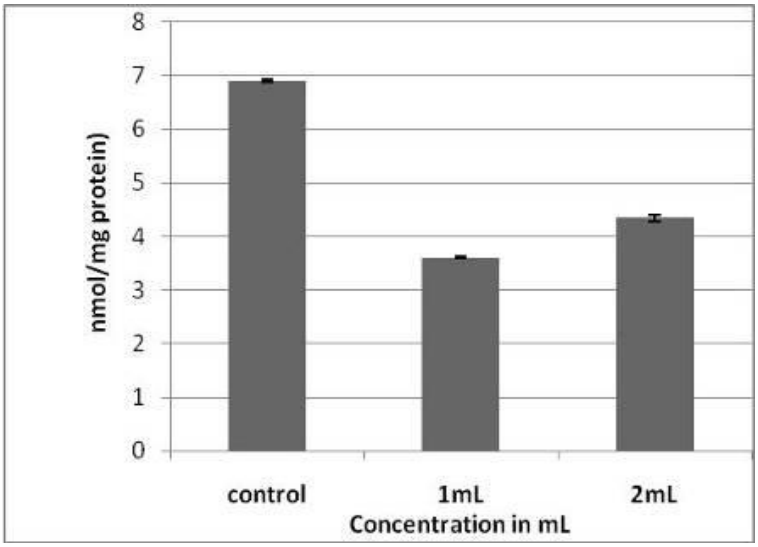


Figure 3 : LPO levels in 10% mice liver homogenate treated with physiological saline (control) and two different concentrations of physiological saline extract of latex incubated for 30, min. Data were represented as mean ± SD. (n=3)

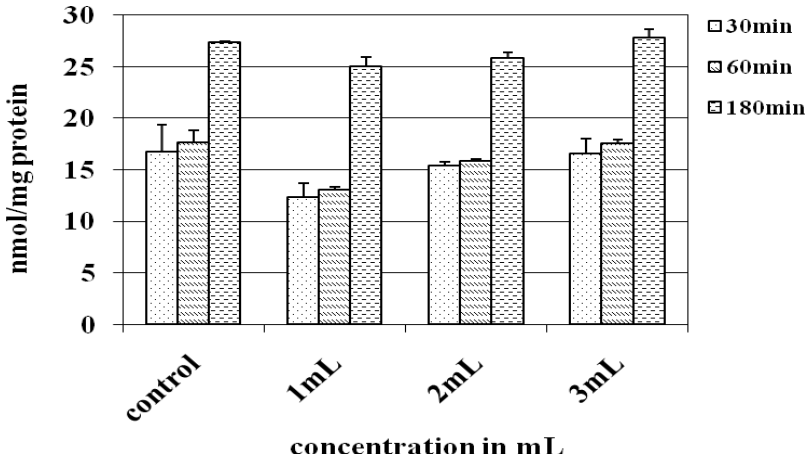


Figure 4: LPO levels in 10% mice liver homogenate treated with physiological saline (control) and three different concentrations of physiological saline extract of fibrin glue incubated for 30, 60 and 180 min. Data were represented as mean ± SD. (n=8)

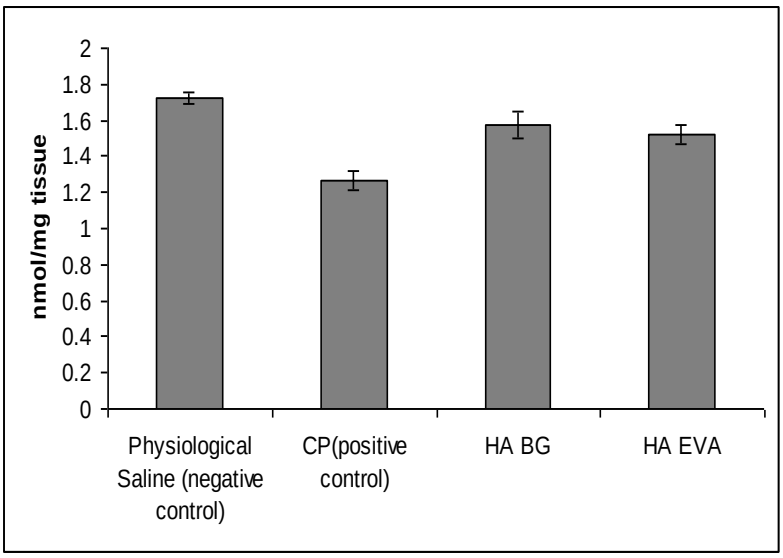


Figure 5 : Reduced glutathione levels in liver homogenate incubated with CP and extracts of HA BG and HA EVA. Values are expressed as mean±S.D, $n = 3$. $p \leq 0$

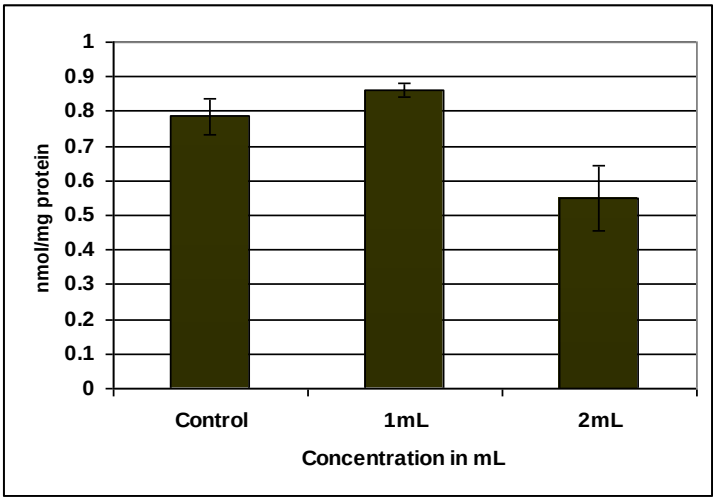


Figure 6 : Reduced glutathione levels in liver homogenate incubated with physiological saline and extracts of Dental material . Values are expressed as mean ± S.D, $n = 3$. $p \leq 0$

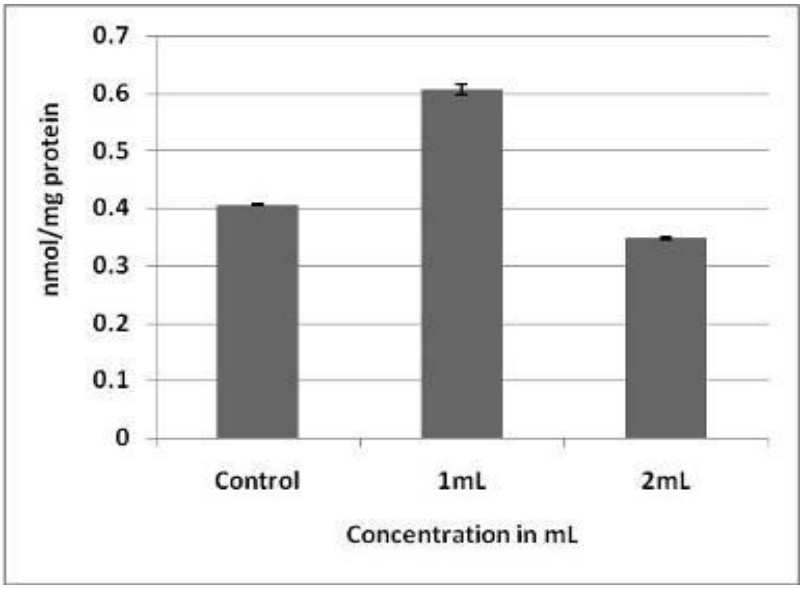


Figure 7: The amount of GSH in 10% mice liver homogenate treated with physiological saline (control) and three different concentrations of physiological saline extract of Latex incubated for 30, min. Data were represented as mean ± SD. (n=8)

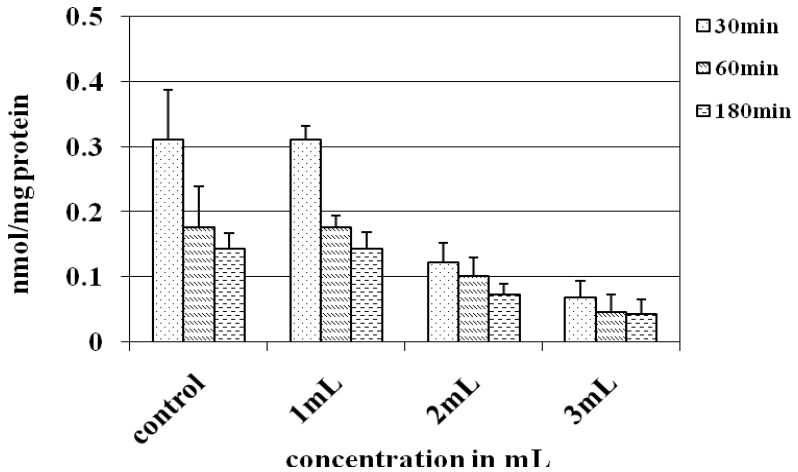


Figure 8: The amount of GSH in 10% mice liver homogenate treated with physiological saline (control) and three different concentrations of physiological saline extract of Latex incubated for 30, min. Data were represented as mean ± SD. (n=8)

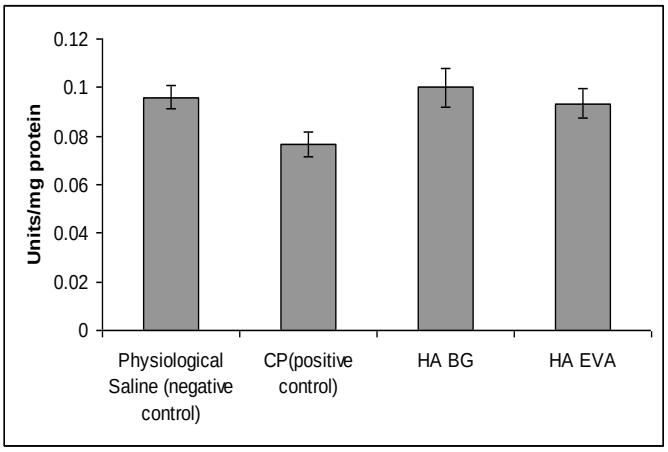


Figure 9 : Levels of Glutathione reductase in liver homogenate incubated with CP and extracts of HA BG and HA EVA. Values are expressed as mean ±S.D, $n = 3$. $p \leq 0.05$.

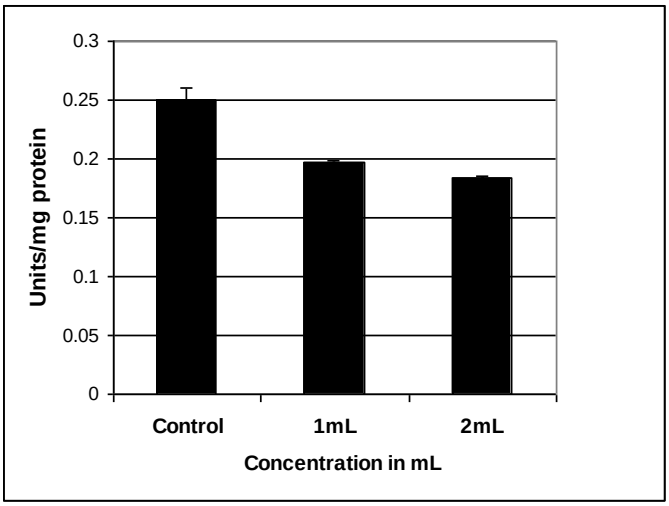


Figure 10: Levels of Glutathione reductase in liver homogenate incubated with physiological saline extracts of Dental material. Values are expressed as mean ±S.D, $n = 3$. $p \leq 0.05$.

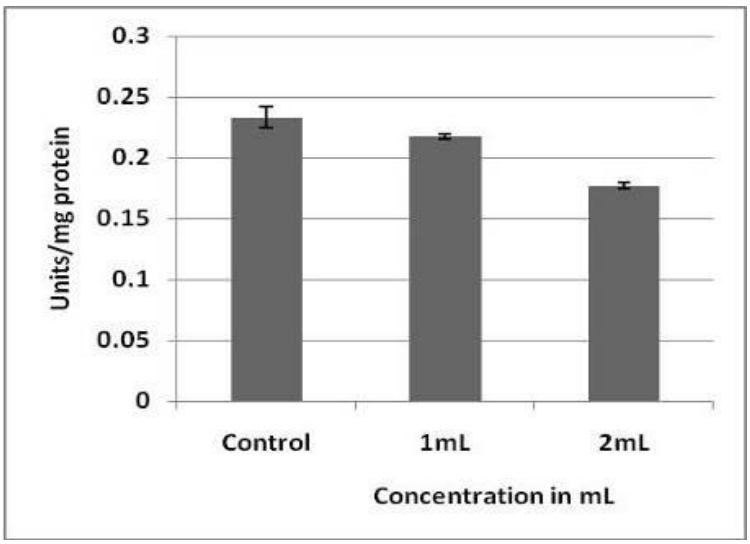


Figure 11: Activity of GR in 10% mice liver homogenate treated with physiological saline (control) and three different concentrations of physiological saline extract of latex incubated for 30, minute. Data were represented as mean ± SD. (n=3)

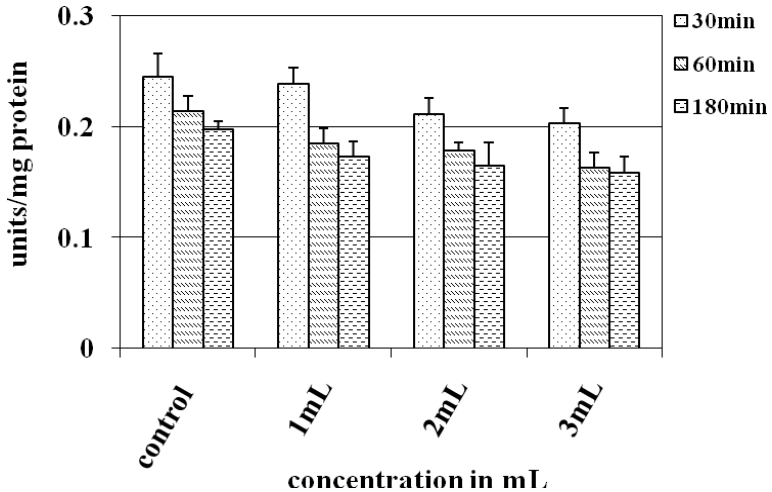


Figure 12 : Activity of GR in 10% mice liver homogenate treated with physiological saline (control) and three different concentrations of physiological saline extract of fibrin glue incubated for 30, 60 and 180min. Data were represented as mean ± SD. (n=8)

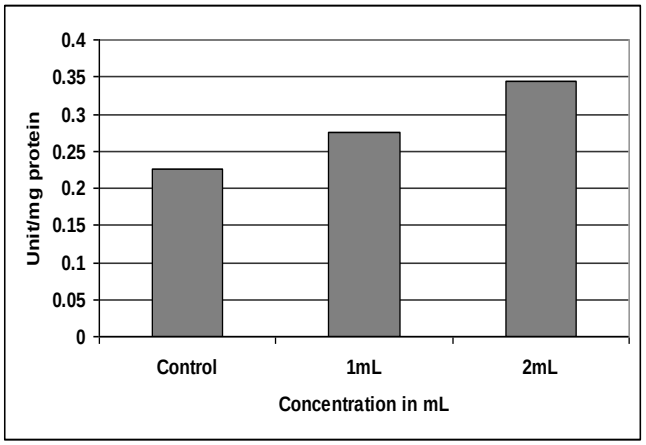


Figure 13 :Concentration of GPx in 10% mice liver homogenate treated with control (physiological saline) and three different concentrations of physiological saline extract of Latex incubated for 30 min. Data were represented as mean ± SD. (n=3).

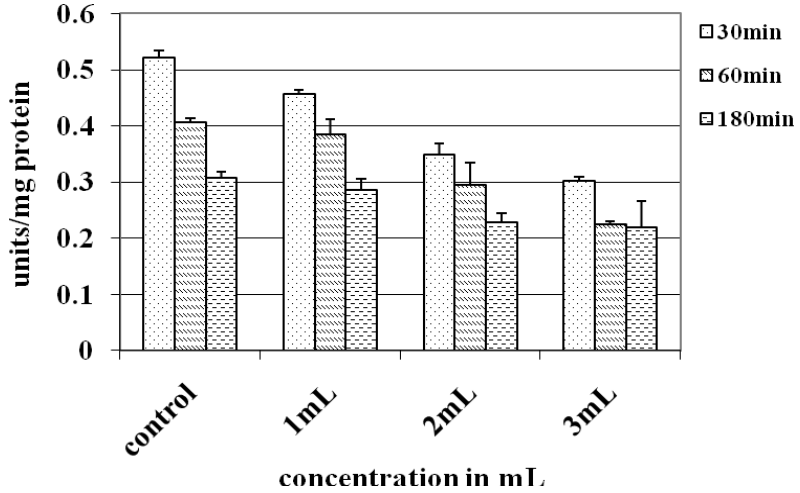


Figure 14 : Concentration of GPx in 10% mice liver homogenate treated with control (physiological saline) and three different concentrations of physiological saline extract of fibrin glue incubated for 30, 60 and 180min. Data were represented as mean ± SD. (n=8).

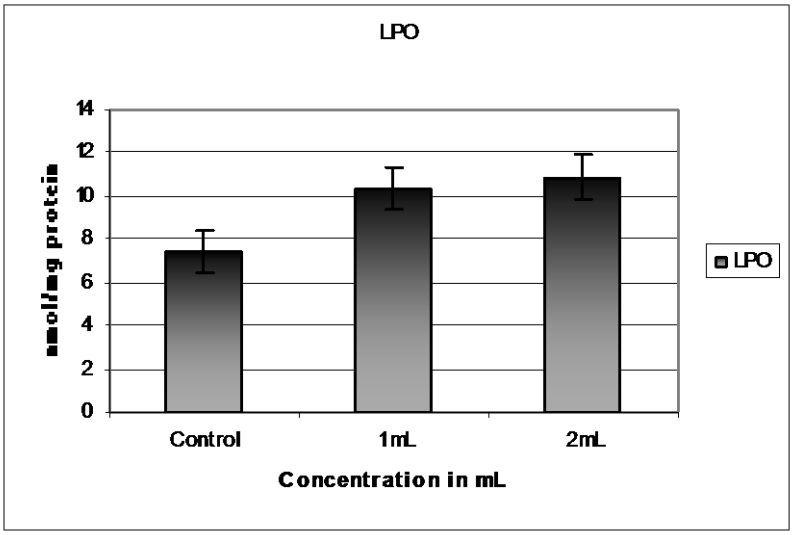


Figure 15: LPO levels in 10% mice liver homogenate treated with artificial saliva (control) and two different concentrations of artificial salival extract of HAP-EVA material incubated for 30 min. Data were represented as mean ± SD. (n=3)

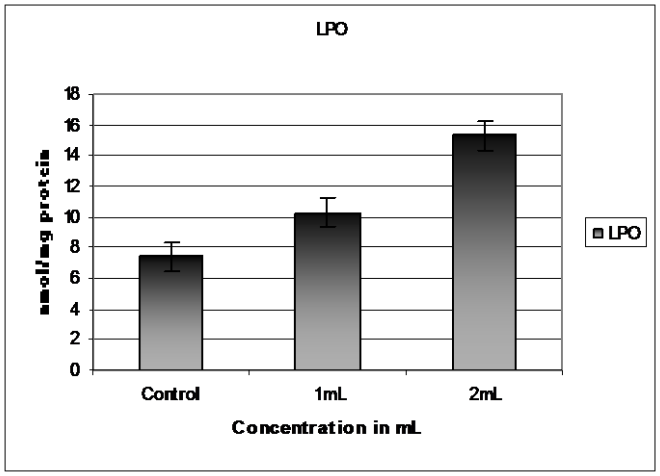


Figure 16: LPO levels in 10% mice liver homogenate treated with artificial saliva (control) and two different concentrations of artificial salival extract of HAP-BGA material incubated for 30 min. Data were represented as mean ± SD. (n=3)

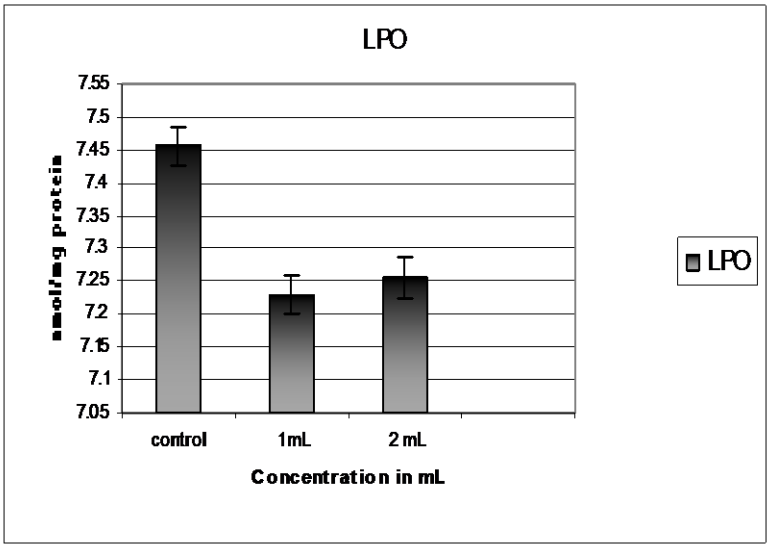


Figure 17: LPO levels in 10% mice liver homogenate treated with artificial saliva (control) and two different concentrations of artificial salival extract of Dental material incubated for 30 min. Data were represented as mean ± SD. (n=3)

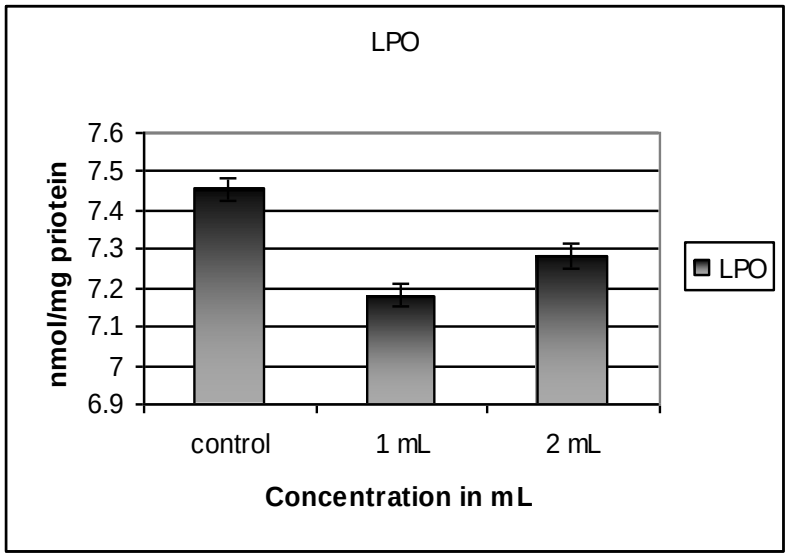


Figure 18: LPO levels in 10% mice liver homogenate treated with artificial saliva (control) and two different concentrations of artificial extract of latex material incubated for 30 min. Data were represented as mean ± SD. (n=3).

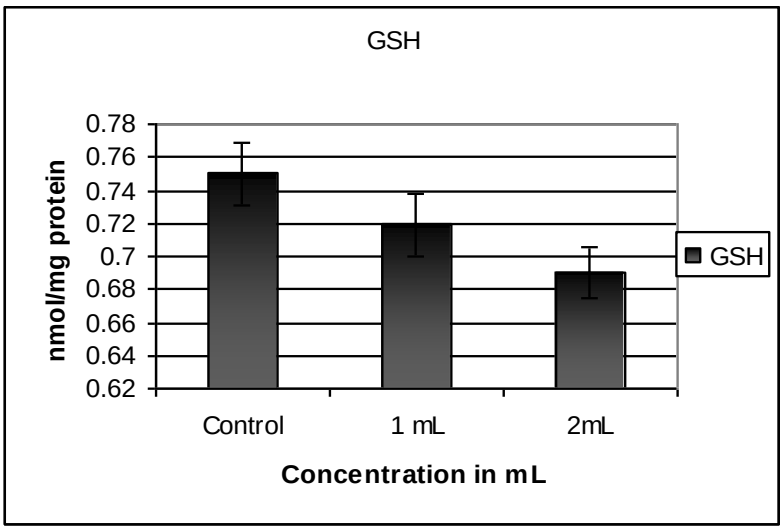


Figure 19: Reduced glutathione levels in liver homogenate incubated with artificial salival extracts of HAP-EVA material. Values are expressed as mean±S.D, $n = 3$. $p \leq 0$

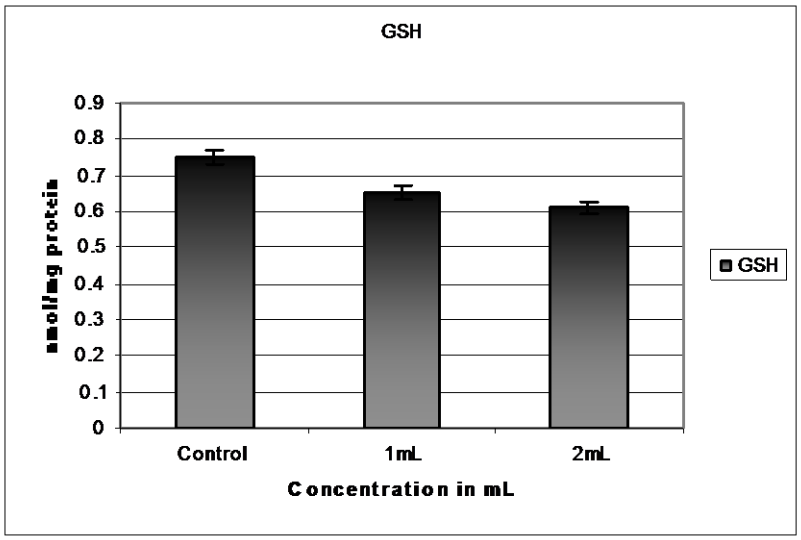


Figure 20: Reduced glutathione levels in liver homogenate incubated with artificial salival extracts of HA-BGA material . Values are expressed as mean±S.D, $n = 3$. $p \leq 0$

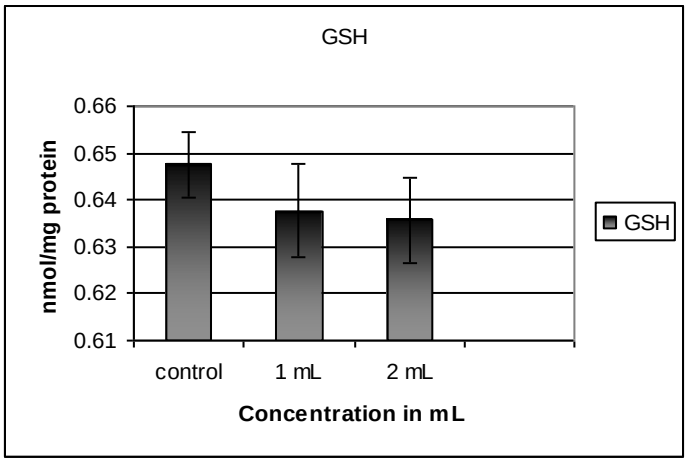


Figure 21: Reduced glutathione levels in liver homogenate incubated with artificial salival extracts of Dental material . Values are expressed as mean±S.D, $n = 3$. $p \leq 0$

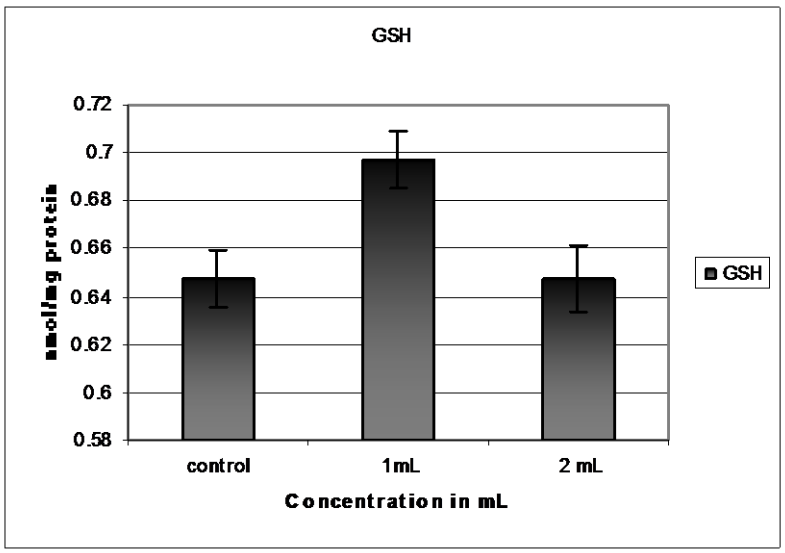


Figure 22: Reduced glutathione levels in liver homogenate incubated with artificial saliva and Latex material. Values are expressed as mean±S.D, $n = 3$. $p \leq 0$

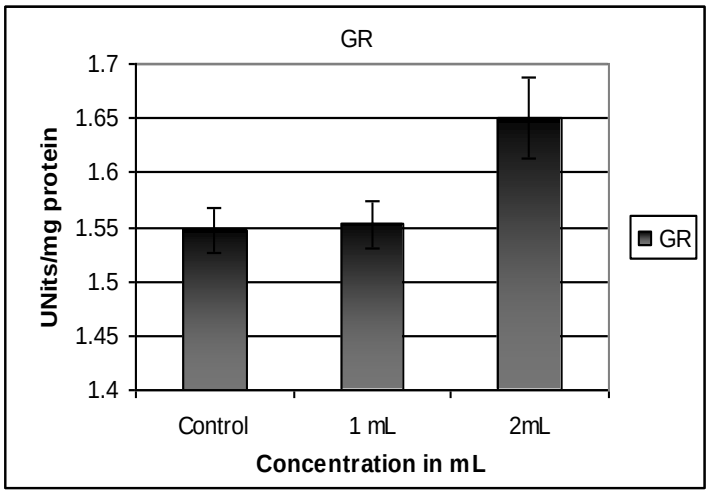


Figure 23: Levels of Glutathione reductase in liver homogenate incubated with artificial saliva and extracts of HAP-EVA. Values are expressed as mean ±S.D, $n = 3$. $p \leq 0.05$.

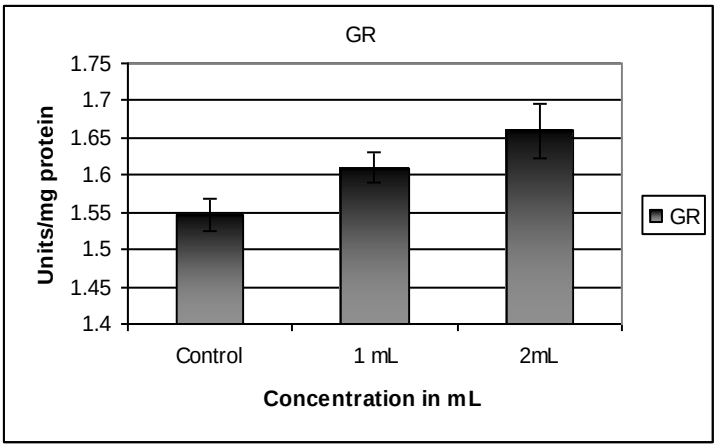


Figure 24 : Levels of Glutathione reductase in liver homogenate incubated with artificial saliva and extracts of HABGA. Values are expressed as mean ±S.D, $n = 3$. $p \leq 0.05$.

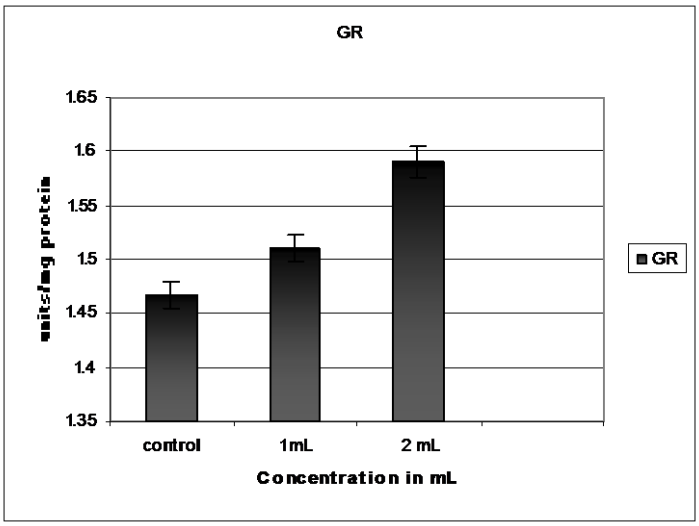


Figure 25: Levels of Glutathione reductase in liver homogenate incubated with artificial saliva and extracts of Dental material . Values are expressed as mean ±S.D, $n = 3$. $p \leq 0.05$.

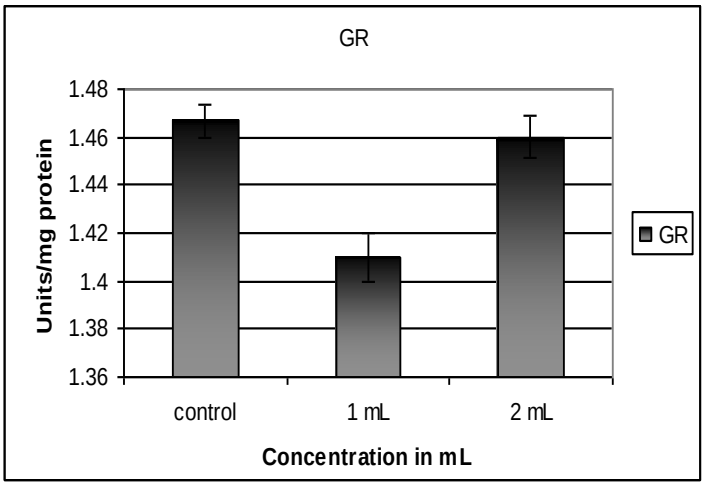


Figure 26: Levels of Glutathione reductase in liver homogenate incubated with artificial saliva and extracts of Latex. Values are expressed as mean ±S.D, $n = 3$. $p \leq 0.05$

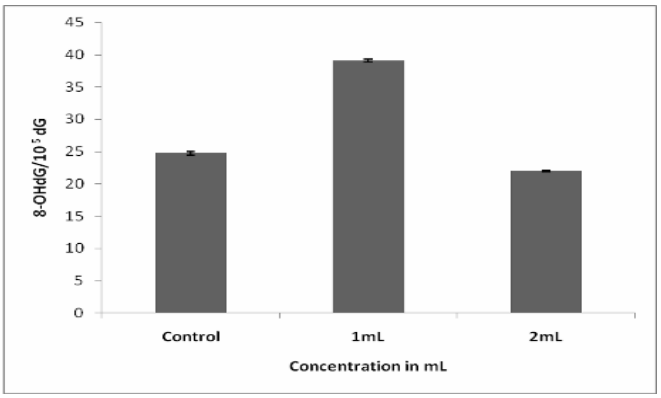


Figure 27: Comparison of 8-OHdG formation on rat brain genomic DNA when treated with physiological saline (control) and two different concentrations of physiological saline extract of latex material. Data were represented as mean ± SD. (n=3)

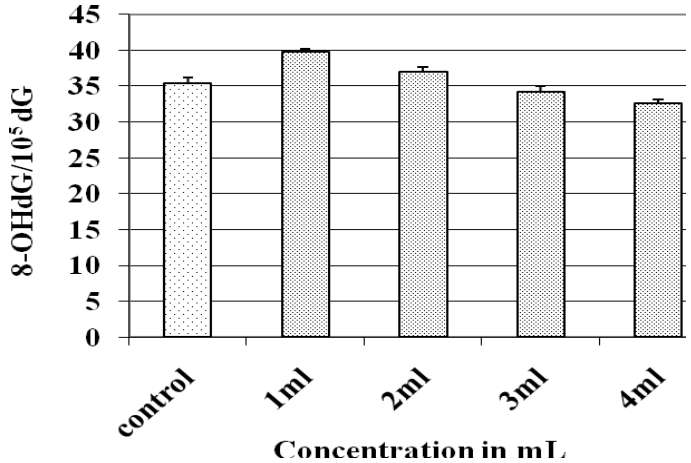


Figure 28: The comparison of 8-OHdG formation on rat brain genomic DNA when treated with physiological saline (control) and four different concentrations of physiological saline extract of fibrin glue. Data were represented as mean ± SD. (n=8)

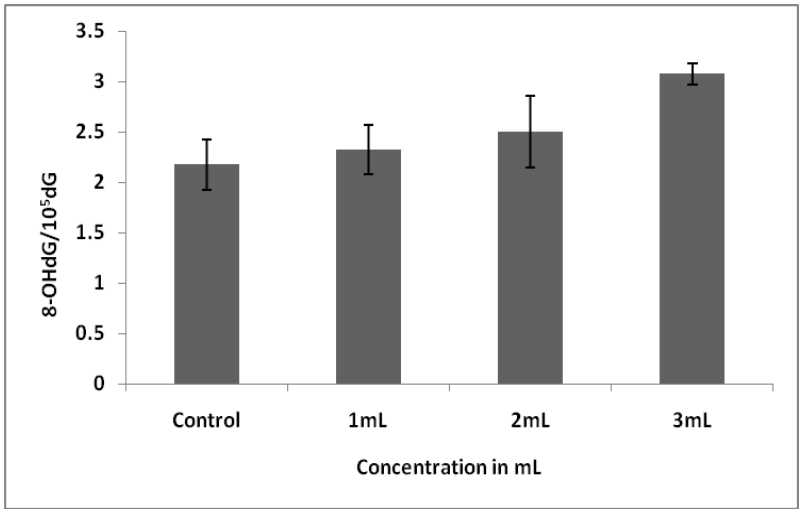


Figure 29. Comparison of 8-OHdG formation on rat brain mitochondrial DNA when treated with physiological saline (control) and three different concentrations of physiological saline extract of HAP-EVA incubated for 30 minutes. Data were represented as mean ± SD. (n=6)

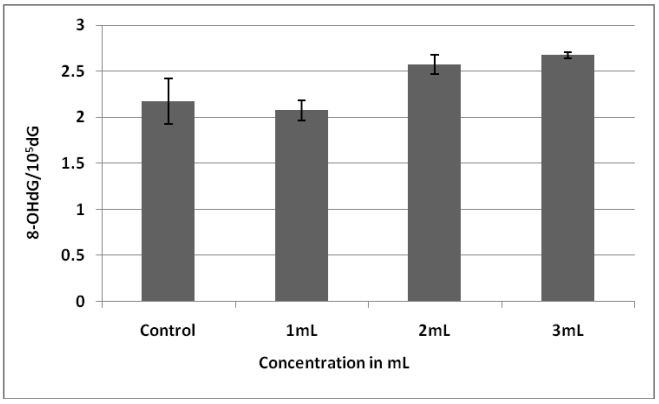


Figure 30: Comparison of 8-OHdG formation on rat brain mitochondrial DNA when treated with physiological saline (control) and three different concentrations of physiological saline extract of fibrin glue incubated for 30 minutes. Data were represented as mean ± SD. (n=6)

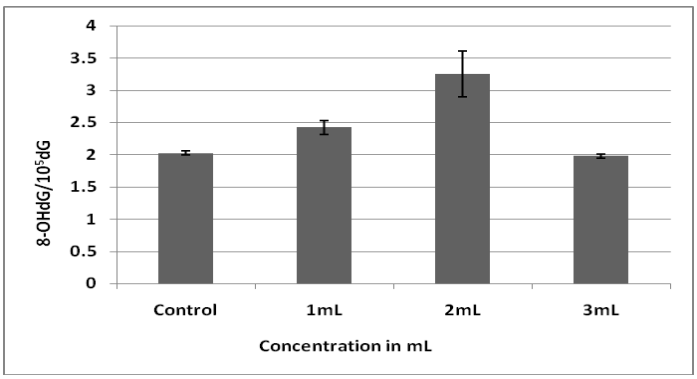


Figure 31: Comparison of 8-OHdG formation on Rabbit bone mitochondrial DNA when treated with physiological saline (control) and three different concentrations of physiological saline extract of HABG incubated for 30 minutes. Data were represented as mean ± SD. (n=6)

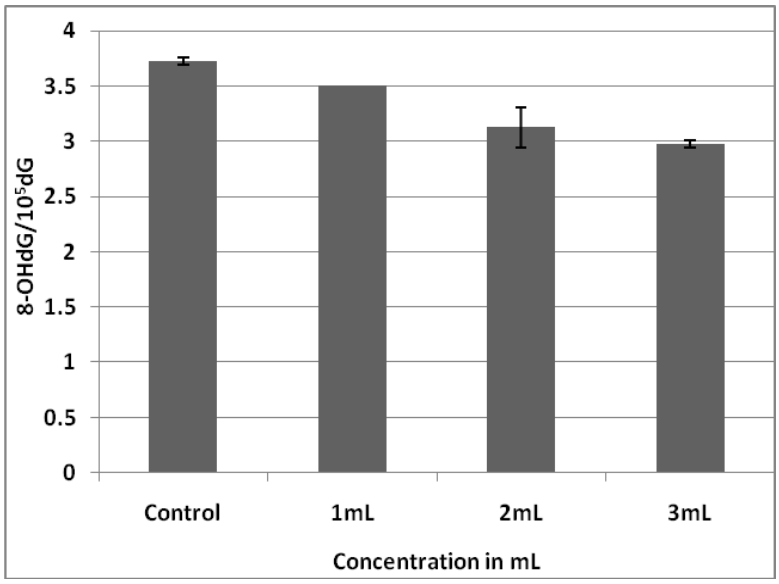


Figure 32: Comparison of 8-OHdG formation on rat muscle mitochondrial DNA when treated with physiological saline (control) and three different concentrations of physiological saline extract of dental material incubated for 30 minutes. Data were represented as mean ± SD. (n=6)

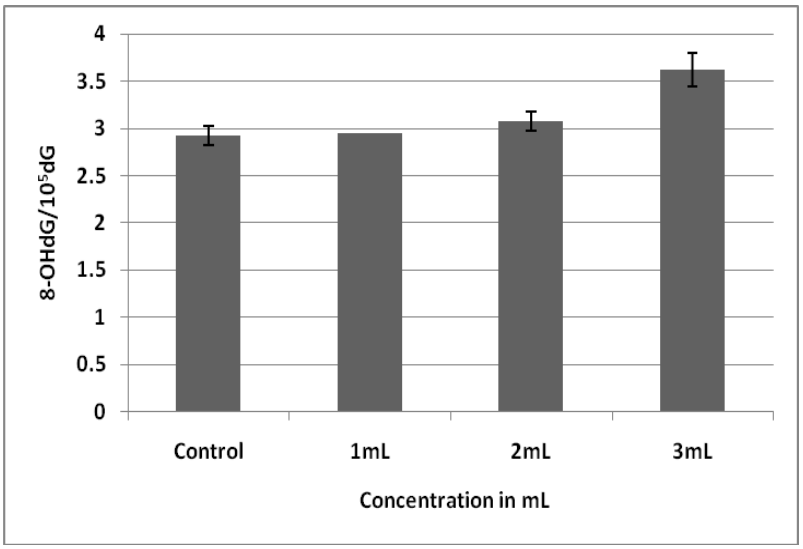
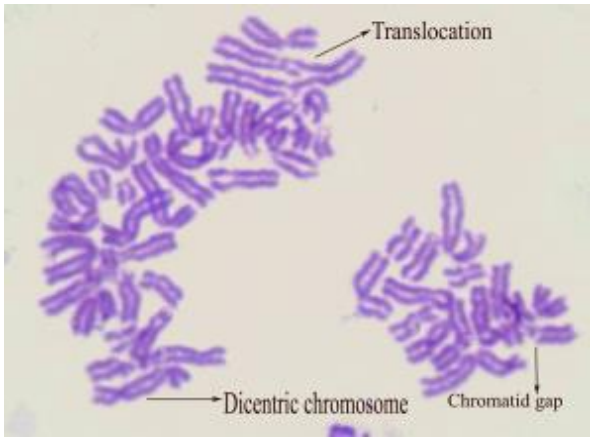
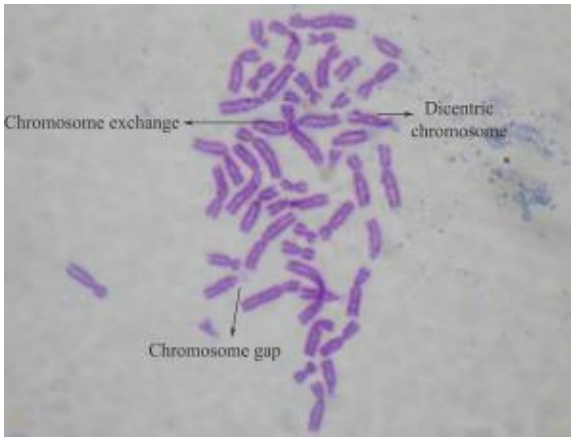


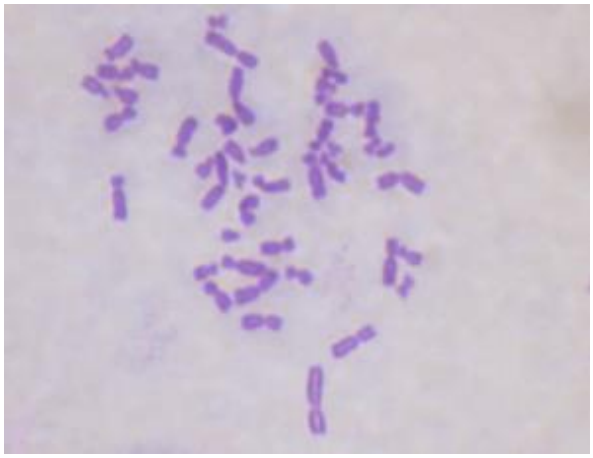
Figure 33: Comparison of 8-OHdG in e subcutaneous tissue mtDNA in physiological saline (control) and three different concentrations of physiological saline extract of Latex material incubated for 30 minutes. Data were represented as mean ± SD. (n=6)


Translocation
Dicentric chromosome
Chromatid gap

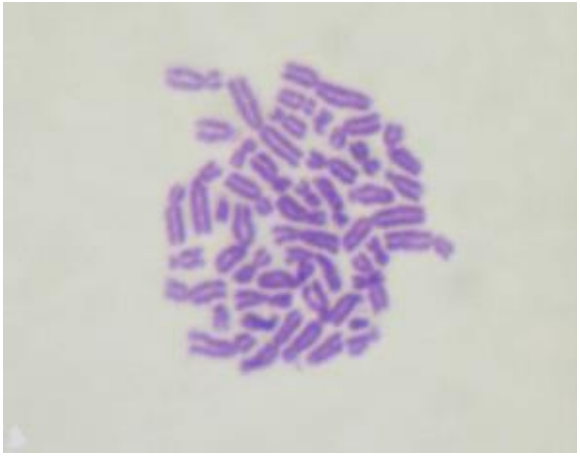
a


Chromosome exchange
Dicentric chromosome
Chromosome gap

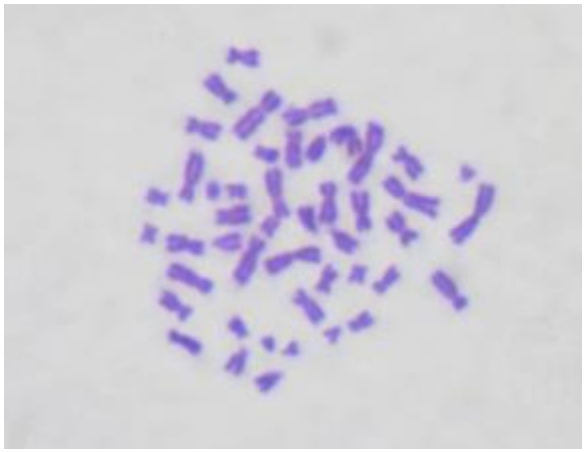
b



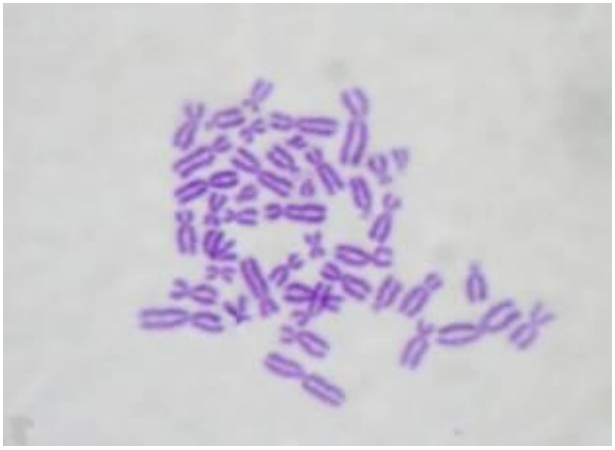
c



d



e



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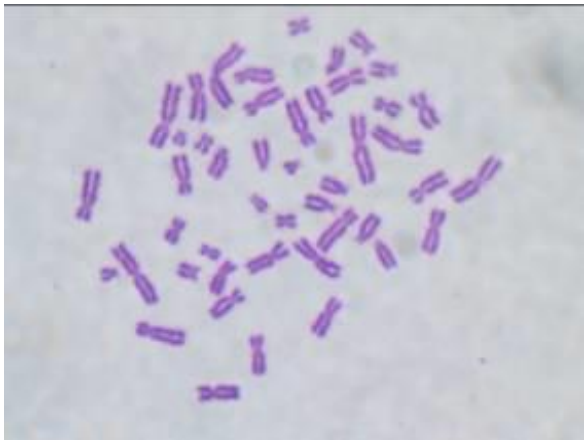
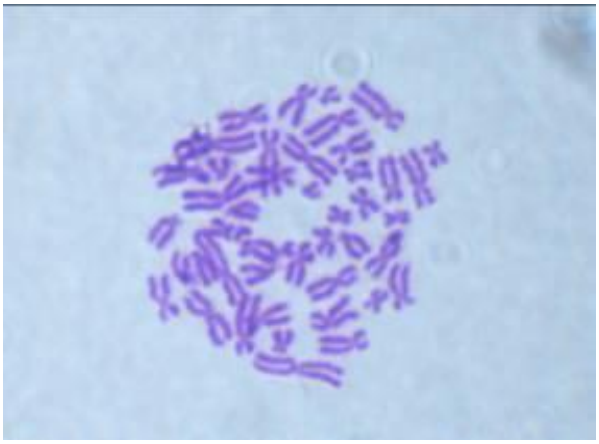
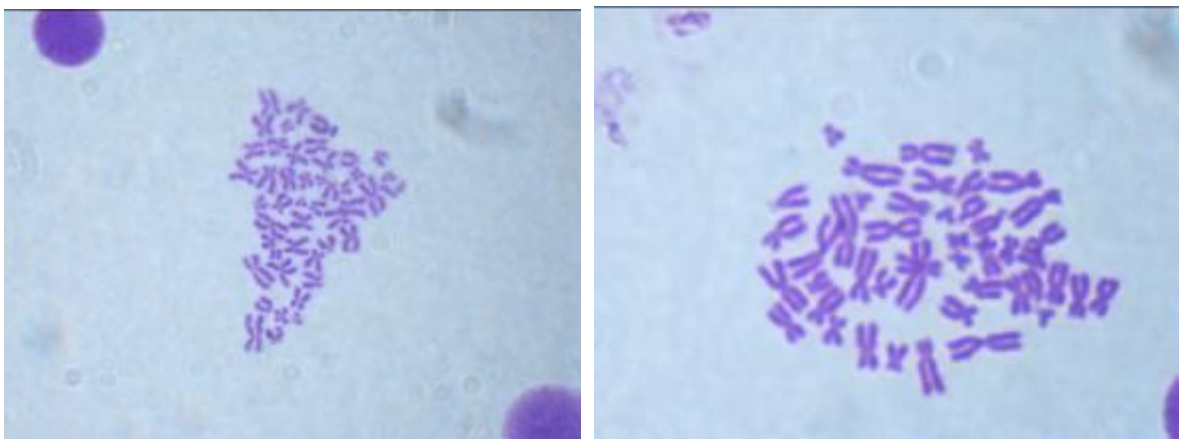


g



h



i **j**

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Figure 34: Conventional Giemsa-stained human chromosomes **a.** Mitomycin C treated **b.** Cyclophosphosphamide treated **c**. HA EV extract treated in the presence of S9 metaboilic activator **d.** HA EV extract treated in the absence of S9 metabolic activator. **e**.HA BG extract treated in the presence of. S9 metabolic activator **f.** HA BG extract treated in the absence of S9 metabolic activator metabolic activator **g**. Latex material extract treated in the presence of S9 metabolic activator **h.** Latex material extract treated in the absence of S9metaboilic activator. **i** .Dental material extract treated in the presence of presence of S9metaboilic activator. **j.** Dental material extract treated in the absence of S9 metabolic activator.

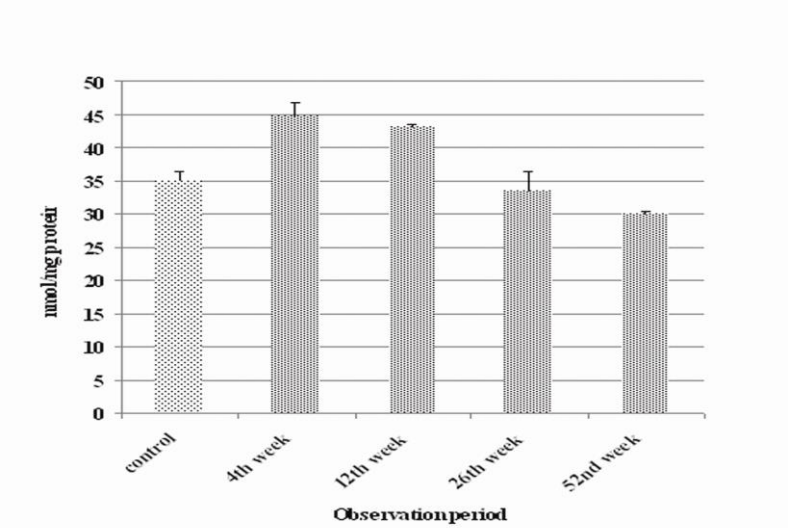

Figure 35 : LPO levels in 10% rat liver homogenate in control (unimplanted) and HAP-EVA implanted at different observation periods. Data were represented as mean ± SD. (n=8)

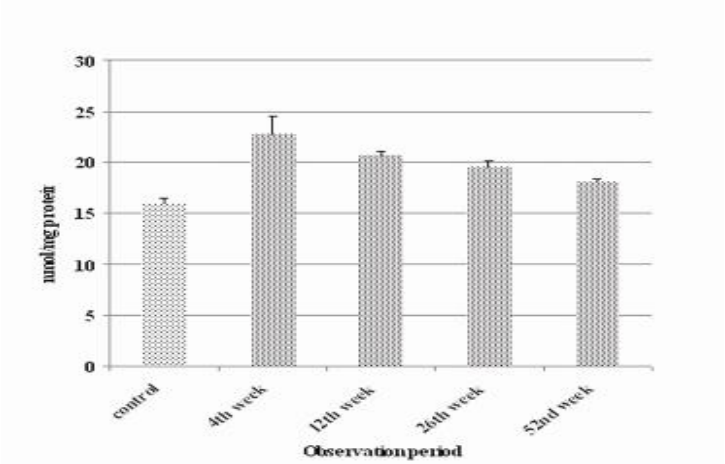

Figure 36 : LPO level in 10% rat liver homogenate in control (unimplanted) and HA BG implanted at different observation periods. Data were represented as mean ± SD. (n=6)

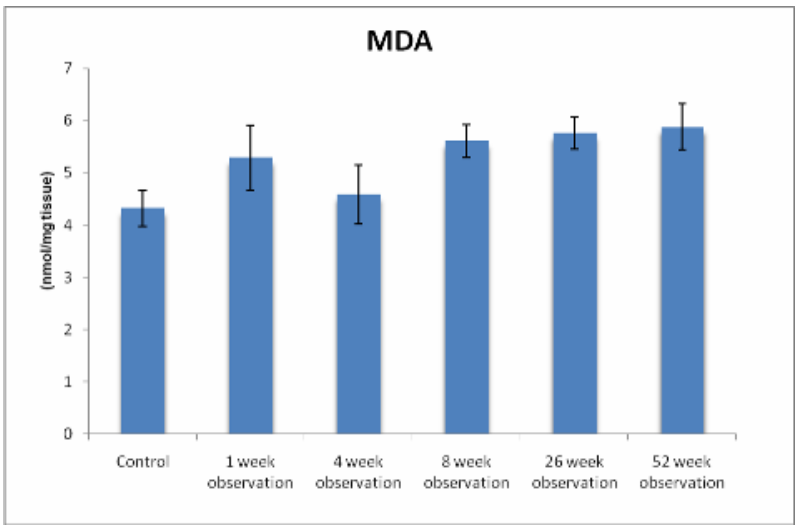

Figure 37: Concentration of LPO in 10% rat liver homogenate in control (unimplanted) and Dental material implanted into muscle at different observation periods. Data were represented as mean ± SD. (n=8)

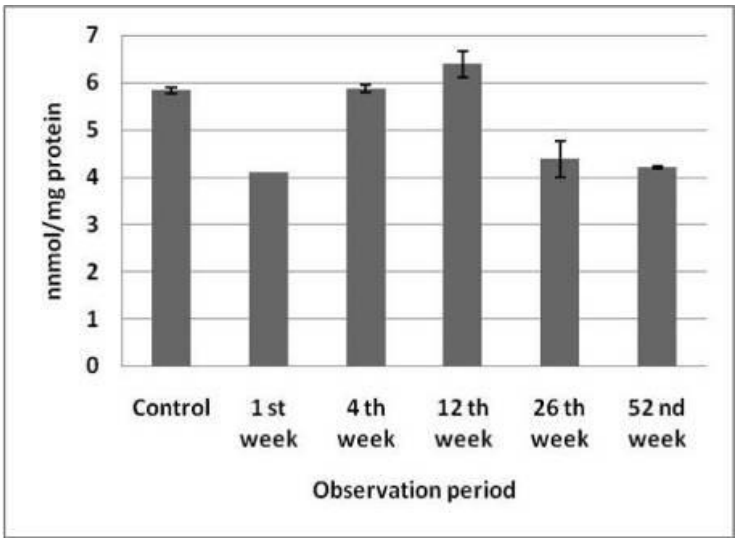

Figure 38: the concentration of LPO in 10% rat liver homogenate in control (unimplanted) and subcutaneously implanted latex at different observation periods. Data were represented as mean ± SD. (n=6)

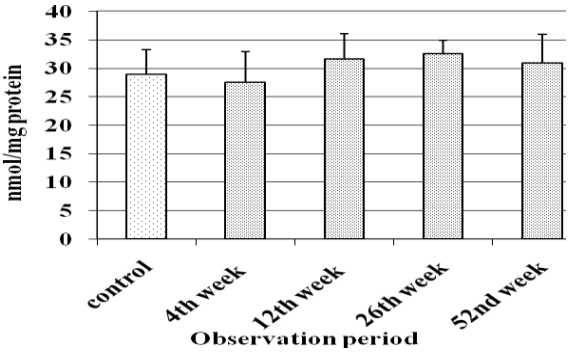

Figure 39 : LPO level in 10% rat liver homogenate in control (unimplanted) and fibrin glue implanted into rat brain at different observation periods. Data were represented as mean ± SD. (n=8)

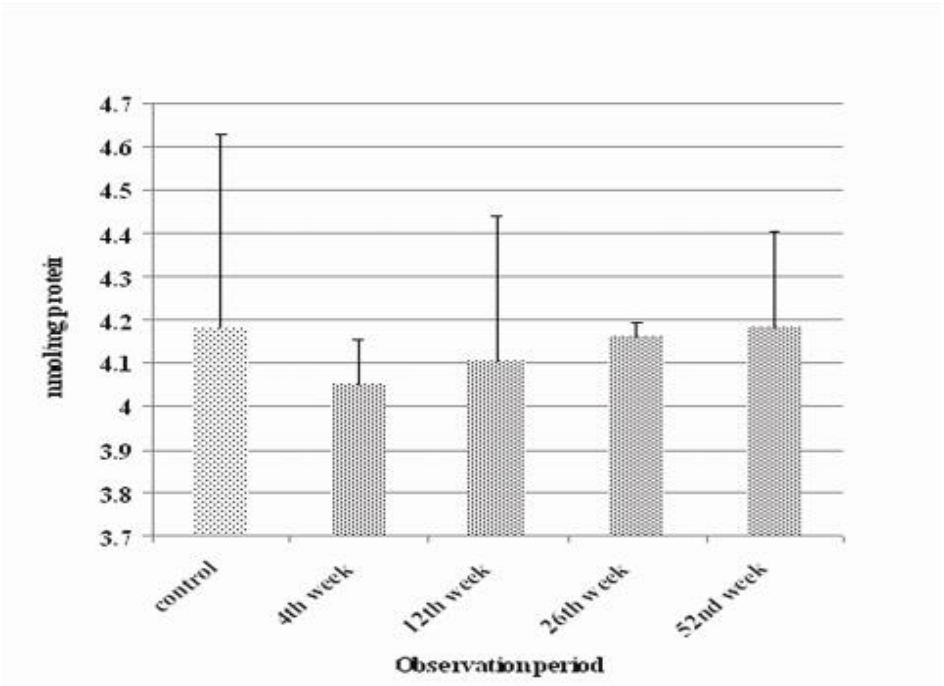

Figure 40 : The amount of GSH in 10% rat liver homogenate in control (unimplanted) and HAP-EVA implanted for different observation periods. Data were represented as mean ± SD. (n=8)

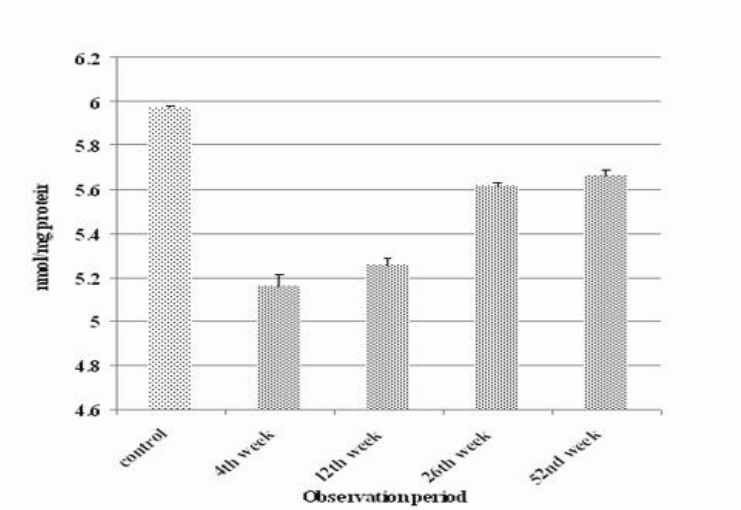

Figure 41: The amount of GSH in 10% rat liver homogenate in control (unimplanted) and HA-BGA implanted for different observation periods. Data were represented as mean ± SD. (n=6)

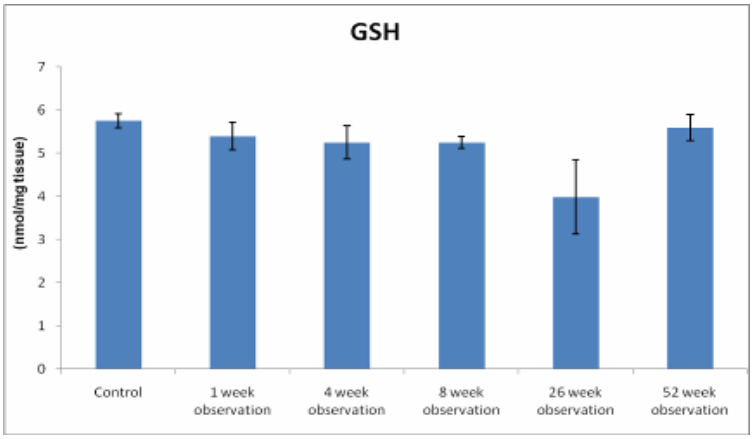

Figure 42: The amount of GSH in 10% rat liver homogenate in control (unimplanted) and Dental material implanted for different observation periods. Data were represented as mean ± SD. (n=8)

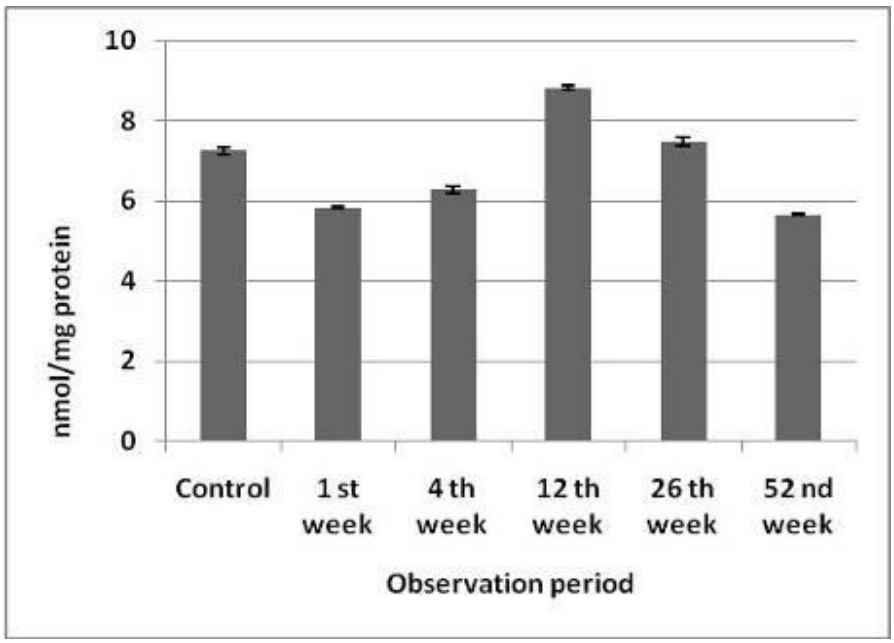

Figure 43: The amount of GSH in 10% rat liver homogenate in control (unimplanted) and Latex material implanted for different observation periods. Data were represented as mean ± SD. (n=6)

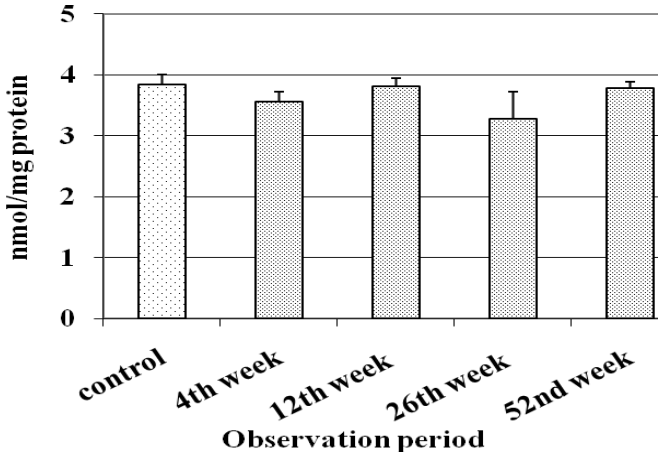

Figure 44: The amount of GSH in 10% rat liver homogenate in control (unimplanted) and Fibrin glue implanted for different observation periods. Data were represented as mean ± SD. (n=8)

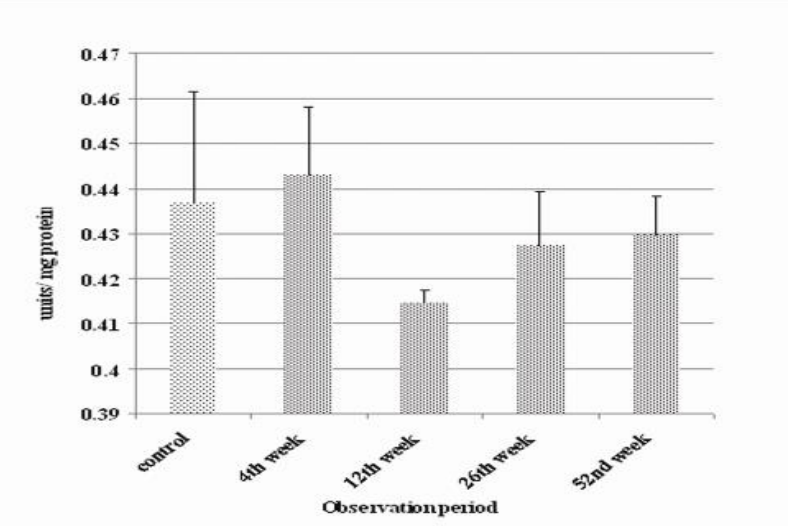

Figure 45: The activity of GR in 10% rat liver homogenate in control (unimplanted) and HAP-EVA implanted for different observation periods. Data were represented as mean ± SD. (n=8)

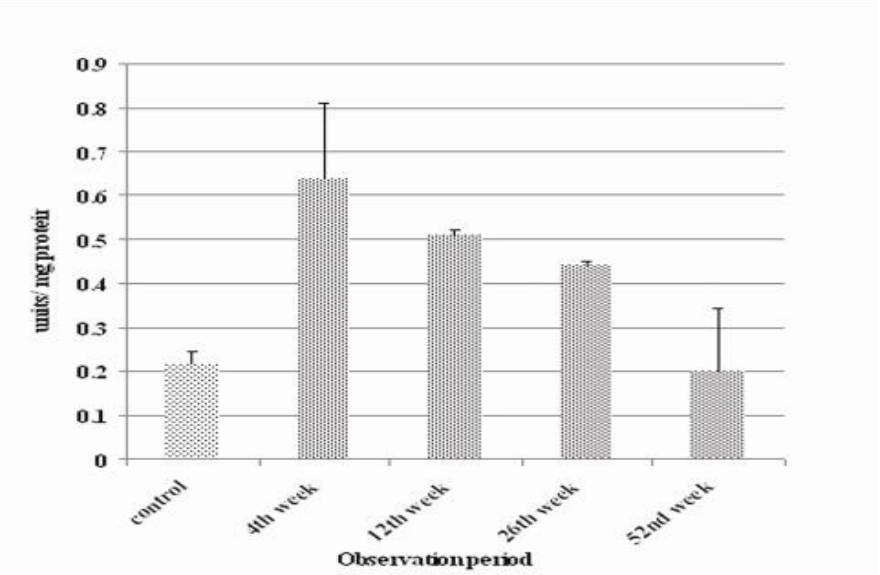

Figure 46: The activity of GR in 10% rat liver homogenate in control (unimplanted) and HABGA implanted for different observation periods. Data were represented as mean ± SD. (n=6)

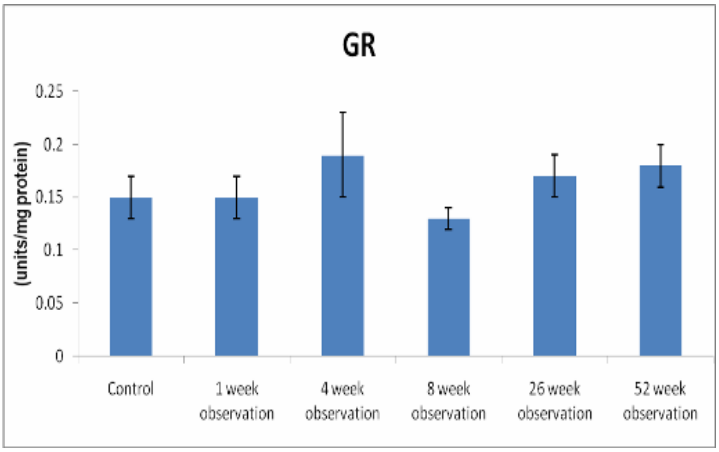

Figure 47: The activity of GR in 10% rat liver homogenate in control (unimplanted) and Dental material implanted for different observation periods. Data were represented as mean ± SD. (n=8)

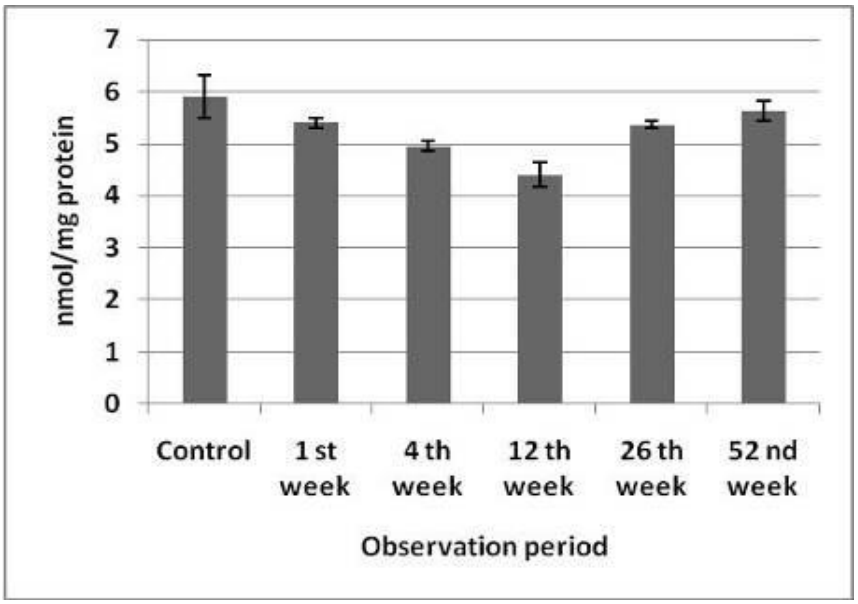

Figure 48: The activity of GR in 10% rat liver homogenate in control (unimplanted) and Latex implanted for different observation periods. Data were represented as mean ± SD. (n=6)

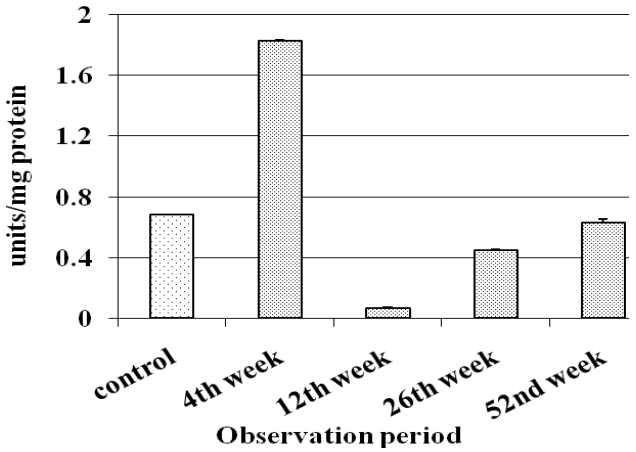

Figure 49: The activity of GR in 10% rat liver homogenate in control (unimplanted) and fibrin glue implanted for different observation periods. Data were represented as mean ± SD. (n=8)

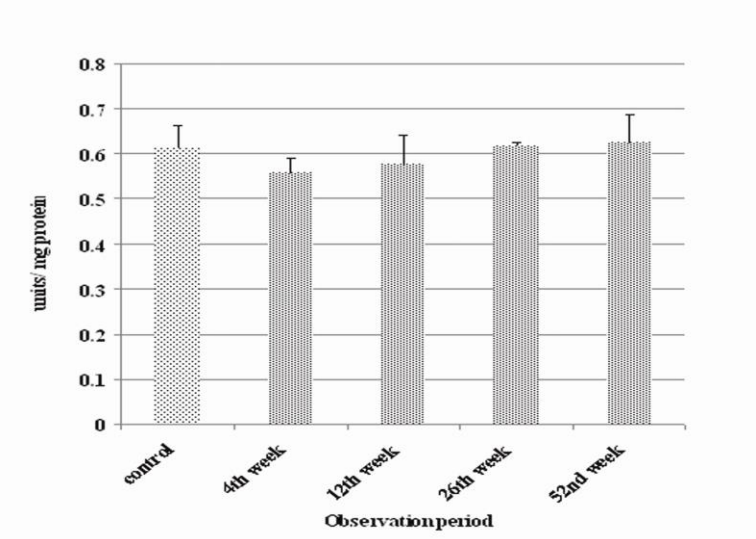

Figure 50 : The concentration of GPx in 10% rat liver homogenate in control (unimplanted) and HAP EVA implanted for different observation periods. Data were represented as mean ± SD. (n=8)

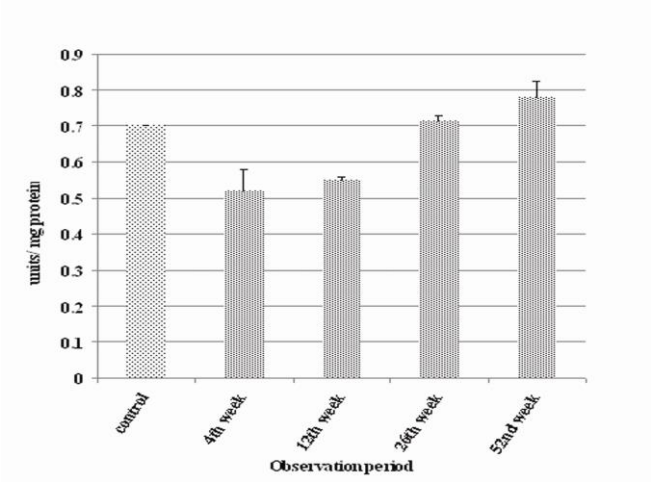

Figure 51: The concentration of GPx in 10% rat liver homogenate in control (unimplanted) and HA BG implanted for different observation periods. Data were represented as mean ± SD. (n=8)

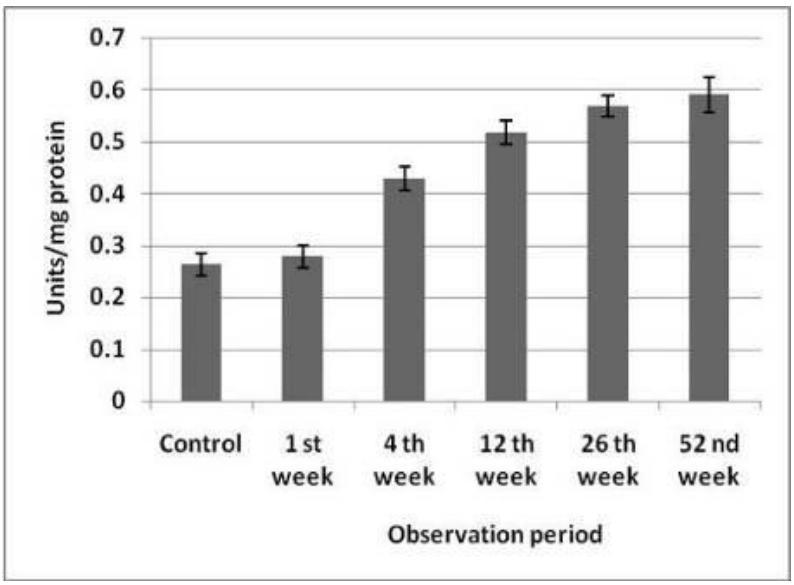

Figure 52: The concentration of GPx in 10% rat liver homogenate in control (unimplanted) and fibrin glue implanted for different observation periods. Data were represented as mean ± SD. (n=8)

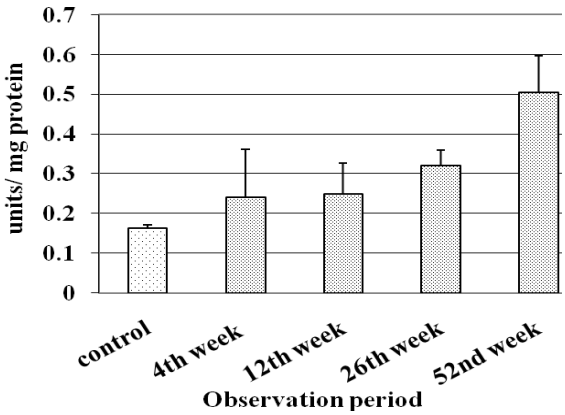

Figure 53: The concentration of GPx in 10% rat liver homogenate in control (unimplanted) and fibrin glue implanted for different observation periods. Data were represented ± SD.

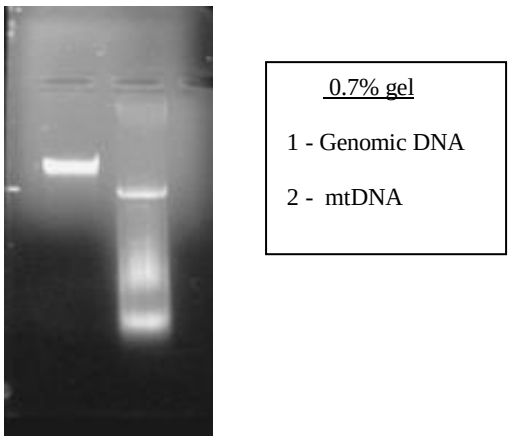

Figure 54: Agarose gel electrophoresis of nuclear and mitochondrial DNA

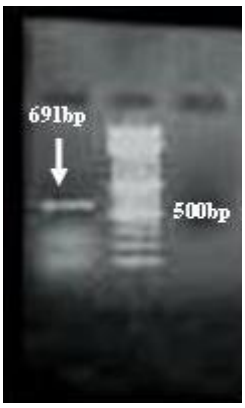

Figure 55: Confirmation of nuclear DNA by using specific primer of β actin gene

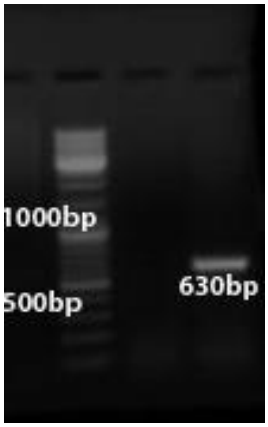

Figure 56: Confirmation of mitochondrial DNA by using specific primer of Cytochrome B gene.

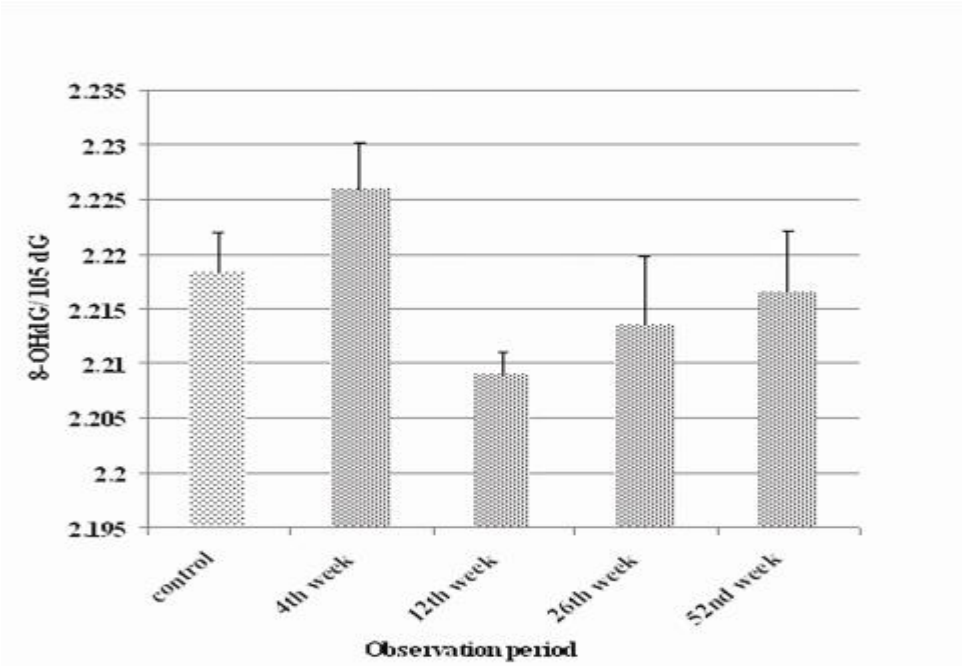

Figure 57: The comparison of 8-OHdG in rat brain genomic DNA in control (unimplanted) and HAP-EVA implanted for different observation periods. Data were represented as mean ± SD. (n=8)

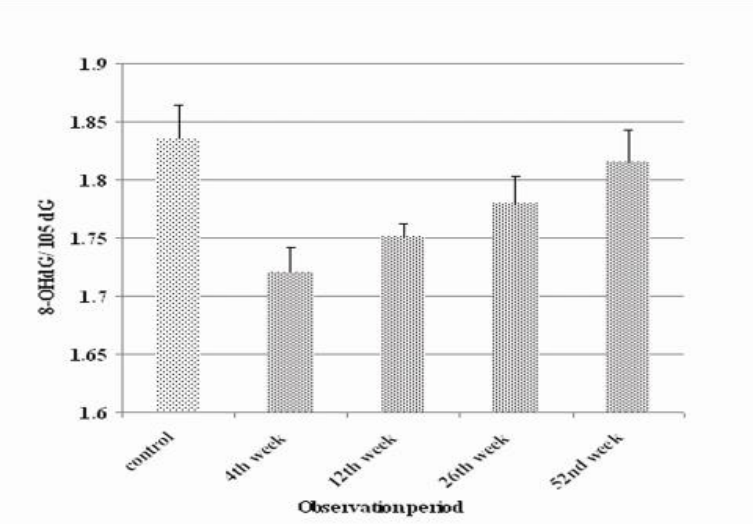

Figure 58: The comparison of 8-OHdG in rabbit bone genomic DNA in control (unimplanted) and HABGA implanted for different observation periods. Data were represented as mean ± SD. (n=6)

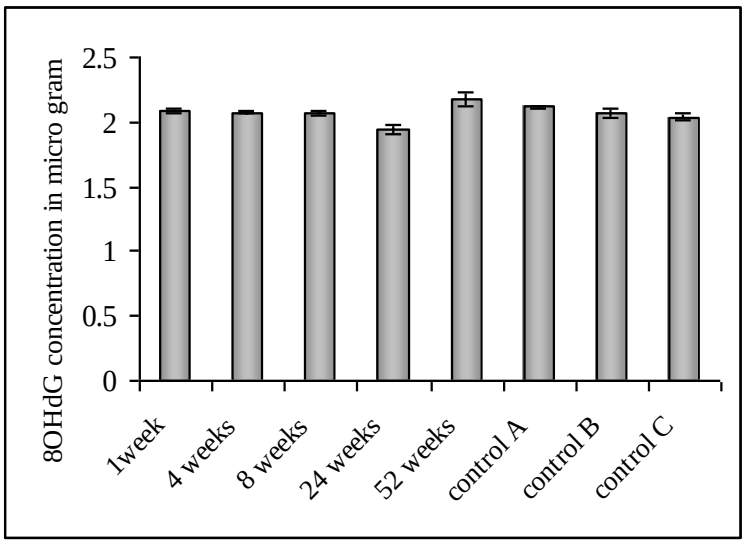

Figure 59: The comparison of 8-OHdG in rat muscle genomic DNA in control (unimplanted) and fibrin glue implanted for different observation periods. Data were represented as mean ± SD. (n=8)

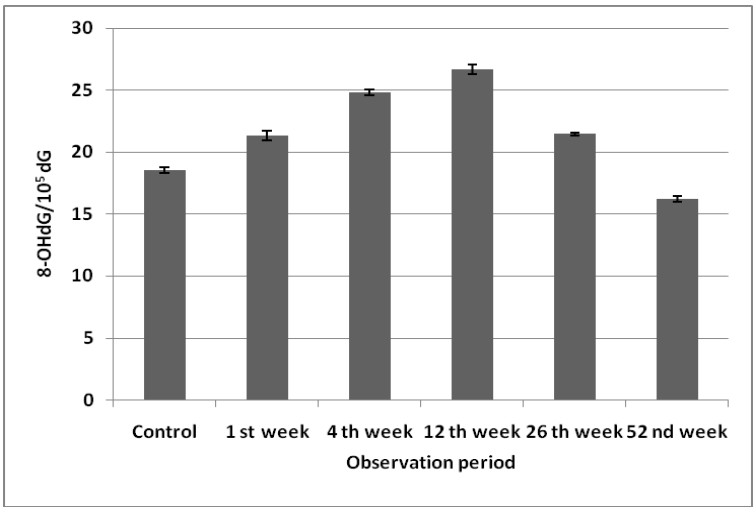

Figure 60: Comparison of 8-OHdG in rat skin genomic DNA in control (unimplanted) and Latex implanted for different observation periods. Data were represented as mean ± SD. (n=8)

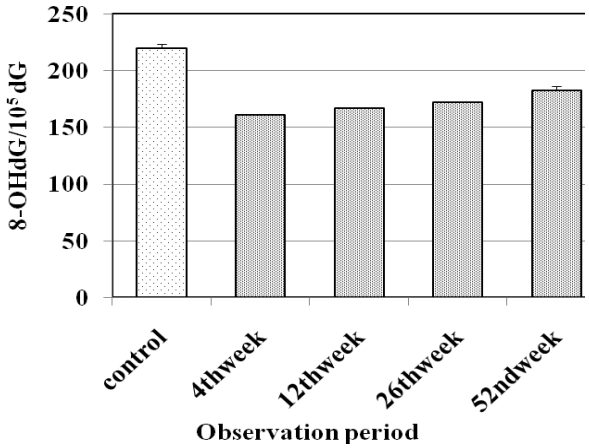

Figure 61: Comparison of 8-OHdG in rat brain genomic DNA in control (unimplanted) and fibrin glue implanted for different observation periods. Data were represented as mean ± SD. (n=8)

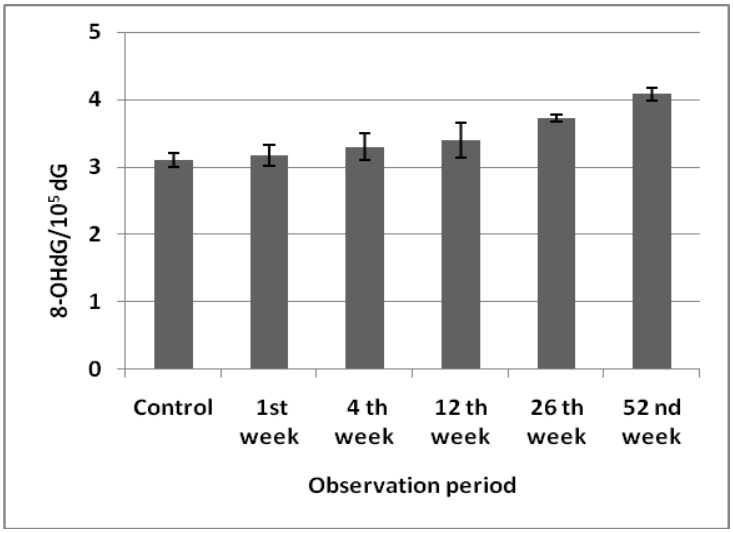

Figure 62: The comparison of 8-OHdG in rat brain mitochondrial DNA in control (unimplanted) and HAP-EVA implanted for different observation periods. Data were represented as mean ± SD. (n=8)

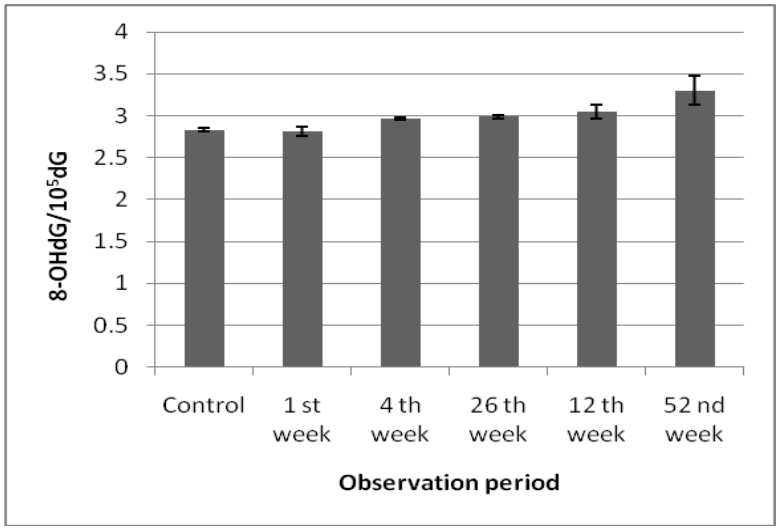

Figure 63: The comparison of 8-OHdG in rat brain mitochondrial DNA in control (unimplanted) and fibrin glue implanted for different observation periods. Data were represented as mean ± SD. (n=8)

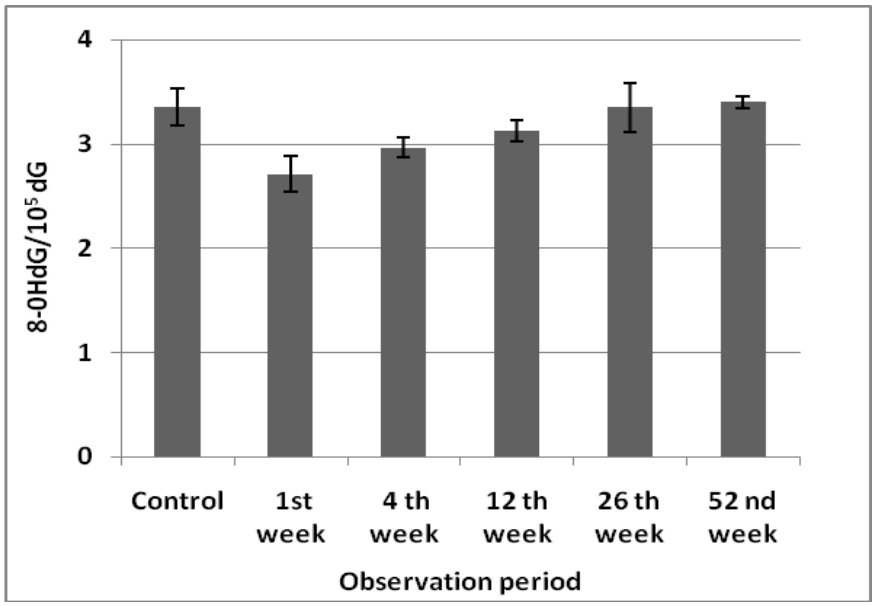

Figure 64: The comparison of 8-OHdG in Rabbit bone mitochondrial DNA in control (unimplanted) and HABG implanted for different observation periods. Data were represented as mean ± SD. (n=6)

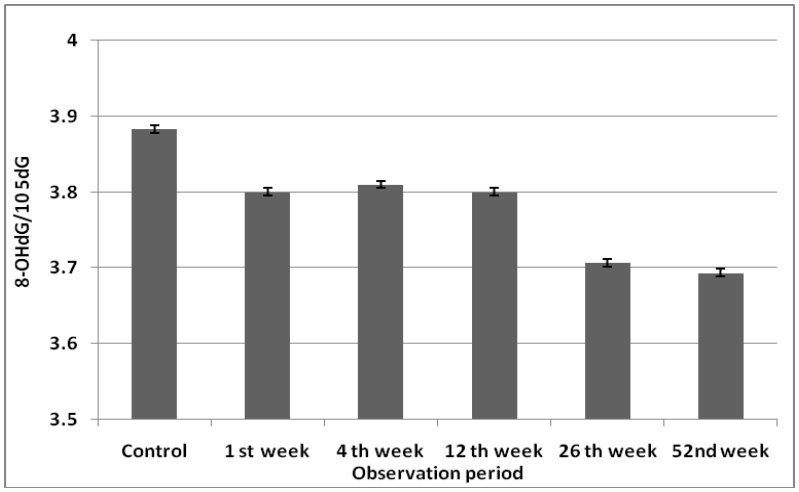

Figure 65: The comparison of 8-OHdG in rat muscle mitochondrial DNA in control (unimplanted) Dental material implanted for different observation periods. Data were represented as mean ± SD. (n=8)

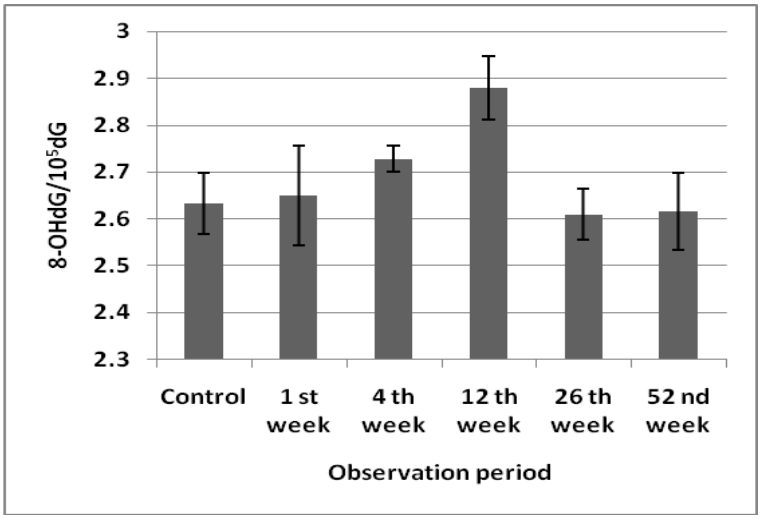

Figure 66: The comparison of 8-OHdG in rat skin mitochondrial DNA in control (unimplanted) and latex implanted for different observation periods. Data were represented as mean ± SD. (n=6)

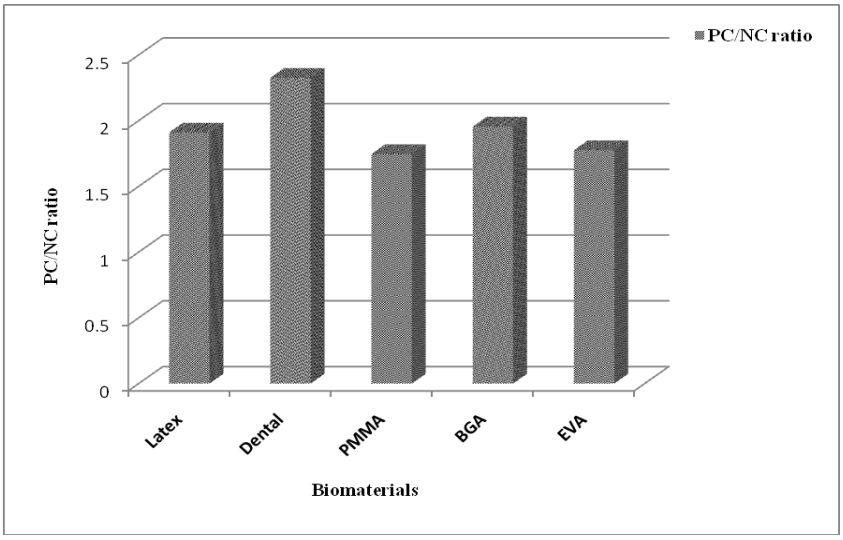

Figure 67: PC/NC ratio in bone marrow cells of mice treated with physiological saline extracts of five different biomaterials (24hr treatment).

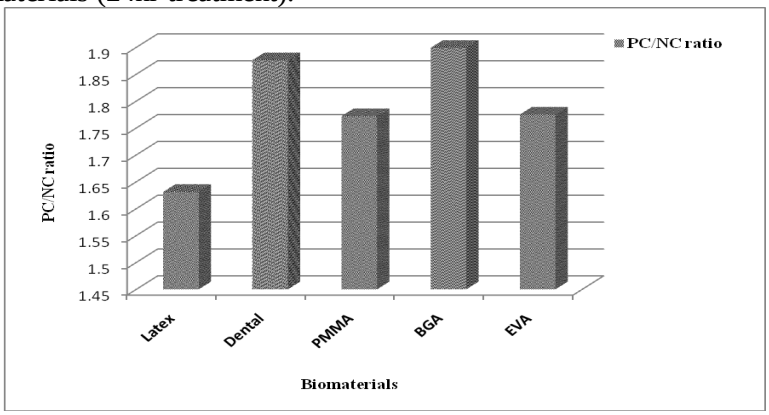

Figure 68: PC/NC ratio in bone marrow cells of mice treated with physiological saline extracts of five different biomaterials (48hr treatment).

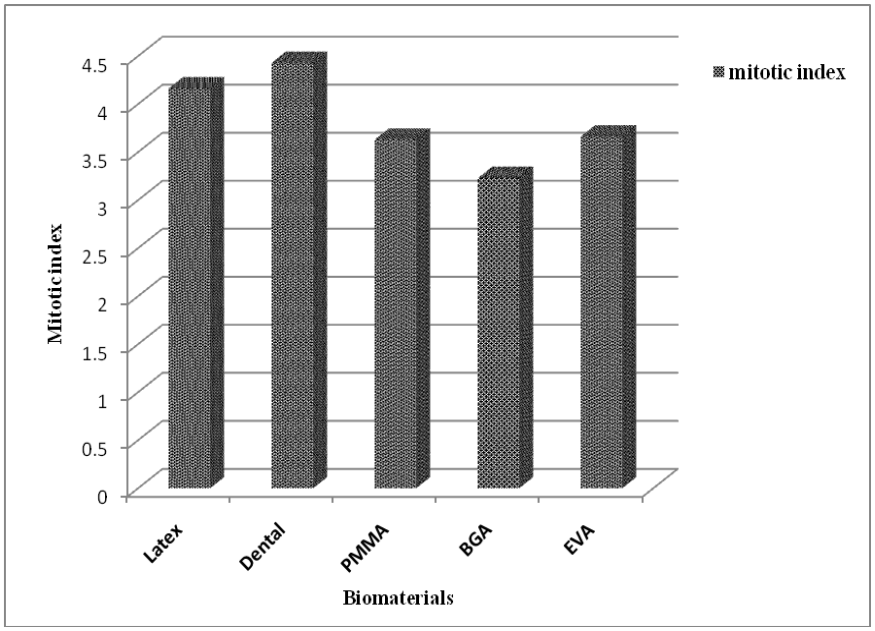

Figure 69: Mitotic index in bone marrow cells of mice treated with physiological saline extracts of five different biomaterials (24hr treatment).

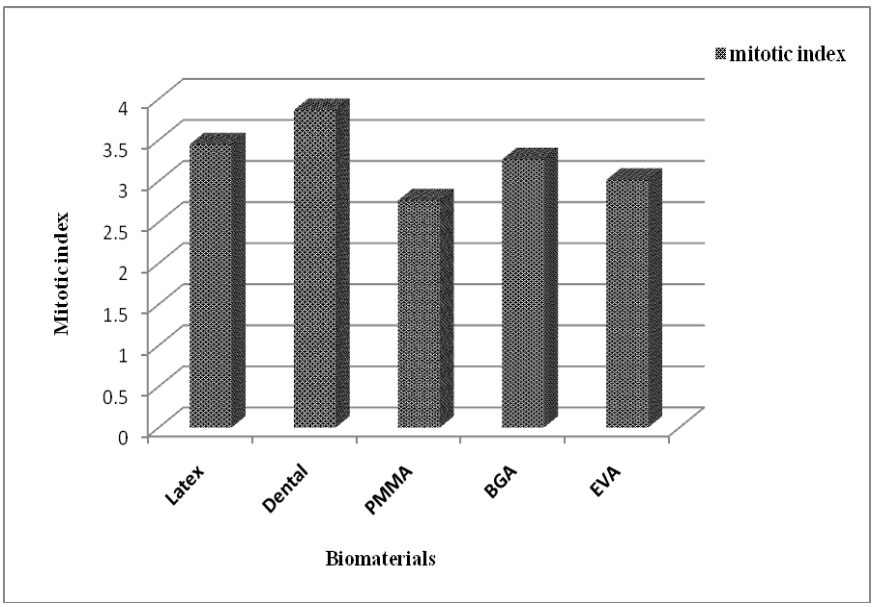

Figure 70: Mitotic index in bone marrow cells of mice treated with physiological saline extracts of five different biomaterials (48hr treatment).

TABLES:

Biomaterials Used	Clinical uses	Departments
Hydroxyapatite- ethylene vinyl acetate co-polymer	Cranioplastic surgery	Polymer Processing Lab & Bio Ceramics Lab
Bioactive ceramic composite	Bone scaffold	Bio Ceramics Lab
Polymethylmethacrylate-Hydroxyapatite composite	Bone apposition	Bio Ceramics Lab
Dental composite	Dental filling	Dental processing lab
Non toxic latex material	Catheters, gloves	Polymer Processing Lab
Fibrin glue	Neurosurgery, wound healing	Thrombosis Research Lab

Table 1: List of biomaterials used in the present study

Animals	Weight
New Zealand Rabbit	Not less than 2kg
Wistar albino rat	180-230g
Swiss albino mice	17-23g

Table 2: List of experimental animals used in the present study

Implantations on	Observation Period	Materials
Rabbit femur bone	1, 4, 12, 26, 52	HA-BGA, PMMA-HAP
Rat brain tissue	1, 4, 12, 26, 52	HAP-EVA
Rat gluteal muscle	1, 4, 12, 26, 52	Dental composite
Rat subcutaneous tissue	1, 4, 12, 26, 52	Nontoxic latex material
Rat brain tissue	1, 4, 12, 26, 52	Fibrin glue

Table 3: List of implantation of different materials in experimental animals

Groups	Number of cells scored	Chromosomal aberrations		Chromatid aberrations	
		+ S9	-S9	+ S9	-S9
CP(positive control)	100	93.00±5.67	-	15.00±1.41	_
Physiological saline	100	3.50±0.71	7.50±0.71	4.00±1.14	4.50±2.12
HA EVA	100	5.50±0.71	9.50±0.70	5.00±2.83	6±2.83
HA BG	100	7.00±1.41	6.50±2.12	5.50±3.54	4.50±3.54
Dental material	100	4.34±1.15	2.00±1.00	3.34±1.13	2.34±0.57
Latex	100	4±0.82	3.00±1.00	4.50±0.58	3.34±0.56
S9 alone	100	3.00±1.41	_	1.50±0.71	_

Table 4 . Metaphase scored from lymphocytes treated with CP, Physiological saline, HA BG and HA EVA, Latex and Dental material in the presence and absence of S9 metabolic activator**.** Values are expressed as mean±S.D, $p \leq 0.05$

Group	**Dose (mg/kg)**	**No. of animals**	**PC with MN(%) Mean± SE [1]**	**NC with MN (%) Mean± SE [2]**	**Total % of MN in PC and NC cells Mean ±SE [3]**	**Average No. of NC in 6 animals Mean±SE [4]**
Physiological Saline (control)	50ml/kg	6	0.083 ± 0.017	0.113 ± 0.043	0.197 ± 0.038	1039± 66.86
Cyclophosphamide (positive control)	50mg/kg	6	2.592± 0.221	0.527± 0.142	3.119± 0.191	1212.83± 82.03
Latex material	50ml/kg	6	0	0	0	1055.67± 39.98
Dental Composite	50ml/kg	6	0.0257 ± 0.024	0.0174± 0.0159	0.049± 0.018	894.17± 86.05
PMMA- HAP	50ml/kg	6	0.008 ± 0.008	0.0271 ± 0.0259	0.035± 0.032	1149.17± 29.35
HA- BG	50ml/kg	6	0.008± 0.008	0.016± 0.014	0.024± 0.016	1029.17± 35.98
HAP- EVA	50ml/kg	6	0	0	0	1143.33 ± 93.75

Table 5: Effect of physiological saline extract of five different biomaterials on micronuclei (24hr treatment). All the values are represented in the form of mean ± SD.

Group	Dose (mg/kg)	No. of animals	PC with MN (%) Mean± SE [1]	NC with MN (%) Mean±SE [2]	Total No. of MN in PC and NC cells (%) Mean ±SE [3]	Average No. of NC in 6 animals Mean± SE [4]
Physiological Saline (Control)	50ml/kg	6	0.083± 0.017	0.113± 0.043	0.197± 0.038	1232.67± 119.001
Cyclophosphamide (positive control)	50mg/kg	6	2.333± 0.274	0.125± 0.017	3.784± 0.391	1620.33± 49.36
Latex material	50ml/kg	6	0.025± 0.011	0.024± 0.015	0.049± 0.018	1255.67± 92.77
Dental Composite	50ml/kg	6	0	0.029± 0.018	0.029 ± 0.018	1095.50± 75.04
PMMA- HAP	50ml/kg	6	0.025± 0.017	0.029± 0.029	0.054± 0.029	1150.17± 67.86
HA- BG	50ml/kg	6	0.008± 0.008	0	0.008± 0.008	1075± 64.82
HAP- EVA	50ml/kg	6	0	0	0	1387.33± 339.85

Table 6: Effect of physiological saline extract of five different biomaterials on micronuclei (48hr treatment). All the values are represented in the form of mean ± SD.

Group	Dose (mg/kg)	No. of animals	Total number of metaphases counted =100/animal					
			Chromatid Breaks (%) Mean ± SE	Chromatid Gaps (%) Mean ± SE	Chromosome Breaks (%) Mean ± SE	Chromosome Gaps (%) Mean ± SE	Total Breaks (%) Mean ± SE	Total Gaps (%) Mean± SE
Physiological Saline (Control)	50ml/kg	6	0	0	0	0	0	0
Cyclophosphamide(positive control)	50mg/kg	6	0.296± 0.049	0.108± 0.027	0.083± 0.008	0.0047± 0.0047	0.379± 0.052	0.113± 0.025
Latex material	50ml/kg	6	0.096± 0.035	0.083± 0.031	0.004± 0.004	0	0.089± 0.037	0.083± 0.031
Dental Composite	50ml/kg	6	0.004± 0.004	0.171± 0.101	0.021± 0.021	0	0.024± 0.024	0.171± 0.102
PMMA- HAP	50ml/kg	6	0.004± 0.004	0.067± 0.039	0	0	0.004± 0.004	0.067± 0.039
HA- BGA	50ml/kg	6	0	0	0	0	0	0
HAP- EVA	50ml/kg	6	0	0	0	0	0	0

Table 7: Effect of physiological saline extract of five different biomaterials on chromosomes (24hr treatment). All the values are represented in the form of mean ± SD.

Group	Dose (mg/kg)	No. of animals	Total number of metaphases counted =100/animal					
			Chromatid Breaks (%) Mean ± SE	Chromatid Gaps (%) Mean ± SE	Chromosome breaks (%) Mean ± SE	Chromosome Gaps (%) Mean ± SE	Total Breaks (%) Mean ± SE	Total Gaps (%) Mean± SE
Physiological Saline (Control)	50ml/kg	6	0.006± 0.003	0	0.012± 0.007	0.002± 0.002	0.018± 0.009	0.002± 0.002
Cyclophosphamide(positive control)	50mg/kg	6	0.622[c]± 0.066	0.275[c]± 0.045	0.426[c]± 0.071	0.482[c] ± 0.065	1.047[c]± 0.073	0.758[c]± 0.090
Latex material	50ml/kg	6	0.025± 0.025	0.079± 0.079	0.021± 0.016	0.042± 0.033	0.046± 0.031	0.121± 0.078
Dental Composite	50ml/kg	6	0	0.079± 0.053	0	0	0	0.079± 0.053
PMMA- HAP	50ml/kg	6	0	0.404± 0.131	0.004± 0.004	0.133± 0.133	0.004± 0.004	0.538± 0.222
HA- BGA	50ml/kg	6	0.004± 0.004	0.113± 0.069	0	0	0	0.113± 0.069
HAP- EVA	50ml/kg	6	0	0.154± 0.102	0	0.008± 0.008	0	0.163± 0.105

Table 8: Effect of physiological saline extract of five different biomaterials on chromosomes (48hr treatment). All the values are represented in the form of mean ± SE.