In Vitro Toxicity of BisGMA (Bowen´s resin)

Master thesis by **Jamini Kathirgamanathan**,
Dental Faculty, University of Oslo
2015/2016

Supervisor: Professor **Dr. Odont Jon E. Dahl**,
Dental Faculty, University of Oslo

Content

1. Abstract 3
2. Introduction 4
2.1 Restorative biomaterials 4
2.2 Leakage of composite 5
2.3 Exposure of methacrylate 5
2.4 Allergic reactions in patients and dental personnel 6
2.5 Recent studies about methacrylate monomers 6
2.6 Cell biology 6
2.6.1 Toxicity 6
2.6.2 Cellcycle 6
2.6.2.1 The control system 7
2.6.2.2 DNA damage 7
2.6.3 Cell death 9
3. Aim of the study and hypothesis 10
4. Materials and methods 11
4.1Materials 11
4.1.1 Monomers 11
4.1.2 Other chemicals 11
4.1.3 Equipment 11
4.2 Methods 12
4.2.1 Cell culture 12
4.2.2 Cell treatment 12
4.2.3 Morphology of the cells 12
4.2.4 Cell toxicity assay, MTT 12
4.2.5 Evaluation of apoptosis and necrosis With Hoechst and PI 13
4.2.6 Flow cytometer 13
4.2.6.1 Staining with annexin V and PI 13
4.2.6.2 Cell cycle analysis 15
4.2.7 Western blot- protein- analyse 15
4.2.8 Statistic 16
5.Results 17
5.1The morphology of the cells 17
5.2 The viability of the cell 18
5.3 Cell death mechanism with Hoecsth 33332 and PI 19
5.4 Cell death mechanism with FITC-Annexin V and PI 20
5.5 DNA- damage response and apoptosis 22
6. Discussion 23
7. Conclusion 24
8. Reference 24
9. Acknowledgement 26

1. Abstract

Restorative dental materials, like composite, are widely used in dentistry. 2,2-bis[4(2-hydroxy-3-metharyloxypropoxy)-phenyl]propane (BisGMA) is one of the most used methacrylate monomer in composite materials. Methacrylate monomers can leach from the tooth and into oral cavity. Also, it has been found methacrylate monomers in the air in the dental practice. Those monomers indicate to enter the human body and may enter the respiratory organs. The purpose of this study was to investigate the effect of BisGMA on human bronchial epithelial cells (BEAS-2B cells). Phase contrast microscope and MTT assay were used to detect the effect of BisGMA on BEAS-2B cell viability. In addition, Hoechst 33342 and PI staining assay, flow cytometer and western blot were used to analyze the cause of changes in cell viability and the DNA damage response activity. Exposure to BisGMA reduced the viability of the BEAS-2B cells as a result of increased necrosis. There were no alteration of the cell cycle and no increase in the activity of the DNA-damage response proteins. The results indicate that BisGMA is cytotoxic to BEAS-2B cells in a concentration and time dependent manner. This study could not detect DNA-damage in the exposed cells, but membrane damage is suggested as an initial event.

2.Introduction

2.1 Restorative biomaterials

Restorative biomaterials are widely used in dentistry. The tooth is a mineralized tissue, which can be fractured or demineralized because of trauma or dietary. The restorative biomaterials are used in dentistry to replace and repair the injured and fractured tooth. The most known restorative dental materials are amalgam and composite.

Amalgam is the silver-grey colored material, which generally contains 50% mercury. This material has been banned in Norway and Sweden since year 2010 because of the mercury pollution to the environment[1]. The material is also being phased out in several other countries after the Minimita convention in January 2013[2].

Composite is a tooth-colored restorative material. In the last decade this material has been widely used in dentistry, especially in Norway. Composite is a composition of two or more distinct materials, which we can divide in three groups:

- Filler particles
- Resin matrix
- Coupling agents

The filler particles are mixture of glass, silica, crystalline, metal oxide or more of other ceramic particles. These particles will emphasize the resin matrix. The resin matrix consist several of resin monomers, which will be polymerized by the curing of light. The coupling agents are the connection fibers between the matrix and particles[3] .

The resin monomers consist different kind of monomers, one of them are methacrylate monomers. Methacrylate monomer constitutes one of the main components in the composite. There are different kinds of methacrylate monomer. Each methacrylate monomer consists at least one methacrylate group and they are distinguished by their chemical structure and properties. HEMA, UDMA, TEGDMA and BisGMA are examples on resin-monomers.

2,2-bis[4(2-hydroxy-3-metharyloxypropoxy)-phenyl]propane (BisGMA) is one of the most used dimethacrylate monomer in composite materials (Figure 1). BisGMA have rigid Bisphenol A core and two pendant hydroxyl groups. This monomer is lipophilic and gives high viscosity and filling load for the composite. The composite is also mixed with monomers like TEGDMA or UDMA to neutralize the viscosity.
HEMA is another resin monomer, which is most used in adhesives and bonding. This molecule is amphiphilic [4] (Figure 2).

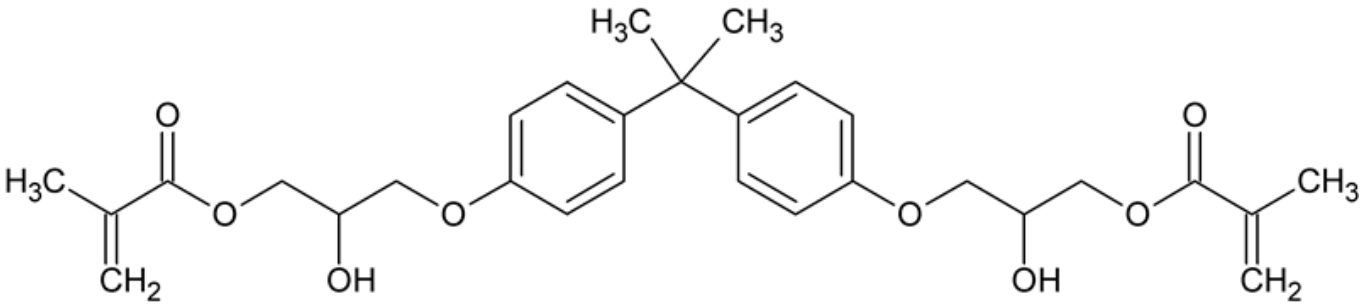


***Figure 1**: Molecular structure of BisGMA*

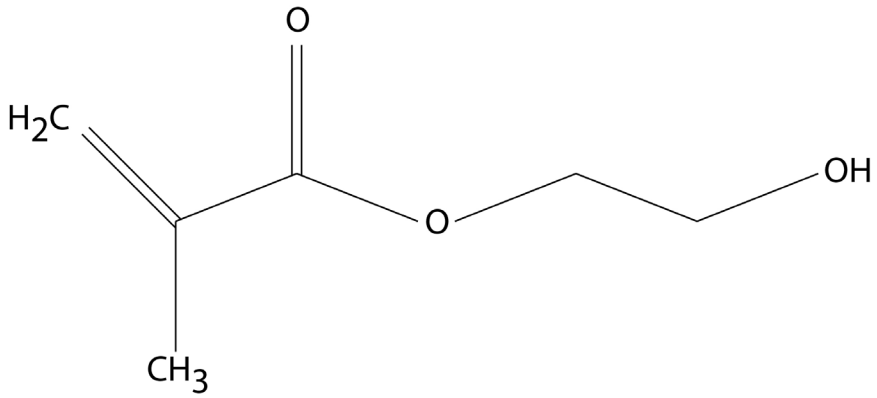


***Figure 2**: Molecular structure of HEMA*

2.2 Leakage of composite

Methacrylate monomers can leach out of the tooth restoration and into the oral cavity. The polymerization of resin monomers is never complete. Conversion of monomers to polymer may vary from 50% to 90% [5]. And the un-polymerized monomers may remain in the material after the curing process. The composite can be degraded by salivary enzymes in the oral cavity and by chewing function [6-9]. As a consequence, monomers leakage may occur.

2.3 Exposure of methacrylate

There has been found measurable concentrations of methacrylate monomers in the air of dental practice[10, 11]. Also leakage of methacrylate monomers from patients treated with resin- based restorative materials in saliva has been observed. Quantified BisGMA monomer has been found in saliva after treatment with resin based composite [12]. The leached BisGMA monomers can penetrate into the oral environment and be absorbed through the gastrointestinal to the whole body [13]. The lipophilic property of this monomer gives possibility to penetrate into the cell.

2.4 Allergic reactions in patients and dental personnel

Dental personnel works daily with uncured dental materials. In addition to the patients, they are also at the risk of exposure to the monomers [10, 11, 14]. There have been observed allergic reactions in patients and dental personnel, who have been treated and worked daily with methacrylate-contained materials. The patients had symptoms like intraoral inflammatory symptoms; dermatitis and some patients also had respiratory problems. In the same line, dental personnel also had these symptoms. Those studies concluded that methacrylate monomers could cause these symptoms, and BisGMA and HEMA were some of these methacrylate monomers [3, 15, 16].

2.5 Recent studies about methacrylate monomers

Methacrylate monomers are indicated to enter the human body[10-14] and may enter to the respiratory organs. In vitro studies show that monomers induce cytotoxicity, causing DNA damage and formation of apoptotic cells in different cell lines[17-27].
Recently Ansteinsson et al (2013) studied the response of epithelial lung cells to different methacrylate monomers. Compared to the other methacrylate monomers, BisGMA induced cell death in lower concentration. Further, BisGMA didn't increase cellular oxidative stress and didn't cause DNA damage, as the other methacrylate monomers[28].

2.6. Cell biology

2.6.1Toxicity

Human body is always receptive for xenobiotic. Xenobiotic is defined as a foreign compound, which is not usually present in the biological system of an organism. Drugs, chemicals are examples for xenobiotic. When dental materials release a substance in the oral cavity, it is able to enter the human body. It can enter by the swallowing of saliva and inhalation or through subsequent passage of the epithelial barriers in the gastrointestinal tract or the lungs. The responses from the tissue and the cells depend on the concentrations, the chemical properties of the xenobiotic and the exposure time. Xenobiotic can either bind or go through the cells and affect the DNA, enzyme activities, protein synthesis and etc. The cells will either be capable to repair the damages caused by the xenobiotic or they will die [29].

2.6.2 Cellcycle

Cell cycle is a process where the cell reproduces itself. The cell will duplicate its contents and divide into two. There are 4 main phases in this cycle(figure 3):

- G1 phase- Gap 1 phase
- S phase – Replication of DNA (duplication)
- G2 phase- Gap 2 phase
- M-phase- Mehta phase- mitosis (division)

DNA duplicates in the S-phase (DNA-synthesis phase). This process is called DNA replication. Later in the M-phase (mitosis) the cell divides into two daughter cells and the replicated DNA will be distributed equally to each cells. This process is called Mitosis.
Between S-phase and M-phase there are Gap phases (G1 and G2). In these phases, the cell gets time to grow and make proteins (Figure 3).

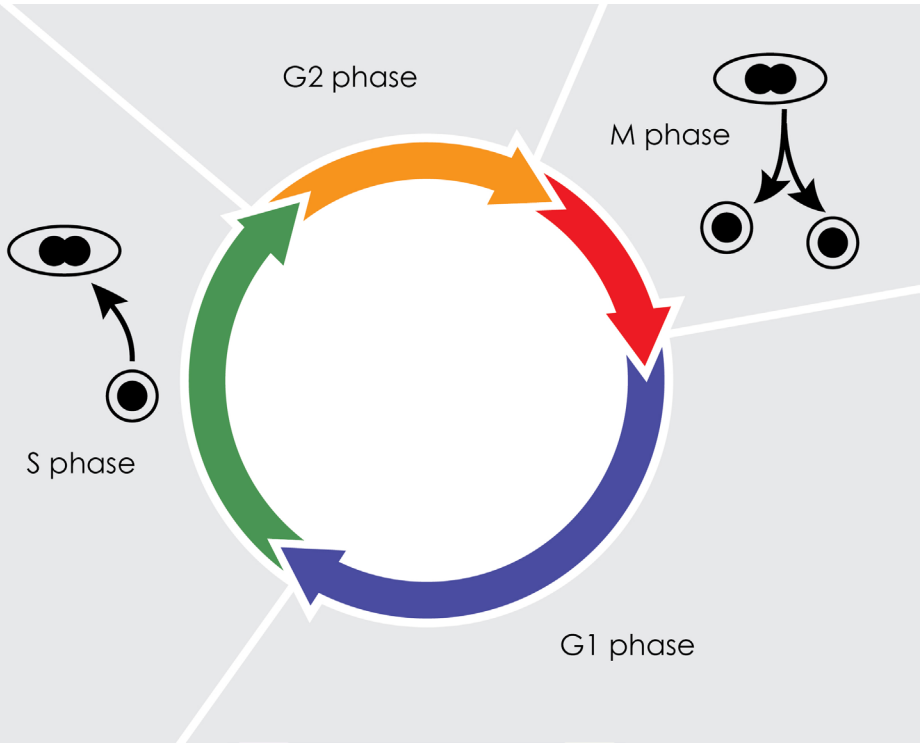


Figure 3: *Cell cycle process is where the cell reproduces itself. This process has 4 phases; S phase (Synthesize Phase), M-phase (Mitosis phase) and G-phases (Gap phases). In S-phase the DNA will duplicate, and in M-phase the cell will divide into two separated daughter cells and the DNA will be distributed equally to each cell. In G-phases, the cell gets time to grow and make proteins.*

2.6.2.1 The control system

The cell cycle has several control system. These systems consist of proteins, enzymes, kinases and etc. that regulate the cycle. The system decides whether the cell shall go to the next phase or stay in the current phase. This is dependent on how the extracellular and intracellular environment is. In the cycle there are checkpoints, where the control system regulates this process.

2.6.2.2 DNA damage

DNA damage is one of the important cell injuries that influence the control system. When DNA damage occurs, a signaling pathway will be initiated. Protein kinases ATM and ATR will be activated and with their association a downstream of various proteins will be activated and phosphorylated thus leads to cell cycle arrest. One of the known signal pathway s when ATM and ATR activate protein kinases called Chk1 and Chk2 by phosphorylation and a gene regulatory protein p53 will also get phosphorylated. Phosphorylated-p53 (P-p53) will then affect gene transcription of proteins that will cause cell cycle arrest. The cell will be in the current phase until the DNA gets repaired or it will go into apoptosis [30]. (Figure 4)

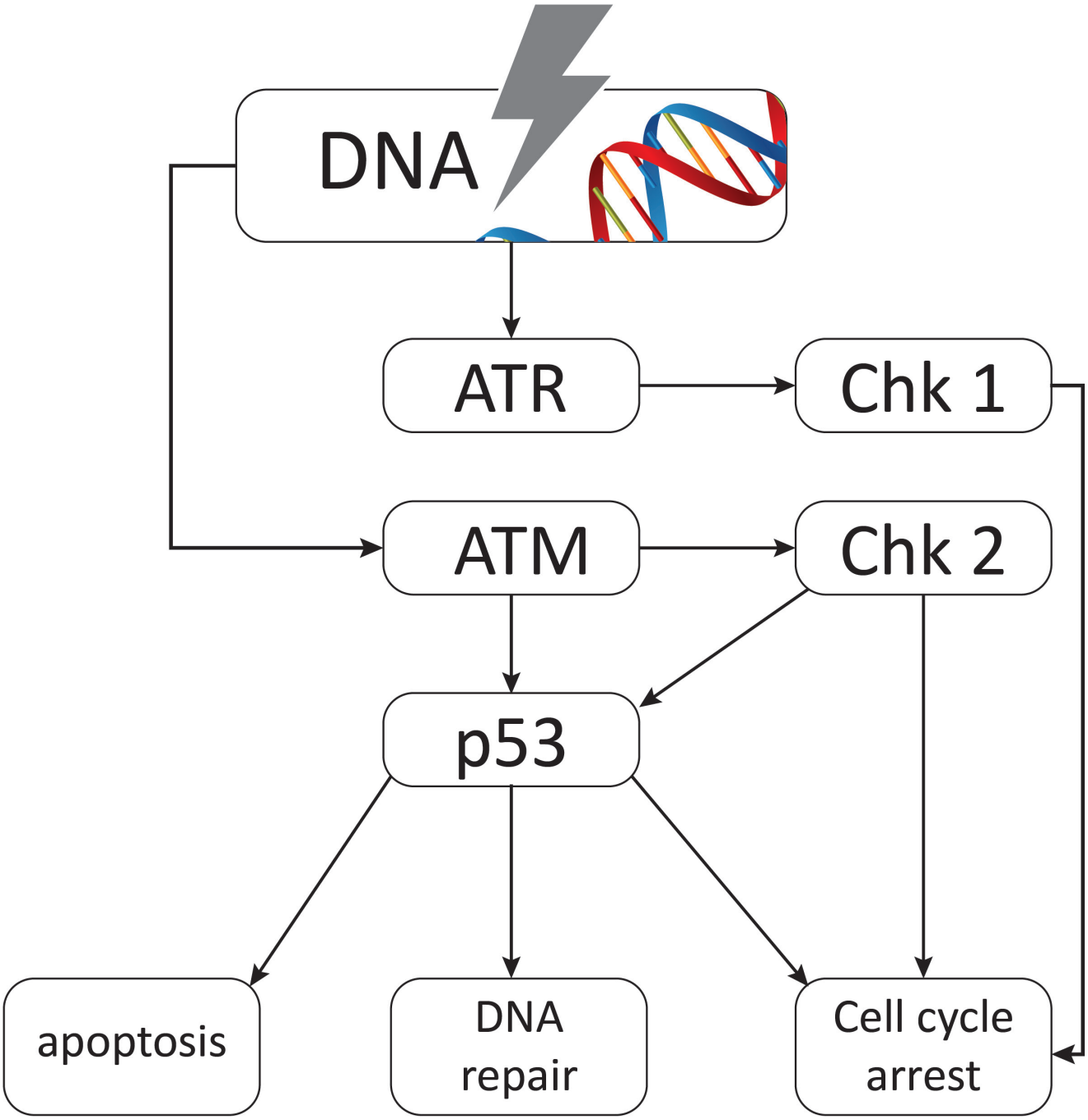
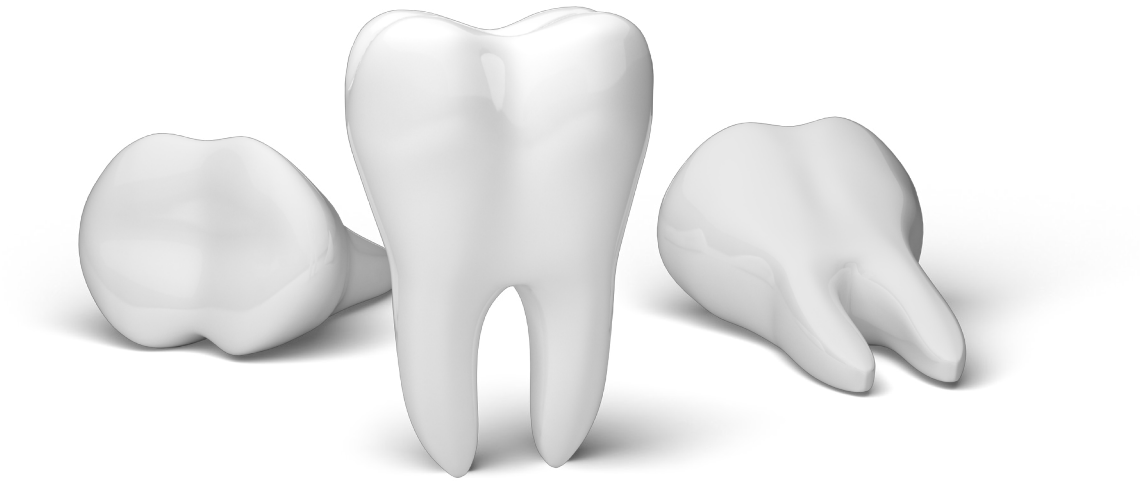


Figure 4: *A simplified illustration of the signaling pathway when DNA damage occurs. Various proteins will be phosphorylated and activated. ATM and ATR activate protein kinases called Chk1 and Chk2 by phosphorylation and a gene regulatory protein p53 will also get phosphorylated. Phosphorylated-p53 (P-p53) will then stimulate gene transcription of proteins that will cause cell cycle arrest, DNA reparation or apoptosis (cell death).*

2.6.3 Cell death

Apoptosis and necrosis are two known mechanism of cell death. Apoptosis is an active programmed cell death that gives morphology changes in the cells, such as cellular and nuclear shrinkage, chromatin condensation, DNA fragmentation and formation of apoptotic bodies. When a cell can't repair its injury; it will go through an apoptotic process to remove the damaged cell. This process can be activated by intracellular and extracellular signals. The programmed cell death is dependent on an intracellular proteolytic enzyme cascade known as Caspases. In a normal living cell, this proteolytic enzyme is inactive precursors called procaspases. During on apoptotic process, the procaspases will be cleaved to active caspases, which will further activate downstream caspases and other enzymes. This is called caspases cascade and will break down intracellular proteins and cause cell death.
In contrast to apoptosis, necrosis is a passive process. Necrotic cell death is the unexpected or accidental cell death that may occur as a result of physical or chemical exposure that disrupts cellular structure or activity. During necrosis, cells swell and cellular content leak due to loss of membrane integrity [31-33].



3. Aim of the study and hypothesis

The specific aim of this study is to:

- Measure the effect of BisGMA on BEAS-2B cell viability
- Analyze the cause of changes in cell viability (cell death and cell growth)
- Analyze the DNA damage response activity

Our hypothesis is that BisGMA is cytotoxic to human bronchial epithelial cells (BEAS-2B cells). Further we hypothesize that DNA damage is not an initial cause of a toxic response.

4. Materials and methods

4.1Materials:

4.1.1 Monomers

Bisphenol A-glycidyl dimethylcrylate(BisGMA)(CAS.no 1565-94-2) was from Poly sciences. Inc Warrington. BisGMA stock solution(0.3mM) was prepared in Dimethyl sulfoxide (DMSO) and further diluted in cell culture medium.
2- hydroxyethyl methacrylate (HEMA) (CAS no.868-77-9) was from Fluka Chemie AG, Buchs Switzerland. HEMA were directly diluted in cell culture medium.

4.1.2 Other chemicals

- Serum free Lechner and Laveck (LHC9) medium was purchased from Invitrogen-life Technologies (Camarillo, CA, USA).
- Hepes buffered saline (HBS) was purchased from Lonza Verciers
- Collagen (PureCOL TM was Inamed Biomaterials (Fremont , CA USA)
- Trypsin was purchased from Lonza , Verviers, Belgium
- Phosphate buffered physiological saltwater (PBS) was purchased from Lonza, Verviers, Belgium
- MTT- salt was purchased from Sigma- Aldrich, St. Louis, USA
- Hoecsth 33342 from Sigma –Aldrich, St. Louis USA
- PI Sigma- Aldrich, St Louis, USA)
- Annexin V- FITC Apoptosis Detection Kit eBioscience (Vienna, Austria)

All other chemicals where purchased from commercial sources at highest purity available.

4.1.3 Equipment

- Phase contrast microscopy, Olympus CKX41, with Olympus C7070 camera, Olympus Europe, Hamburg, Germany
- Fluorescence microscope, Olympus BX51 with Olympus DP 70 camera, Olympus Europe, Hamburg, Germany
- Flow cytometer, Cell Lab Quanta SC, Beckman Coulter, Florida USA
- Spectrophotometer, Synergy H1, BioTek Instruments Inc ., Vermont, USA
- Centrifuge , Rotina 35R, Hettich, Tuttlingen, Germany

4.2 Methods

4.2.1 Cell culture

BEAS-2B cells are SV40 hybrid (Ad12SV40)- transformed human bronchial epithelial cell line [34]. The cell line was purchased from ECACC, the European Tissue Type Culture Collection.
The cells were cultured in LHC9 medium and were replaced every second day. The cells were passaged when they had reached >85% confluence. The culture flasks were pre- coated in Hepes- buffered saline (HBS) with collagen (30µg/mL), The cells were cultured at 37˚C, in air atmosphere containing 5% CO_2 and >95% relative humidity. The cells where seeded on plates or in single wells (Falcon®) precoated with HBS with collagen, one day before the assays were done.

4.2.2 Cell treatment

BisGMA stock solution was diluted in cell culture medium (LHC9) into various concentrations. The exposure was done by exchanging the medium with cell in the single wells and plates with BisGMA-containing medium.

4.2.3 Morphology of the cells

600 000 cells were seeded in each wells with 5mL medium. Exposure of the cell culture was done the next day after seeding. BisGMA was diluted with fresh medium to 200µM, 100µM, 50µM, 25µM and 10 µM. Phase-contrast microscope picture of the wells were taken with different concentration before exposure and 30min, 1 hour and 2 hours after exposure of BisGMA.

4.2.4 Cell toxicity assay, MTT

The assay was carried out according to Denizot et al. (1985)[35]. BEAS-2B cells were seeded at a concentration of 60 000 cells/ wells. The MTT assay was done in two different periods, 30 minutes and 1hour after the exposure. The medium was then exchanged in each well with MTT solution (0,5mg/mL PBS), followed by 1hour incubation at 37˚C. Then the MTT solution was removed and 500µL of DMSO was added in each well to solubilize the formazan product. The plate was then shaken to ensure the solubilization of the blue formazan for about 10 to 15 minutes. The plate was then measured by the spectrophotometer in the absorption at 570nm.

4.2.5 Evaluation of apoptosis and necrosis With Hoechst and PI

Hoechst 33342 is a fluorescent DNA-intercalating probe that is membrane-permeable. This gives the blue dye in chromatin in viable and apoptotic cell nuclei. During apoptosis, chromatin condensation occurs. Condensed chromatin can be visualized by fluorescence microscopy of Hoechst stained cells. A clearly more intense fluorescence is seen in apoptotic cells. In contrast, Propidium iodide (PI) cannot go through intact cell membranes like Hoechst. It is a red fluorescent probe, which is only permeable in necrotic cells [36].

The assay was done according to Alexander et al (2011). The cells were first exposed to 100µM, 50µ, 25µM. After 1hour, the cells were trypsinized. The tubes were centrifuged with cells and medium in 250g for 10minutes. The medium was then removed from the tubes. The cells were re-suspended in 50µL FBS containing 10µg/mL Hoechst 33342 and 10µg/mL PI. After incubation for 30min in dark at room temperature, the cells were spread onto microscope slides. At least 300 cells were counted using a fluorescence microscope with an excitation filter 340- 380nm, and cells were classified as apoptotic, necrotic and viable cells.

4.2.6 Flow cytometer

Flow cytometer is used to measure fluorescence of single cells in a cell suspension. First the cells will be stained with flour chrome, which binds to the component we want to study in or on the cell. The cells will go inn a flow system, by passing a light from UV or laser. The light and fluorescence from the cell will be recorded in the system. There are plenty of study spaces by using the flow cytometer. We can study molecules on the cell surface, cytoplasm or the nucleus by using the flow cytometer. The data will be presented as a histogram or cytogram. The cytogram is presented with dot, contour or density. Each dot is represented as one single cell. [37]

4.2.6.1 Staining with annexin V and PI

Flow cytometer staining with Annnexin V conjugated with FITC and Propidium iodide (PI) detects apoptosis and necrosis.

A viable cell has an asymmetric cell membrane, where Phosphatidylserine (PS) is on the cytosol side of the membrane. In an apoptotic process the PS will be trans-located to the surface of the cell. Annexin V is a protein, which binds to PS. This protein is not permeable to intact cell membrane (viable cells). Annexin V only has access to bind to PS on apoptotic and necrotic cells. PI is only permeable to necrotic cells membrane. That's why PI is used to distinguish between apoptotic and necrotic cells. Annexin V is conjugated with FITC to be used in flow cytometer [36, 38]. (Figure 5)

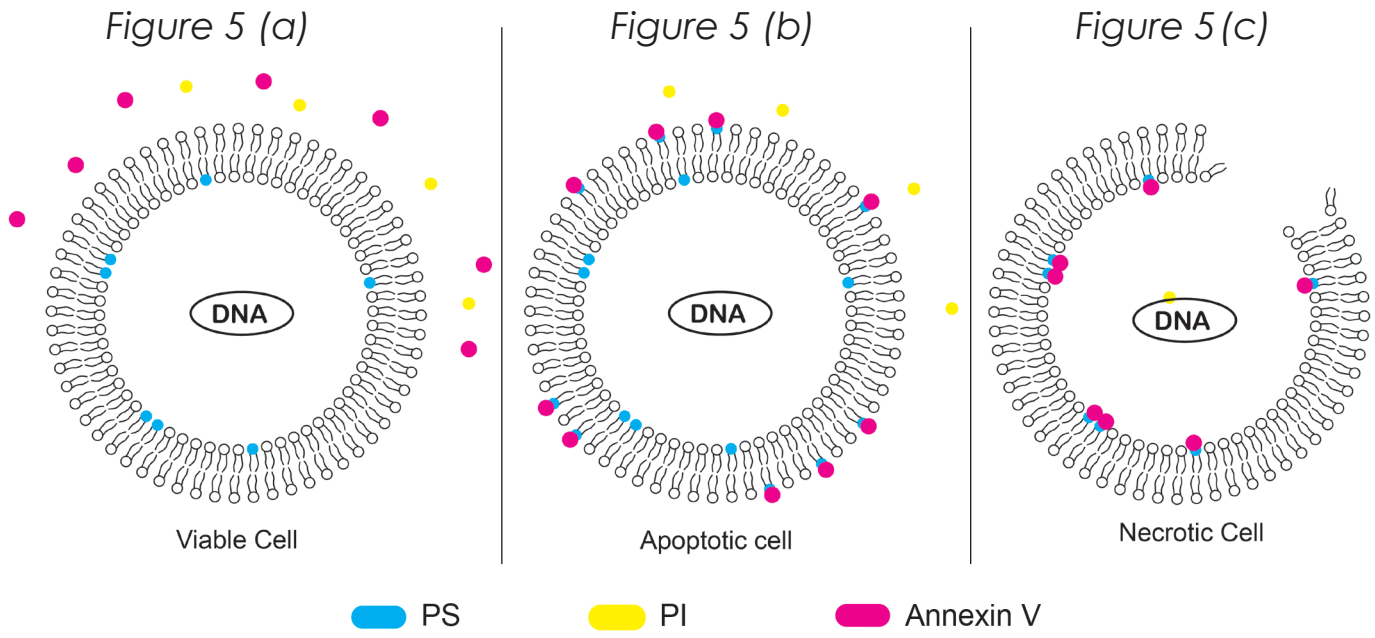


Figure 5: *Simplified illustration of how Annexin V and PI response to Viable and Apoptotic cells and Necrotic cells. Annexin V is a protein, which binds to Phosphatidylserine (PS). PI will bind to DNA and can´t go through intact membrane.*
(a) A viable cell has an asymmetric cell membrane, where PS is on the cytosol side of the membrane.
(b) In an apoptotic process the PS will be trans- located to the surface of the cell. That's why Annexin V is able to bind apoptotic cells.
(c) On necrotic cells the membrane will be injured and PI and annexin V are able to bind to their substrate [38]

The assay was done according to Alexcander E. et al. The cells were exposed with 10µM, 25µM, 50µM and 100µM BisGMA and 5mM HEMA. The cells with HEMA were exposed for 24 hours ,while cells with BisGMA were exposed for 1 hour. After exposure, the cells were trypsinized, centrifuged and washed with PBS twice. 400µL 1xBiniding buffer, 5µL FITC-Annexin V and 10µL PI were added in each tube. The tubes were mixed gently and then incubated in 15 min at room temperature in the dark. Samples were measured using Flow cytometer and the results are presented in cytogram. Viable, apoptotic and necrotic cells were determined as described by Alexander et al (2011).

4.2.6.2 Cell cycle analysis

The cells were exposed to 0 - 25µM BisGMA in 24 hours. Then the cells were trypsinized and diluted in citrate buffer before staining with nuclear staining solutions (detergent and DAPI). The cells were analyzed on a flow cytometer.

DNA- histogram was analyzed by Multicycle software (Phoenics Flow Systems, San Diego, CA). T he different cell-cycle phases were identified based on the DNA content (Figure 6).

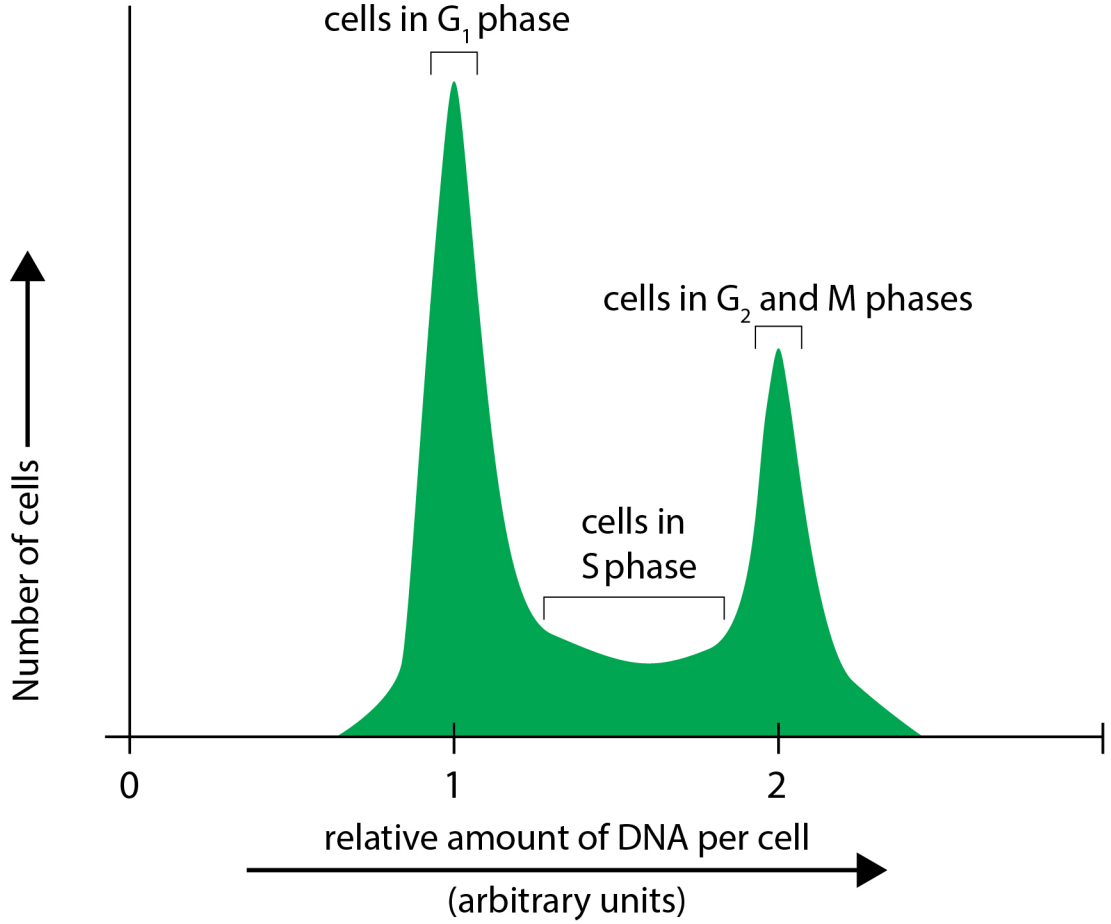


Figure 6: *DNA histogram as recorded on a flow cytometer: The histogram shows distribution of cells in different phases in the cell cycle.*

4.2.7 Western blot- protein- analyse

Western blot is used to detect and quantify specific proteins in cell and tissue and extracts.
The cells where exposed for up to 100 µM BisGMA for 1, 2 and 6 hours.
After the exposure, the cells were washed with PBS and dissolved in SDS-sample buffer – (150mM TRIS, 12% SDS, 30% glycerol, pH 7.0).
For the protein- analyse Tricine- SDS-PAGE was used. After electrophoresis and blotting onto nitrocellulose membrane, the membrane was stained with Ponceau-S to verfy equal protein application in each well.
The membranes were blocked with 3% Bovine Serum Albumin (BSA) in tris buffered saline (TBS)- Tween (0.1% [vol/vol] (TBS-T)Tween) for 1 hour. It was incubated with primary

antibody diluted in TBS-T containing 1% BSA overnight at 4°C. Next day the membrane was washed three times for 5min with TBS-T before incubation for 1 hour at room temperature in 1% BSA in TBS-T containing secondary antibody. The membrane-bound antibody was washed 3 times for 5 minutes in TBS-T. To detect the protein, 1 X SignalFire ECL Reagent was prepared and was incubated with the membrane for 1 minute. The excess solution was removed and the membrane was wrapped with plastic and exposed to X-ray film.

Phosphorylated p53 (Ser15), active- caspases 3 and phosphorylated-Chk1 and phosphorylated Chk2 were used as primary antibodies. Each membrane was exposed with one primary antibody.

4.2.8 Statistic

All analyses were performed using Graph Pad Prism software (GraphPad Software, CA, USA) data and were analyzed using Student´s t-test to evaluate the differences between control groups and BisGMA exposed groups. P-values $\leq 0,05$ were considered significant. ¨*¨Indicates statistical different from control ($p \leq 0,05$).

5. Results

5.1The morphology of the cells

The effect of BisGMA exposure was monitored by phase contrast microscopy up to 2 hours. Morphological changes were observed in cell cultures exposed with BisGMA compared to the control cell culture. The changes in the cells are more clearly to be seen, as the concentration of BisGMA and the time increased. (Figur 7)

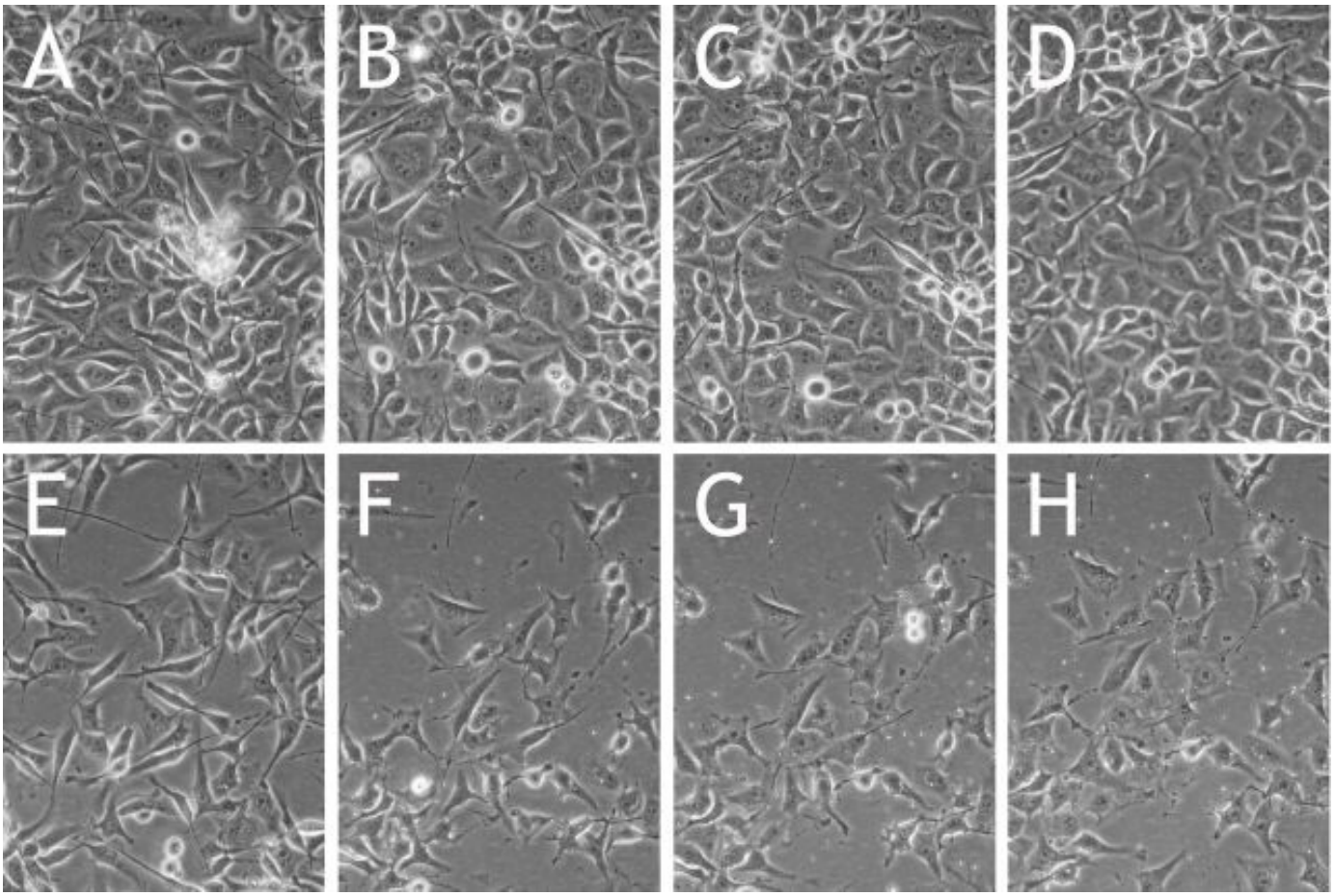


Figure 7: *Phase contrast microscopy picture of BEAS-2B cells. Picture was taken before and after exposure of BisGMA, from 0-2 hours. Picture A was taken before exposure of 25µM and B-D was taken 30 minutes, 1hour and 2 hours after the exposure of BisGMA in BEAS-2B cellls. Picture E was taken before exposure of 50µM and F-H was taken after 30 minutes, 1hour and 2 hours after the exposure of BisGMA in BEAS-2B cells. Morphological changes can be seen, as the concentration of BisGMA and the exposure time increased.*

5.2 The viability of the cell

MTT- assay measures only viable cells. The tetrazolium salt MTT (3-(4,5-dimethulthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) register the activity of succinate dehydrogenase activity (SDH-activity) in the mitochondria of the cells [35, 39]. BisGMA reduces the SDH- activity on bronchial epithelia cells. The results show a concentration and time dependent decrease in the percentage of SDH activity of the control. However, the BEAS-2B cells exposed with 25µM after 30 minutes and 200µM after 1 hour were not significantly. (Figure 8)

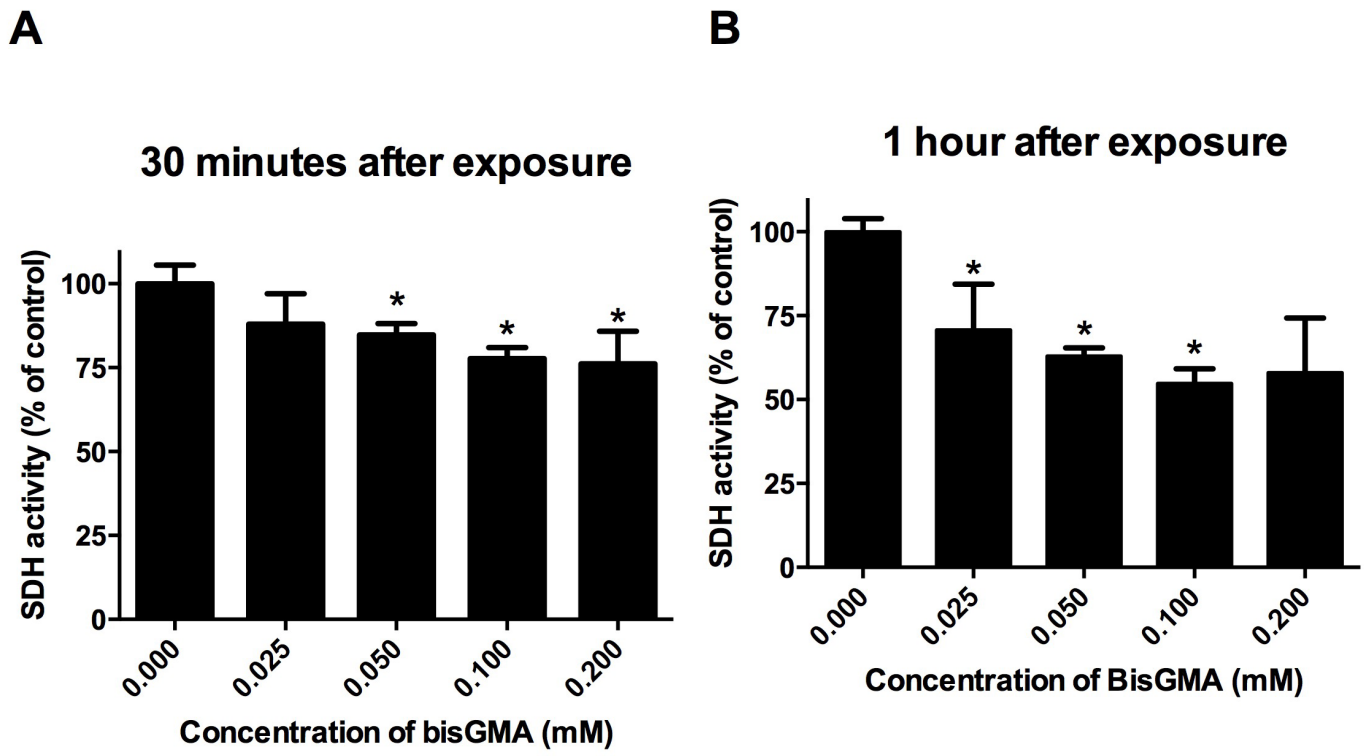


Figure 8: *MTT-assay measures the SDH-activity, which is only detectable on viable cells. Figure 9A: MTT-assay results after 30 minutes exposure of BisGMA. As the concentration of BisGMA increased above 25 µM, the SDH-activity decreased significantly. Figure 8B: MTT-assay results after 1hour exposure. Compare to figure A, the SDH-activity is further decreased. However, the SDH activity in cells exposed to 200 µM were not significantly different from control.*

5.3 Cell death mechanism with Hoecsth 33332 and PI

The evaluation assays of apoptosis and necrosis, present what this toxicity is caused by. Staining with Hoecsth 33332 and PI assay gave results that show a concentration dependent on the increase in the percentage of necrosis cells after exposure to BisGMA (significant at 25 and 100µM) (Figure 9). Figure 10 shows the references used to count the cells.

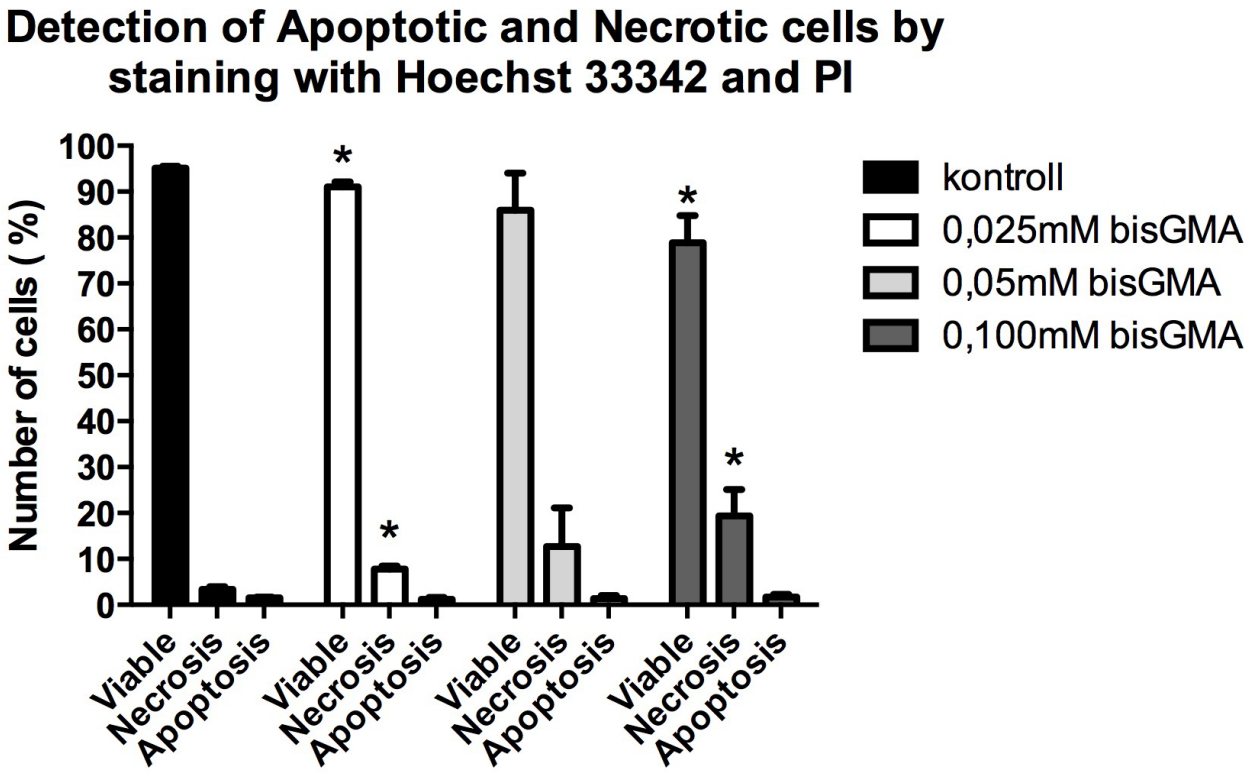
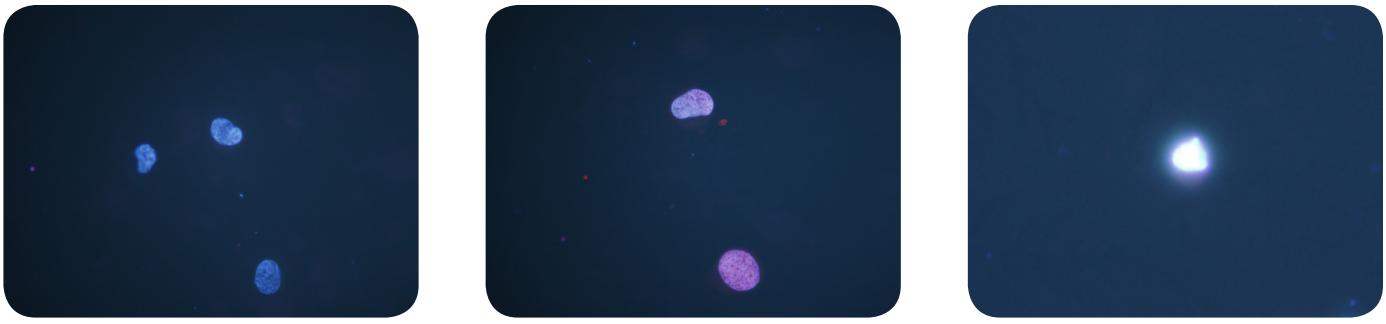


Figure 9: *Results from Hoecsth 33332 and PI assay. There was increased percentage of necrotic cells (significant at 25 and 100 µM) and decrease level of viable cells (significant at 25 and 100 µM) as the concentration of BisGMA was increased. No change in the number of apoptotic cells was seen.*



Viable cell *Necrotic cell* *Apoptotic cell*

Figure 10: *The Hoecsth 33332 and PI assay, references used when counting the cells on fluorescence microscope.*

5.4 Cell death mechanism with FITC-Annexin V and PI

The flow cytometer show a concentration dependent increase in the necrotic cells. The results are presented in cytogram. FITC- Annexin V is on the x- axis and PI is on the y-axis. Cytogram shows in dot plots where each dot represents one single cell [36, 37]. This cytogram has 4 regions. The lower left region contains viable cells (Annexin V and PI negavtive), the lower right quadrant contains apoptotic cells (annexin V postitive and PI negative), the upper right quadrant contains necrotic cells (annexin V positive and PI negative) (Figure 11).

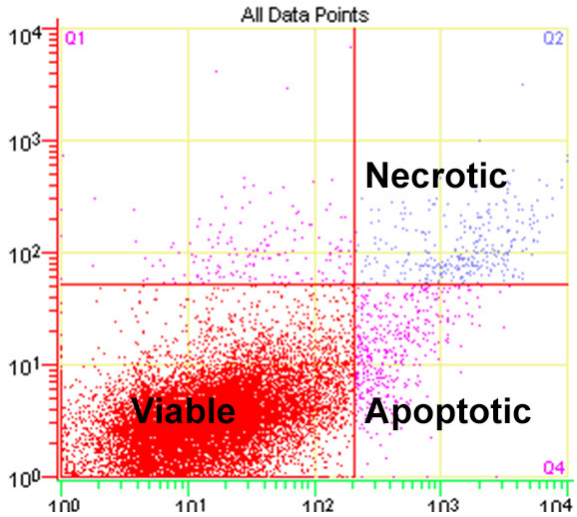


Figure 11: *Cytogram shows in dot plots where each dot represents one single cell.[36, 37] This cytogram has 4 regions. The lower left region contains viable cells (Annexin V and PI negavtive), the lower right quadrant contains apoptotic cells (annexin V postitive and PI negative), the upper right quadrant contains necrotic cells (annexin V positive and PI negative)*

The control had most percentage of viable cells. When concentration of BisGMA increases, there are a decreased percentage of viable cells and increased death cells.
However, in lower concentrations from 10µM to 25µM BisGMA, this assay shows increased percentage of apoptosis compared to the control. But the difference between the percentages of apoptosis in 10µM BisGMA and control is not high. In 25µM exposed cell culture, there are significantly no differences between percentages of apoptotic and necrotic ((PI positive and annexin V negative) + necrosis) cells.
There was increased percentage of necrotic cells as the concentrations BisGMA increases.
Cell culture exposed with 5mM HEMA, gave increased percentage of apoptotic cells compared to the control, but there was no significant increase in the percentage of necrotic cells compared to control was observed (figure 12)

Figure 12 (a)

Control

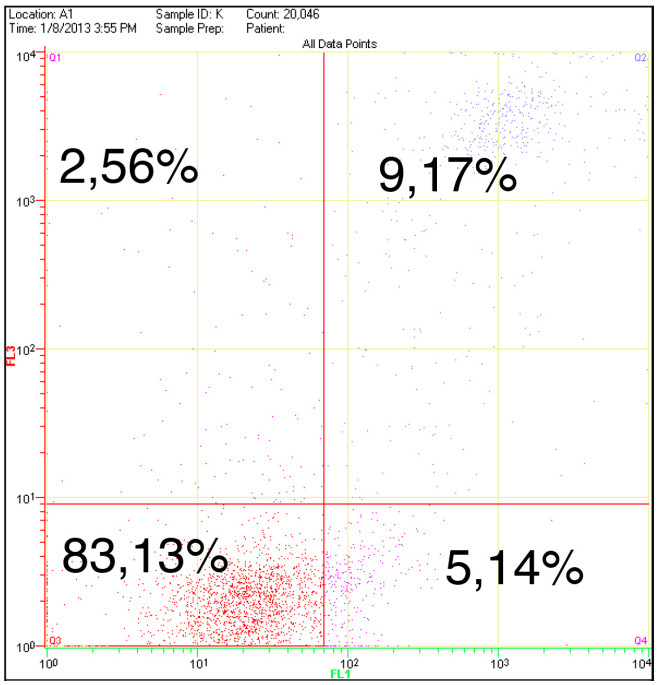


Figure 12 (b)

10µM bisGMA

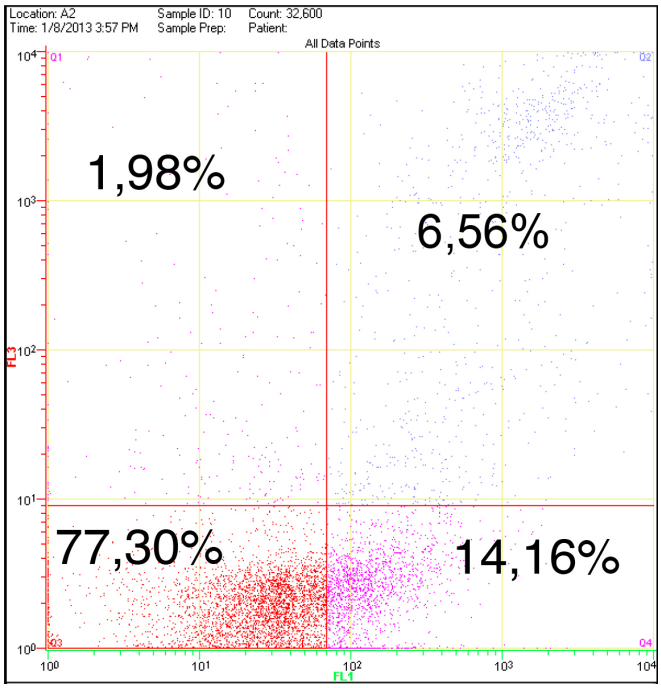


Figure 12 (c)

25µM bisGMA

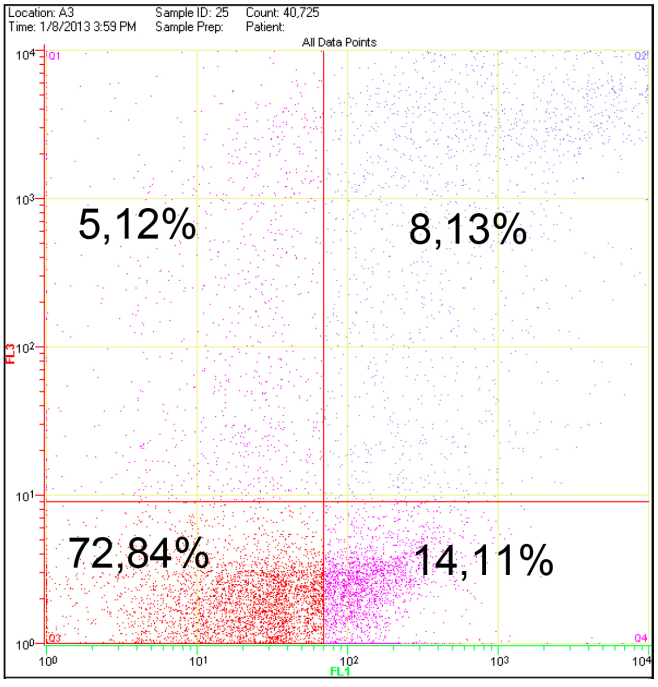


Figure 12 (d)

50µM bisGMA

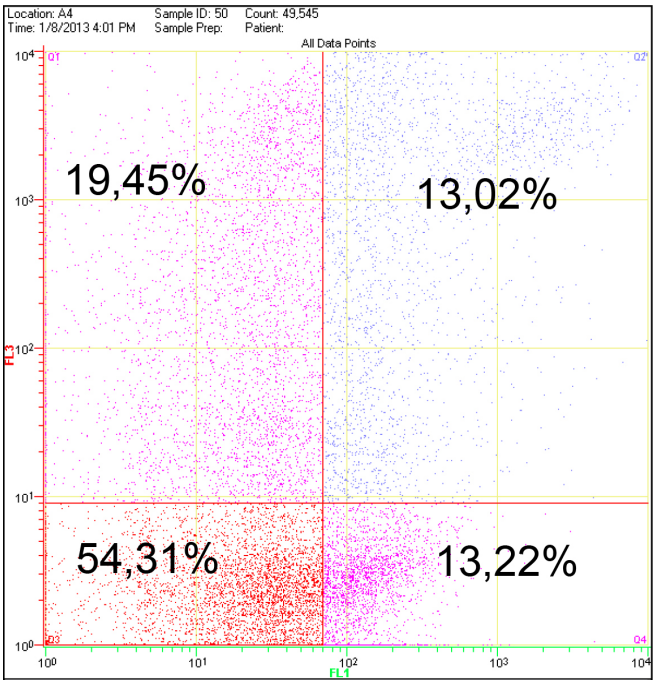


Figure 12 (e)

100µM bisGMA

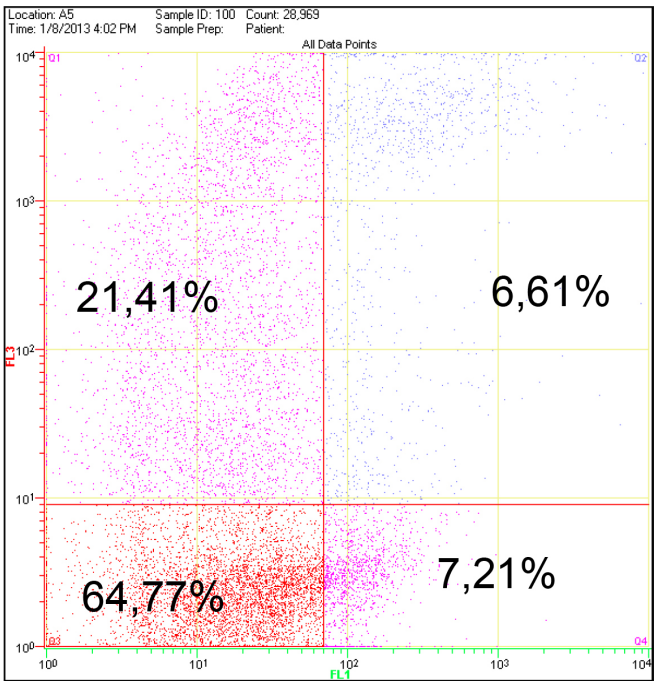


Figure 12 (f)

HEMA

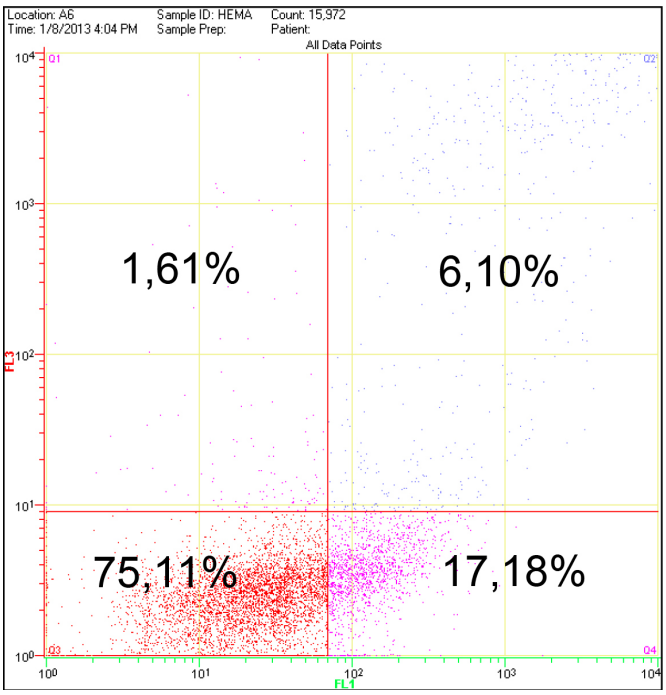


Figure 12 *(a, b, c, d, e, f)**:** The control had most percentage of viable cells. Compared to the control, when concentration of BisGMA increases, there are a decreased percentage of viable cells and increased death cells. In 25µM exposed cell culture, there are significantly no differences between percentages of apoptotic and necrotic ((PI positive and annexin V negative) + necrosis) cells. In 50µM and 100µM BisGMA there were increased PI positive and Annexin V negative cells and necrotic cells. HEMA had more percentage of apoptotic cells than necrotic cells.*

5.5 DNA- damage response and apoptosis

The cell cycle analysis did not show any alterations in cells exposed with BisGMA. There was no increase in the activity of the DNA-damage response proteins p53 and Chk-1/Chk-2 as measured by Western blotting (data not shown). Caspase -3 is a protein which is activated in apoptotic cells [33]. The level of active caspase -3 was not altered by BisGMA exposure (data not shown).

6. Discussion

The effect of BisGMA has been studied in different cell lines. In those studies, it was observed that BisGMA induce cytotoxicity in vitro [18-26]. Although BisGMA is cytotoxic in many systems, the mechanisms underlying this response may vary among cell types. In contrast to our results, BisGMA induce apoptosis on human gingival fibroblasts, dental pulp cells and macrophages [18-20]. The differences may result from use of different treatment, concentration and duration.

The current study shows that BisGMA induce toxicity on BEAS-2B cells dependent on time and concentration. Microscope pictures show that BisGMA induce morphology changes in cells as the concentration and time increases. The most clearly changes in the cells were seen after exposure of 50μM and higher concentrations (figure 7). In addition, the MTT-assay show that the SDH-activity decrease on BEAS-2B cells as the concentration of BisGMA increase. Compare to 30 minutes exposure of BisGMA on BEAS-2B cell, the 1- hour exposure of BisGMA cells have more decrease percentage of SDH-activity (figure 8). These results conclude that the toxicity of BisGMA depend on time and concentration.

The evaluation of cell death assay, staining Hoecsth 33332 and PI, shows that BisGMA exposure cause necrotic cell death in BEAS-2B cells. Most necrotic cells can be seen when the cells were exposed with 50μM BisGMA and further up (figure 9). The flow cytometer assay with Annexin V and PI results show roughly the same. The cells treated with 50μM and 100μM BisGMA had increased PI positive cells with no evident Annexin V staining. As seen on the figure 5, PI only stain cells lacking membrane integrity, such as necrotic cells. Annexin V is also expected to bind PS in non-apoptotic cells when the cell membrane is damaged. The lack of Annexin V staining in the PI positive cells may suggest "semi-permeable" membranes. The Annexin V protein is relative large (~34 kDa) compared to PI (~0.7 kDa). During normal necrosis where the cells swell and burst, the cell membrane is permeable to Annexin V. Hence; our results indicate a different mechanism. One possible explanation may be that the lipophilic BisGMA-molecule damages the plasma membranes directly, thereby inducing toxicity.

BisGMA didn't affect the cell cycle profile of BEAS-2B cells. The western blot assay detected p-p53, but there were no measurable increase of this protein. As earlier described, p-p53 is a gene regulatory protein, and its activity is altered in cells with DNA damage. These results indicate that BisGMA do not induce DNA damage in BEAS-2B cells at the concentrations used in the current study. The lack of change in Chk-1 and Chk-2 activity supports this assumption.

In contrast to BisGMA, HEMA induces apoptosis on BEAS -2B cells (figure 12). Studies on the mechanism of HEMA toxicity in BEAS-2B cells support this result [27, 40]. The studies about HEMA toxicity [27, 40] and the current study indicate that the properties and the chemical structure of methacrylate monomers influence the response to cells. This may explain that the response of BisGMA to BEAS-2B cells is different compared to HEMA.

7. Conclusion

The present study shows that BisGMA is cytotoxic to BEAS-2B cells in a concentration and time dependent manner. This study could not detect DNA-damage in the exposed cells, but membrane damage is suggested as an initial event.

8. Reference

1. Arenholt-Bindslev, D., Environmental aspects of dental filling materials. Eur J Oral Sci, 1998. 106(2 Pt 2): p. 713-20.
2. http://www.mercuryconvention.org/.
3. Schmalz, G., Resin-Based Composites, in Biocompatibility of Dental Materials. 2009, Springer-Verlag: Berlin Heidelberg. p. 99-137.
4. Santerre, J.P., L. Shajii, and B.W. Leung, Relation of dental composite formulations to their degradation and the release of hydrolyzed polymeric-resin-derived products. Crit Rev Oral Biol Med, 2001. 12(2): p. 136-51.
5. Alshali, R.Z., N. Silikas, and J.D. Satterthwaite, Degree of conversion of bulk-fill compared to conventional resin-composites at two time intervals. Dent Mater, 2013. 29(9): p. e213-7.
6. Jaffer, F., Y. Finer, and J.P. Santerre, Interactions between resin monomers and commercial composite resins with human saliva derived esterases. Biomaterials, 2002. 23(7): p. 1707-19.
7. Qvist, V., The effect of mastication on marginal adaptation of composite restorations in vivo. J Dent Res, 1983. 62(8): p. 904-6.
8. Finer, Y., F. Jaffer, and J.P. Santerre, Mutual influence of cholesterol esterase and pseudocholinesterase on the biodegradation of dental composites. Biomaterials, 2004. 25(10): p. 1787-93.
9. Finer, Y. and J.P. Santerre, Biodegradation of a dental composite by esterases: dependence on enzyme concentration and specificity. J Biomater Sci Polym Ed, 2003. 14(8): p. 837-49.
10. Hagberg, S., et al., Exposure to volatile methacrylates in dental personnel. J Occup Environ Hyg, 2005. 2(6): p. 302-6.
11. Marquardt, W., et al., Volatile methacrylates in dental practices. J Adhes Dent, 2009. 11(2): p. 101-7.
12. Michelsen, V.B., et al., Detection and quantification of monomers in unstimulated whole saliva after treatment with resin-based composite fillings in vivo. Eur J Oral Sci, 2012. 120(1): p. 89-95.
13. Reichl, F.X., et al., Distribution and excretion of BisGMA in guinea pigs. J Dent Res, 2008. 87(4): p. 378-80.
14. Henriks-Eckerman, M.L., et al., Exposure to airborne methacrylates and natural rubber latex allergens in dental clinics. J Environ Monit, 2001. 3(3): p. 302-5.
15. Kanerva, L., et al., Increase in occupational skin diseases of dental personnel. Contact Dermatitis, 1999. 40(2): p. 104-8.
16. Connolly, M., et al., Allergic contact dermatitis from bisphenol-A-glycidyldimethacrylate during application of orthodontic fixed appliance. Contact Dermatitis, 2006. 55(6): p. 367-8.

17. Schweikl, H., G. Spagnuolo, and G. Schmalz, Genetic and cellular toxicology of dental resin monomers. J Dent Res, 2006. 85(10): p. 870-7.
18. Li, Y.C., et al., The role of DNA damage and caspase activation in cytotoxicity and genotoxicity of macrophages induced by bisphenol-A-glycidyldimethacrylate. Int Endod J, 2012. 45(6): p. 499-507.
19. Yang, M.L., The effects of cytotoxicity and genotoxicity induced by 2,2-bis[4-(acryloxypropoxy)phenyl]propane via caspases in human gingival fibroblasts. Toxicol Ind Health, 2014. 30(8): p. 755-64.
20. Engelmann, J., et al., Effects of BisGMA on glutathione metabolism and apoptosis in human gingival fibroblasts in vitro. Biomaterials, 2004. 25(19): p. 4573-80.
21. Schweikl, H., G. Schmalz, and T. Spruss, The induction of micronuclei in vitro by unpolymerized resin monomers. J Dent Res, 2001. 80(7): p. 1615-20.
22. Issa, Y., et al., Resin composite monomers alter MTT and LDH activity of human gingival fibroblasts in vitro. Dent Mater, 2004. 20(1): p. 12-20.
23. Kleinsasser, N.H., et al., Genotoxicity and cytotoxicity of dental materials in human lymphocytes as assessed by the single cell microgel electrophoresis (comet) assay. J Dent, 2004. 32(3): p. 229-34.
24. Moharamzadeh, K., et al., Cytotoxicity of resin monomers on human gingival fibroblasts and HaCaT keratinocytes. Dent Mater, 2007. 23(1): p. 40-4.
25. Chang, M.C., et al., The role of reactive oxygen species and hemeoxygenase-1 expression in the cytotoxicity, cell cycle alteration and apoptosis of dental pulp cells induced by BisGMA. Biomaterials, 2010. 31(32): p. 8164-71.
26. Urcan, E., et al., Induction of DNA double-strand breaks in primary gingival fibroblasts by exposure to dental resin composites. Biomaterials, 2010. 31(8): p. 2010-4.
27. Morisbak, E., V. Ansteinsson, and J.T. Samuelsen, Cell toxicity of 2-hydroxyethyl methacrylate (HEMA): the role of oxidative stress. Eur J Oral Sci, 2015. 123(4): p. 282-7.
28. Ansteinsson, V., et al., Cell toxicity of methacrylate monomers-the role of glutathione adduct formation. J Biomed Mater Res A, 2013. 101(12): p. 3504-10.
29. Schmalz, G.A.-B., D., Basic Aspects, in Biocompatibility of Dental Materials. 2009, Springer-Verlag: Berlin Heidelberg. p. 1-12.
30. Bruce Alberts, A.J., Julian Lewis, Martin Raff, Keith Roberts, Peter Walter., The cell cycle, in The cell. 2008, Garland science. p. 1053-1114.
31. Kam, P.C. and N.I. Ferch, Apoptosis: mechanisms and clinical implications. Anaesthesia, 2000. 55(11): p. 1081-93.
32. Samejima, K. and W.C. Earnshaw, Trashing the genome: the role of nucleases during apoptosis. Nat Rev Mol Cell Biol, 2005. 6(9): p. 677-88.
33. Bruce Alberts, A.J., Julian Lewis, Martin Raff, Keith Roberts, Peter Walter., Apoptosis, in The cell. 2008, Garland science. p. 1115-1130.
34. Roger R. Reddel, Y.K., Brendal I. Gerwin, et al., Transformation of Human Bronchial Epithelial Cells by Infecton with SV40 or Andenovirus-12 SV40 Hybrid Virus, or Transfection via Strontium Phosphate Copreciptation with a Plasmid Containing SV40 Early Region Genes. Cancer Research, 1988. 48: p. 1904-1909.
35. Denizot, F. and R. Lang, Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods, 1986. 89(2): p. 271-7.

36. Alexander E. Kabakov, V.A.K., Vladimir L. Gaba, Determination of Cell Survival or Death, in MOLECULAR CHAPERONES, T.L.P. Stuart K. Calderwood, Editor. 2011, Humana Press. p. 231-244.
37. Available from: http://flowbook-wiki.denovosoftware.com/Flow_Book/Chapter_1%3a_Introduction.
38. van Engeland, M., et al., Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry, 1998. 31(1): p. 1-9.
39. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 1983. 65(1-2): p. 55-63.
40. Ansteinsson, V., et al., DNA-damage, cell-cycle arrest and apoptosis induced in BEAS-2B cells by 2-hydroxyethyl methacrylate (HEMA). Mutat Res, 2011. 723(2): p. 158-64.

9. Acknowledgement:

I would like to express my gratitude to my supervisor professor Dr. Odont Jon E. Dahl, Co-supervisors Jan Tore Samuelsen Dr.philos and Vibeke Ansteinsson phd, for providing me an opportunity to do my master thesis in NIOM, and for the patient guidance, enthusiastic encouragement, support and useful critiques of this project work.

I also wish to express my sincere appreciation to Else Morisbak (Bioengineer MSc) for her brilliant help and guidance in lab, her advice, encouragement and support and in keeping my progress on schedule. Her willingness to give her time so generously has been a vital part in the success of my project

I thank to all other staff member of NIOM who provided tremendous help during the period of my project work.

Finally I want to thank Mr Kalex Ratnam for contribution of his Graphic Design skills in the areas of sketching figures and designing the layout for the final output as well as providing the illustrations and images *(from http://www.billionphotos.com)*.