Toxicity of methacrylate monomers in dental biomaterials

in vitro comparison of the cellular effects of HEMA and TEGDMA

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Toxicity of methacrylate monomers in dental biomaterials – *in vitro* comparison of the cellular effects of HEMA and TEGDMA

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Abstract

Resin-based biomaterials are commonly used materials during dental treatments. 2-hydroxyethyl methacrylate (HEMA) and triethylene glycol dimethacrylate (TEGDMA) are the major exposures during and after treatment from these materials, as unpolymerized methacrylates leaks from the cured resin. Both HEMA and TEGDMA are shown to cause cytotoxicity *in vitro*. It has been suggested that both methacrylates induce toxicity through the same mechanisms. Other studies, however, indicate that there are different cellular responses when human monocytes are exposed to each substance. In these studies, HEMA is suggested to mainly affect cells through electrophilic stress, while TEGDMA is suggested to affect cells through oxidative stress.

Immortalized human leukemia monocytic cell line THP-1 was exposed to HEMA (0.5-15 mM) and TEGDMA (0.25-5 mM) individually and in combinations. Cell viability was measured with MTT assay. Combination concentrations were chosen from the results of the individual exposure on the MTT assay. Glutathione (GSH) and reactive oxygen species (ROS) levels were measured using flow cytometry. Western blotting was used to quantify selected proteins.

Individual and combined exposures of HEMA and TEGDMA resulted in a dose-dependent decrease in cell viability. Similarly, the GSH level also decreased in a dose-dependent manner, although significant at much lower concentrations than the viability loss. Only slight, mostly not significant, increases in ROS levels were measured. Level measurement of the selected proteins, Sequestosome 1 (p62), Heme oxygenase 1 (HO-1), and Pirin, showed an increase in cells exposed to HEMA and TEGDMA, both individually and in combinations.

Based on the similar responses on the different cellular events, the study concluded that there were no data supporting the hypothesis that HEMA and TEGDMA induced toxicity by different mechanisms. The combination experiments indicated that the substance had an additive effect on each other. Further studies are needed to strengthen this new hypothesis, however.

Keywords: *HEMA, TEGDMA, oxidative stress, electrophilic stress, GSH, ROS, cytotoxicity, mechanism, protein, additive interaction*

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Abbreviations

ANOVA	Analysis of Variance
APS	Ammonium persulfate
ARE	Antioxidant response element
ATP	Adenosine triphosphate
Bcl-3	B cell leukemia-3
Bis-GMA	Bisphenol A-glycidyl methacrylate
BSA	Bovine serum albumin
DCF	2',7'-dichlorofluorescein
DCFH	Dichlorodihydrofluorescein
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC50	Effective Concentration values for 50% of population
ECACC	European Collection of Authenticated Cell Cultures
FBS	Fetal bovine serum
FSC	Forward scatter
GSH	Glutathione (reduced state)
GSR	Glutathione-disulfide reductase
GSSG	Glutathione (oxidized state)
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
HEMA	2-Hydroxyethyl methacrylate
HO-1/HMOX1	Heme oxygenase 1
HO	Heme oxygenase

Keap1	Kelch-like erythroid cell-derived protein with Cap'n'collar homology-associated protein 1
LC3	Microtubule-associated protein 1A/1B light chain 3
LED	Light-emitting diode
MAPK	Mitogen-activated protein kinases
mBBr	Monobromobimane
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor-kappa B
NFE2L2	Nuclear Factor, Erythroid 2 Like 2
Nrf2	Nuclear factor erythroid 2-related factor 2
p62/SQSTM1	Sequestosome-1
PBS	Phosphate-buffered saline
PIR	Pirin
PMMA	Polymethyl methacrylate
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RMGIC	Resin-modified glass ionomer composite
ROS	Reactive/Radical oxygen species
SAPK	Stress-activated protein kinases
SD	Standard deviation
SDH	Succinate dehydrogenase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel-electrophoresis
SILAC	Stable isotope labelling by amino acids in cell culture
SSC	Side scatter
TBS	Tris buffered saline
TBST	Tris buffered saline containing 0.1 % Tween® 20

TEGDMA	Triethylene glycol dimethacrylate
TEMED	N,N,N’,N’-Tetramethyl-ethylenediamine

1 Introduction

1.1 History of biomaterials

With time people generally gain a better understanding of the different products they use. They learn how to use them more efficiently, how to produce the product more efficiently, and other aspects depending on what the product consists of. In biomedical science, the learning process is generally about how a substance can be used to improve human (or animal) health. However, there is a big issue in this area as described by Swayne [Swayne, 2012]: "*The narrow focus of biomedical research, for all its achievements, cannot do justice to its complexity*". This means that while research can give indications for how different effects or mechanisms would work, it cannot replicate the complexity of the body. The scientific results may not reflect realistic effects. Biomaterials suffers from this, in that while they are created to improve one's health, it's usage presents several possible issues (anatomy, aesthetics, compatibility, etc.) [Metcalfe & Ferguson, 2006].

The biomaterial-tissue interface is one of these issues when it comes to biomaterials. The material used has to be nontoxic in addition to physically strong enough to support the applied area [Hench, 1980]. Anderson and co. [Anderson *et al.,* 2007] compiled a list of potential injuries that could occur when a biomaterial is in contact with the body, for example inflammation and unwanted development. To avoid these reactions, the biomaterials must be biocompatible with the body. Williams [Williams, 2008] define biocompatibility as: "*the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effect….*". Articles such as those performed by Williams [Williams, 2014] have created templates of specifications that biomaterials need to follow to decrease the biomaterials toxic effect.

Biocompatibility is important since biomaterials are used in a multitude of medical areas, including dental applications. Dental materials also have to resist the harsh environment in the oral cavity, with the material being exposed to varying temperatures, acidic and basic ingestible, bacteria, and more [St. John, 2007]. To achieve this, multiple composites have been used in an attempt to find the most optimal dental material [Hossain *et al.,* 2017].

Metals as dental materials

The idea of using metals or metal alloy in the dental material became common by the beginning of the 1700s [Ferracane, 2001]. Metals were used as filling materials since they were easy to use, durable, and cost-effective (depending on the metal/alloy used) [Roach, 2007]. The use of various

metals continued until the early 1800s, when dental amalgam was introduced [Ferracane, 2001; Kingston, 2013].

Amalgam is very durable, cost effective, and easy to apply. The amalgam used in dentistry is a mixture of mercury, silver, tin, and copper (sometimes zinc, palladium, indium) [Kefi *et al.,* 2011]. This was a long time before people began to realize the toxic potential of mercury, which would come to our attention in more recent years [Ferracane, 2001]. However, studies that have compiled the results of several individual experiments (such as St. John, 2007) indicates that the mercury in amalgam had no adverse effect on the majority of the patients. Amalgam is still the most commonly used dental material world-wide, however in countries such as Norway they have been banned [Shenoy, 2008] and been replaced by resin based composite [Skjelvik, 2012].

Resin based dental materials

The introduction of resin based materials partially came as a result of aesthetics [Chan *et al.*, 2010], as amalgam left dark spots on the teeth where it was applied. The first material was polymethyl methacrylate (PMMA), which was introduced in 1936 under the name Vernonite [Rueggeberg, 2002]. PMMA dental material was very durable and looked similar to teeth [Kumar & Ali, 2020]. The material worked as intended but had a major problem in that the polymerization caused the resin to shrink [Pratap *et al.,* 2019]. The shrinkage could be repressed by using quartz as a filler material, with filler taking up to 70% of the composite's volume [Riva & Rahman, 2019], however, it did not work clinically.

In an attempt to reduce the shrinkage, the hybrid monomer bisphenol-A glycidyl methacrylate (Bis-GMA) was introduced in the 1960s. Bowen [Bowen, 1982] describes the process, with Bis-GMA causing less shrinkage than previous resin materials. Bis-GMA has since become the most commonly used resin material [Barszczewska-Rybarek & Jurczyk., 2015].

Monomer resin composite

The resin composites of today are mostly based on the methacrylate monomer system. Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) defines a monomer as a "*substance capable of forming covalent bonds with a sequence of additional like or unlike molecules under the conditions of the relevant polymer-forming reaction used for the particular process*" [ECHA, 2012]. High water resistance and reactivity are main reasons for why methacrylate monomers are used in dental materials. Modern dental materials use Bis-GMA with other monomers mixed in like 2-Hydroxyethyl methacrylate (HEMA) and/or Triethylene glycol dimethacrylate (TEGDMA) [Stansbury, 2012]. Bis-GMA alone is highly viscous which makes it unfit to be used as dental material. TEGDMA in

particular helps with this, as it lessens the viscosity, making it possible to apply the Bis-GMA/TEGDMA mixture as a dental material [Alrahlah *et al.,* 2021]. However, the system still contains drawbacks. Many studies, as described by Peutzfeldt [Peutzfeldt, 2010], mentions similar issues such as polymerization shrinkage and degree of conversion. The degree of conversion means how much of the monomer is hardened during the polymerization process [Tarle & Par, 2017].

1.2 Motivation for studying methacrylate monomers

Dental restoratives are defined as medical devices and not as drug. Because of this, the tests performed on the substances are different compared to those drugs would go through. Gelijns [Gelijns, 1990] describes this difference clearly, as medical devices criteria include factors such as user acceptability or ease of use. The clinical relevancy of these tests has also been questioned [Mjör, 2007]. Drugs on the other hand included more trials on and for patients, that took account for different people and different disabilities [Gelijns, 1990]. To compare, the estimated time it takes a drug or medical device to go from development to market, based on the U.S. Food and Drug Administration (FDA), is 12 years for a drug [Dickson & Gagnon, 2009], while it is 3-7 years for a medical device [Fargen *et al.*, 2013].

During dental treatment, monomers leak from the resin based biomaterials, as not all is polymerized, Studies have shown that in general, conversion rates rarely exceed 75% [Santerre *et al.*, 2001]. The monomers needs to be very reactive to ensure good polymerization [Stansbury, 2012], but this may also include reactions towards biological components [Samuelsen *et al.,* 2019; Nilsen *et al.,* 2018]. In addition to the patients, there is also the occupational exposure that the workers like dentists are exposed to. Monomers have been proven to cause allergies in dental workers [Aalto-Korte *et al.*, 2007], as well as other respiratory diseases, like adult-onset asthma [Jaakkola *et al.,* 2007]. However, the mechanisms behind these toxic effect of methacrylate monomers is still unclear.

Since the mechanism of the toxic effect has been questioned, the “true” toxic potential of the monomers may not be fully understood [Samuelsen & Dahl, 2016]. Monomers classification as medical devices do not help this, considering their effects are comparable to a drug’s effect. Unfortunately, because of polymer’s low density, as well as high mechanical strength and flexibility, they are optimal for use near the soft tissue in the oral region [Hasirci & Hasirci, 2018]. While Bis-GMA is one of the main components, because it has a high hydrophobic nature [Sideridou *et al.*, 2011], it is not easily dissolved in the saliva, thus not easily absorbed by the cell either. That is not the case for HEMA and TEGDMA, which are more easily dissolved [Michelsen *et al.*, 2012].

1.3 Toxic responses

Most knowledge on methacrylate effects is based on *in vitro* studies. Previous studies have shown that exposure to HEMA and TEGDMA monomers have caused reduced cell proliferation and altered gene expression. These effects have generally been linked to increased levels of reactive/radical oxygen species (ROS) in exposed cells [Schweikl *et al.*, 2007].

1.3.1 Oxidative stress

Oxidative stress is caused by an imbalance between antioxidants and oxidants [Pizzino *et al.*, 2017]. Oxidants are needed for normal cell responses, such as signaling pathways [Kurutas, 2016]. Oxidants can come from either an internal metabolism [Navarro-Yepes *et al.*, 2014] or an external exposure, which can lead to the uncontrolled production of even more oxidants [Pizzino *et al.*, 2017]. Oxidative stress can cause damages to cell components, resulting in possible disease development [Liguori *et al.*, 2018]. To counteract this oxidation, a complex antioxidant defence system is needed, to make sure that cells are protected against uncontrolled oxidation.

Nrf2/ARE signaling pathway

Nuclear factor erythroid 2-related factor 2 (Nrf2), encoded by the gene Nuclear Factor, Erythroid 2 Like 2 (NFE2L2), is an important transcription factor specifically focused on protecting against electrophilic and oxidative stress [Navarro-Yepes *et al.*, 2014; Itoh *et al.*, 2003]. The pathway regulates transcription of several antioxidant and phase II biotransformation enzymes [Buendia *et al.*, 2015; Rushmore *et al.*, 1991]. Upon activation, Nrf2 translocate into the nucleus where it binds to the antioxidant response element (ARE) [Velichkova & Hasson, 2005]. Under normal conditions, the Nrf2 is regulated by Kelch-like erythroid cell-derived protein with Cap'n'collar homology-associated protein 1 (Keap1). Through a ubiquitin-proteasomal pathway, Keap1 supresses Nrf2 by degradation. [Itoh *et al.*, 2003].

The substances effects on this pathway are possibly connected to the mechanisms they work through. Studies like Samuelsen and co. [Samuelsen *et al.*, 2019], and Nilsen and co. [Nilsen *et al.*, 2018] looked at a variety of proteins, with different functions, but mostly connected to the Nrf2 pathway. There were differences between their results, with some proteins showing an effect on HEMA but not on TEGDMA, and vice versa. For example, HEMA increased p62 and Pirin levels, while TEGDMA increased p62, HO-1, and to a lesser extent, Pirin levels.

Heme oxygenase 1 (HO-1) is encoded by the HMOX1 gene and regulated by the Nrf2 pathway as a cytoprotective gene [Araujo *et al.*, 2012]. It is one of the isoforms of Heme oxygenase (HO) enzymes, that (in addition to multiple other functions) degrades heme, a potent oxidant [Chen *et al.*, 2003;

Loboda *et al.,* 2016]. The heme is cleaved into several substances, that potentially can help with the redox-balance [Araujo *et al.*, 2012]. Willis and co-workers [Willis *et al.,* 1996] has suggested that HO-1 is a protective gene that is majorly activated by inflammation.

The p62 protein plays an important role in autophagy activity. This occurs through a ubiquitin-binding directly to the Microtubule-associated protein 1A/1B light chain 3 (MAP1LC3 or LC3) proteins, which are central in the autophagy pathway. The protein can affect both the autophagy and Nrf2 because it contains a LC3- and a Keap1-interacting region, respectively [Komatsu & Ichimura, 2010]. The Keap1 region allows p62 to bind with Keap1 protein, which leads to Nrf2 activation [Johansen & Lamark, 2010]. At the same time, p62 and its associated substrates are degraded themselves by autophagy [Carroll *et al.,* 2018]. The protein, which is encoded by the human Sequestosome-1 (SQSTM1) gene, also acts as a positive feedback activation of Nrf2, where an accumulation of p62 will release and stabilize Nrf2 [Johansen & Lamark, 2010].

Lastly is Pirin, which is encoded by the PIR gene. Pirin has been known to increase in response to oxidative stress [Liu *et al.,* 2013], showing some indications of the mechanisms exposure can work through. Pirin is also reported to form quaternary complexes with transcription factors nuclear factor-κB (NF-κB) and B cell leukemia-3 (Bcl-3), and target promoter regions of anti-apoptotic genes [Yang *et al.,* 2018]. NF-κB being a family of transcription factors that affect a diverse array of gene expressions [Franzoso *et al.*, 1993]. NF-κB is induced by a myriad of exogenous or endogenous agents, such as oxidative stress. The NF-κB will then in turn induce genes to remedy this, which includes oxidative stress-related enzymes [Mercurio & Manning, 1999]. Bcl-3 regulates nuclear NF-κB activity by removing a p50 inhibitor from the NF-κB site. The PIR gene has been suggested to work with Nrf2 modulated genes, with the Nrf2 binding to the PIR promoter that contains four antioxidant ARE [Hübner *et al.,* 2009].

While some of Pirin's functions such as its effect on NF-κB has been identified, it is believed all of its functions have yet to be fully understood [Aedo-Aguilera *et al.,* 2019]. While this may discredit its usability to indicate possible mechanisms, the connection to NF-κB is important, as NF-κB has vital functions that may indicate the mechanisms for exposure effects.

1.4 Effects of HEMA and TEGDMA

Both HEMA and TEGDMA have been proven to cause clinical effects such as allergies. *In vitro* studies have further shown that they cause multitude of effects often linked to ROS induced cytotoxicity [Ginzkey *et al.*, 2015]. While no studies have shown their combined effects on toxicity, a study by Gerzina and Hume [Gerzina & Hume, 1996] has shown that a mixture may affect the substances ability to diffuse through dentine.

1.4.1 HEMA

HEMA is most commonly as a dental adhesive, in the form of a bonding material [Nakabayashi & Takarada, 1992]. Because it has both hydrophilic and hydrophobic groups, it is an excellent material for mediating bonding between hydrophobic collagenous dentin and hydrophobic resin material. This increases the strength of the bond for dental restoration materials [Williams *et al.,* 2013]. It is assumed that a Lewis acid-base interaction between HEMA (the electron donor) and the teeth (electron acceptor) is the reason why HEMA creates so strong bonds [Morra, 1993].

Because of its low molecular mass and hydrophilicity, the unpolymerized HEMA is able to diffuse through dental tubules and affect dental pulp cells directly [Hamid *et al.*, 2009]. HEMA exposure (based on *in vitro* studies) to dental pulp tissue has been estimated to be up to 8 mM [Noda et al., 2002]. It has been proven to lessen the healing capabilities of relevant cells (pulp stem cells), through reducing cell proliferation and cell migration [Williams *et al.,* 2013]. The cause for the increased cell death and inhibited cell growth has been connected to the decrease of glutathione, associated with an increase in ROS [Samuelsen *et al.*, 2005]. In response, several proteins connected to Nrf2/ARE had increased, including p62 and Pirin [Samuelsen *et al.,* 2019]. However, the importance of this mechanism alone has been questioned, with studies such as Samuelsen and co. indicating that instead of oxidative stress, electrophilic stress may be the cause for cytotoxicity from HEMA exposure [Samuelsen *et al.,* 2019].

1.4.2 TEGDMA

TEGDMA is a common component of both dental adhesives and dental composite filling material. When mixed with Bis-GMA, TEGDMA causes a viscosity reduction in Bis-GMA that makes the mixture a better composite for dental application. This mix also increases the degree of conversion for the Bis-GMA monomers [Barszczewska-Rybarek *et al.,* 2020].

Not all of TEGDMA polymerize, with some leaking from the dental material [Örtengren *et al.*, 2001]. Similar to HEMA, TEGDMA is considered hydrophilic, thus it is able to dissolve and diffuse [Gerzina & Hume, 1995]. It is shown that TEGDMA causes cytotoxicity *in vitro* [Harorli *et al.,* 2009]. TEGDMA

may be assumed to be more toxic than HEMA because it contains two reactive methacrylate-groups (**Figure 1.2**), compared to HEMAs one group (**Figure 1.1**). After diffusing through dentin in deep cavities, TEGDMA exposure (based on *in vitro* studies) is estimated to be around 4 mM concentration [Noda et al., 2002]. Similar to HEMA toxicity it has been suggested that GSH depletion is the main cause for TEGDMA-induced cytotoxicity. As with HEMA, TEGDMA exposure to cells has been shown to cause increased levels of p62 proteins as well as Pirin, although to a much lesser extent [Nilsen *et al.,* 2018]. In contrast to HEMA, TEGDMA has also been shown to strongly increase HO-1 levels [Nilsen *et al.,* 2018]. The GSH depletion (and consequent ROS increase) is the baseline for the hypothesis by Nilsen and co. [Nilsen *et al.,* 2018], that TEGDMA causes cytotoxicity through oxidative stress.

To understand the toxic potential of HEMA and TEGDMA, it is necessary to confirm the molecular mechanisms causing the toxicity. Studies that have conducted similar experiments on each substance, such as the study performed by Samuelsen and co. for HEMA exposure [Samuelsen *et al.,* 2019] and the study by Nilsen and co. for TEGDMA exposure [Nilsen *et al.,* 2018] can indicate how the mechanisms differ. For HEMA it is suggested that the cytotoxicity is the result of electrophilic stress, while for TEGDMA it is suggested that the cytotoxicity was through oxidative stress. The interaction between the substances may also help determining the mechanism. However, no study so far has been performed on this subject.

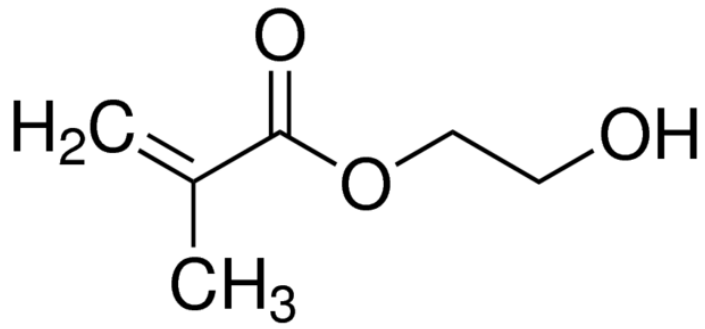

Figure 1.1: Representative figure showing the chemical structure of HEMA [Sigma-Aldrich, HEMA].

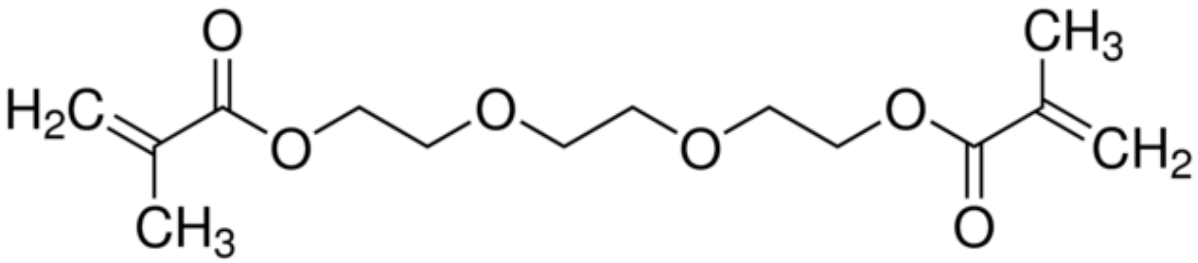

Figure 1.2: Representative figure showing the chemical structure of TEGDMA [Sigma-Aldrich, TEGDMA].

2 Aim of the study

Based on the articles by Samuelsen (and co.) and Nilsen (and co.) [Samuelsen *et al.,* 2019; Nilsen *et al.,* 2018], we hypothesis that HEMA and TEGDMA affects cells in a different manner. Since the studies are similar in many aspects besides the substance, it allows their studies to be more easily compared. We will attempt to test the hypothesis by comparing various endpoints in HEMA and TEGDMA exposed THP-1 cells. The chosen endpoints to measure are:

- Cell viability
- Glutathione- and reactive oxygen species levels
- p62, HO-1, and Pirin levels

We will also observe how combination exposures of HEMA and TEGDMA affect the same endpoints. The type of interaction may also give an indication for their mechanisms.

3 Materials and methods

3.1 Materials

All chemicals, substances, antibodies, kits, and instruments used during this study are listed in **Appendix 1**. the recipes for all solutions and buffers used in this study are listed in **Appendix 2**.

3.2 Cell preparation

For this study, we use a human leukemia monocytic cell line called THP-1, available from the European Collection of Authenticated Cell Cultures (ECACC). In humans there exist several different types of monocytic cells. The THP-1 cell line will then act as a model for those monocytic cells, as the monocytic cells in the mouth and upper respiratory tract are likely to be exposed to our substances. Another reason to use the cell line is that it is reproducible, meaning we can get the same result on individual experiments without variations caused by the differences between sampled cells. This is because the cells are homogeneous, which minimizes the variability in the cells [Chanput *et al.*, 2015]. The cells are derived from the peripheral blood of a 1-year-old infant with acute monocytic leukemia.

3.2.1 Cultivation

THP-1 cells were grown in a culture medium called RPMI-1640. This culture medium was supplemented with 5 % fetal bovine serum (FBS), in addition to Gentamicin, Sodium pyruvate, and 1 M Hepes, with the complete mixture being henceforth called medium. The recipe of the medium is listed in **Appendix 2**. The cells were cultivated in a 75 cm^2 cell culture flasks (FALCON, Corning Incorporated, [Corning, NY, USA]) inside a PHCBI MCO-170AICUV CO_2 Incubator (PHC Corporation, [Tokyo, Japan]) that held a constant 37 °C, 5 % CO_2, and >95 % humidity. All incubation of the cells was performed with the same conditions. To maintain a sufficient supply of nutrients and growth factors, a portion of the cells were transferred into new cell culture flasks containing fresh medium three times a week.

Treatment

The cells were monitored every second day, before and after they were transferred into new cell culture flasks. This was to ensure that the cells did not overgrow, as too much cell culture would lead cell division to stop, and subsequently die. With each passage, we increased the number of the cells'

"phase"-levels. Each "phase" represents how often the cells have gone through a new passage, as each passage leads to the cells becoming less viable. When the "phase"-level is high enough, generally around 30, the cells should be thrown out and new ones should be implemented. All equipment used on cells was sterilized in an autoclave (HV-50, Hirayama, [Tokyo, Japan]) before being discarded. In addition, all work performed on cells were performed in a sterile cell culture hood (SCANLAF - Mars safety Class 2, LaboGene, [Lillerød, Denmark])

Procedure:

1. The medium was preheated in a water bath to 37 °C before being applied to the cells.
2. The concentration of cells was counted by adding 75 µL of the cell suspension to a Mini Automated Cell Counter, Moxi^{TM} Z (Orflo Technologies, [Ketchum, ID, USA]).
3. The cell culture was transferred into a new cell culture flask, and new medium was added to dilute the cell concentration to the wanted concentration, before putting the new flask back in the incubator. The cell concentration in the new flask was set to be 250 000 cells per mL.
4. The rest of the cells from the old cell culture flask could be used for experiments.

3.2.2 Seeding of cells

To obtain the desired concentration of cells for each experiment, we used Moxi^{TM} Z once again to count the cell concentration, before diluting it to the wanted concentration using medium. This was to ensure that each experiment had the same number of cells, as a difference in concentration could give different results.

MTT assay and flow cytometry

The cells were seeded in sterile 12-wells cell culture plates (Costar, Corning Incorporated, [Corning, NY, USA]), with a growth area of 3.8 cm^2. For the MTT assay and Glutathione (GSH) experiments, the cells were concentrated to be 250 000 per mL, with each well holding 1 mL cell culture. For reactive oxygen species (ROS) experiments, the cells were concentrated into 500 000 per mL, in 1 mL. The plates were tilted gently to allow the cell culture to spread over the surface and avoid being collected along the edges of the well. The plates were then put into the incubator for 24 hours.

Western blot

For western blotting, the cells were seeded the day before exposure in sterile tissue culture dishes (FALCON, Corning Incorporated, [Corning, NY, USA]), with a growth area of 21.29 cm^2. Cell concentrations were set to be 250 000 per mL, with the dishes containing 4 mL of cell culture. The cells were incubated for 24 hours.

3.2.3 Substance exposure and harvest

We prepared a stock solution of HEMA (1000 mM) and several different solutions of TEGDMA in a dilution series (**Appendix 2**) within 1 hour before exposure. TEGDMA is (unlike HEMA) quite hydrophobic, so we need to perform the dilution with dimethyl sulfoxide (DMSO). DMSO is however harmful to the cells, so we kept the exposure of the cells to DMSO at a maximum of 2 ‰, to avoid the DMSO harming the cells. This is the reason we need so many different stock solutions for TEGDMA. HEMA stock solution was diluted in medium. The exposure was performed the same way minimum of three times, to possibly provide some consistent data.

MTT assay

The plates mentioned in section 3.2.4 were exposed to several different concentrations of HEMA and TEGDMA. First, they were exposed separately, then they were tested in combinations. For the separate experiments, each plate contained two samples of each concentration, as well as two controls containing medium for HEMA and DMSO for TEGDMA. The DMSO concentration was kept at 2 ‰ for every well it was exposed to. For combinations, we used four plates each with a different TEGDMA concentration, where the wells on the plate contained a different HEMA concentration. The combination plates also contained one medium and one DMSO control. After 24 hours and 48 hours, the MTT assay procedure was performed as per section 3.3.

Flow cytometry

The cells in the plates are exposed to different concentrations for HEMA and TEGDMA, first separately, then in combinations. DMSO kept being exposed to the wells in 2 ‰ concentrations. For GSH experiments, we had one sample for each concentration, in addition to two controls with either medium, DMSO, or one medium + one DMSO for HEMA, TEGDMA, and combinations respectively. For ROS experiments we had the same setup as GSH, barring exchanging one sample with 0.5 mM Hydrogen peroxide (H_2O_2), as a positive control (**Appendix 2**). This positive control was only used to determine the accuracy of the method used and will not be included in figures. The exposure and harvesting method for GSH is described in 3.4.2. The same for ROS is described in 3.4.1.

Western blotting

Dishes were exposed to various concentrations of HEMA and TEGDMA, separately and in combinations. After the exposure, the protein was harvested using the procedure below.

Procedure:

1. The cells were transferred to Eppendorf tubes (Eppendorf, [Hamburg, Germany]), then centrifuged at 50 x g, 20 °C, for 5 minutes. The supernatant was removed.
2. 200 mL of Sample buffer (**Appendix 2**) was added to the pellet, and mixing caused the pellet to dissolve in the Sample buffer.
3. The samples are then left at room temperature for 24 hours, before being placed in a freezer (-20 °C) for storage and further analysis (section 3.5.1)

3.2.4 Calculation of mycoplasma

It is necessary to assure that the cells are not contaminated in any way before being used. This is why we perform a Mycoplasma test on the cells. The mycoplasma bacteria are a common contaminant of cell cultures that can affect the results of the tests, as they take nutrients intended for the cell culture and occupies place intended for cell culture. While unnoticeable, they have been proven to have a considerable effect on several parameters in the cell cultures [Uphoff & Drexler, 2014]. Every cell culture is therefore tested regularly for mycoplasma to ensure that results are accurate.

We use MycoAlert™ Assay Control Set and MycoAlert™ Mycoplasma Detection Kit (Lonza Group AG, [Basel, Switzerland]) to measure mycoplasma contamination. The kits are based on the measured luminescence that is created when the substrate included in the kit reacts to the bacteria in the cell culture. The ratio between the measurement after (read B) and before (read A) the substrate is added determines if there either is contamination, there may be contamination, or there is no contamination.

Procedure:

1. 2 mL cell culture is spun at 50 x g for 5 minutes. 50 µL of supernatant is transferred to one well of a Nunc™ F96 Microwell™ White Polypropylene (Thermo Scientific, [Waltham, MA, USA]).
2. Two separate wells are filled with a negative and a positive control from the MycoAlert™ Assay Control Set.
3. 50 µL of MycoAlert™ reagent diluted in MycoAlert™ assay buffer is added to each well. The plate was left in a dark room for 5 minutes before reading luminescence (read A) in a spectrophotometer (Synergy H1, BioTek/Agilent, [Santa Clara, CA, USA]).
4. Added 50 µL of MycoAlert™ substrate to sample and controls. Left the plate in a dark room for 10 minutes before reading luminescence (read B) in a spectrophotometer.
5. The ratio of read B / read A determines the mycoplasma contamination. >0.9 and <1.2 are negative and positive for mycoplasma respectively, while 0.9-1.2 means the test must be retaken after 24 hours.

3.3 Measurement of cell viability, MTT assay

MTT assays are used to check for the viability of the cells after being exposed to a substance. MTT is also called 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is a yellow tetrazole that is reduced into purple/blue formazan crystals in living cells by succinate dehydrogenase (SDH) activity. This purple/blue wavelength of color is what we will look for when we scan the samples with the plate reader, so wells with a deeper purple color mean that they will have a higher value, meaning that there are more living cells in those wells [Mosman, 1983].

Procedure:

1. 110 µL of a stock solution of 5 mg/mL MTT (diluted in phosphate-buffered saline (PBS)) is added to each well, exposing the cells to 0.5 mg/mL MTT. The plate is then left in the incubator for 4 hours.
2. After the incubation the samples are transferred to tubes, then centrifuged at 50 x g, 20 °C, for 5 minutes. The supernatant is removed.
3. The pellet is dissolved by adding 400 µL of DMSO to the tube and mixing it. The tubes are left at room temperature for 10-15 minutes to let the formazan crystals dissolve in the DMSO.
4. After the crystals are dissolved, we transfer 100 µL of the samples onto a 96-wells microplate (Nunc™ MicroWell, Thermo Fisher Scientific, [Waltham, MA, USA]). The plate is then read at the spectrophotometer with an absorbance of 570 nm.

3.4 Flow cytometry

We can use flow cytometry to examine an individual cell or particle's characteristics through information given by its component's fluorescence. As the light from the light source hits the cell, the light is scattered, where then it and its fluorescence wavelength are recorded. The light typically scatters two directions, where a detector measures the forward scatter (FSC) while another measures the side scatter (SSC). The light source can either be a laser, lamp, or even a light-emitting diode (LED). Lasers are most commonly used, since they are the most sensitive to weak signals, and generally have a small “spot” size, where the light is focused [Ormerod, 2008]. The data gathered from the fluorescence is then gated using the control samples to remove dead cells or debris. Provided by the FSC and SSC plot, only the population of cells with similar characteristics (alive) is included. This means that even cells with different features like size, cycle, cell count, etc., can be included in the analysis

3.4.1 Reactive oxygen species analysis

Reactive Oxygen Species (ROS) are chemicals formed as a by-product of metabolism from O_2 that are very reactive to other chemicals. ROS is a necessity, as it has functions in the cells, such as the cardiovascular or immune system [Patel *et al.*, 2017]. The problem is that ROS is also able to oxidize and modify other separate cellular components, preventing them from doing their original functions. Antioxidants such as GSH work on eliminating ROS, to keep ROS from damaging other components. However, environmental stress (like being exposed to chemicals) can also cause ROS production to increase. Damage caused by ROS/an increase in ROS is called oxidative stress.

ROS can be measured using a substance called 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). The acetate group in H_2DCFDA is cleaved by intracellular esterases and oxidation, which turns it into a fluorescent 2',7'-dichlorofluorescein (DCF). This DCF can then be measured with a flow cytometer (BD Accuri™ C6, BD Biosciences, [Franklin Lakes, NJ, USA]).

Procedure:

1. We start by taking 20 mL of cell culture straight from the flask we use to cultivate them in, into a separate tube. We add 20 µL of dissolved H_2DCFDA (**Appendix 2**) to the tube, mix it gently, then place it back in the incubator for 15 minutes.
2. After that, we centrifuge the tube at 50 x g, 37 °C for 5 minutes, and remove the supernatant.
3. We dissolve the pellet with 10 mL of PBS, while at the same time washing the cells. The cells are again centrifuged at the same settings as before, with supernatant removed again.
4. The pellet is then dissolved in 10 mL medium, then we follow section 3.2.4 for seeding of the cells onto a 12-well plate.
5. The cells are immediately exposed to the substance(s) and are placed in the incubator for 30 minutes. After the exposure, the cells are transferred into Eppendorf tubes and placed on ice to slow down the ROS production. They are then taken to the flow cytometer for measuring.

3.4.2 Glutathione analysis

Glutathione (GSH) are antioxidants in cells used to prevent damage caused by free (reactive) oxygen on cellular components. GSH neutralizes the danger posed by reactive oxygens by letting them oxidize themselves, turning it into its oxidized state, GSSG. Since accumulation GSSG also causes oxidative stress, the stress-activated protein kinase (SAPK)/mitogen-activated protein kinase (MAPK) pathway can notice the decrease in GSH and induce apoptosis to the cells [Filomeni *et al.*, 2003]. By overserving the level of GSH, we can see if the substances cause an effect on the cells that makes the

level of GSH increase or decrease. GSH has a multitude of other roles as well, like acting as a buffer, or transportation, however since we know that our substances cause an increase in reactive oxygen (based on prior research), we will focus on the GSH-ROS effect for this study.

We can measure the GSH levels using flow cytometry (Cell Lab Quanta™ SC, Beckman Coulter, [Brea, CA, USA]) in the cell samples. They are measured by using monobromobimane (mBBr) that binds to the -thiol group of the glutathione, which in turn forms a fluorescent adduct [Morisbak *et al.*, 2015]. This fluorescent can then be measured with the flow cytometer, with a 366/465 nm excitation/emission filter. The monobromobimane method does have an issue with background noise, however, it is an issue dealt with in section 3.6.

Procedure:

1. The exposure described in section 3.2.5 lasts for 4 hours. After the 4 hours have passed, the samples are transferred into tubes, centrifuged at 50 x g, 37 °C for 5 minutes, and have their supernatant removed.
2. 400 µL of a mBBr mixture (**Appendix 2**) is added to the pellets, and we dissolve them in it. The samples are placed somewhere dark place at room temperature for 15 minutes.
3. When this is done, the samples can be taken to the flow cytometry to get the GSH measured. We measure with a 366/465 nm excitation/emission filter and only include the main cell populations (excluding dead cells or debris) when quantifying the mean fluorescence in each sample.

3.5 SDS-PAGE western blot

This method created by Ulrich K. Laemmli, allows us to separate proteins based on their molecular mass. It is called sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE), and it separates charged molecules in a solution. The separated proteins are transferred from the gel to a membrane, where the level of proteins we want to look at is expressed by using antibodies. The signals we get from the following scan will show us if the amount of protein changed, depending on how much substance it was exposed to.

3.5.1 Sonication of samples

The samples prepared in 3.2.4 are thawed and readied to be sonicated. The sonicator (Vibra-Cell™ VCX130, Sonics & Materials, Inc., [Newtown, CT, USA]) is set to ultrasound with an amplitude of 25% maximum power, for 35 seconds (2 seconds pause), and a pulse of 5. This is required as it causes the

cell membranes to be destroyed, which removes long deoxyribonucleic acid (DNA) strands that would prevent the proteins to move freely across the protein electrophoresis.

3.5.2 Gel preparation

A Glycine SDS-PAGE gel is capable of separating proteins with molecular mass between 5 and 250 kDa by isolating and identifying the proteins with SDS, then transferring them through an acrylamide gel with an electric current. The SDS gives the proteins a negative charge and helps with the denaturation of folded proteins of equal mass. The denaturation is necessary, as it disrupts the structure of the proteins, making it easier for the antibodies to bind to them.

The acrylamide-gel itself is created by a polymerization that is catalysed by tetramethylethylenediamine (TEMED), and ammonium persulfate (APS). The gel is divided into a stacking gel and a separation gel. The difference between the gels are concentrations of acrylamide, and the molarity of Tris buffer as this gives them different pore sizes and ionic strengths, respectively. Proteins as collected in the stacking gel, which is given a negative charge by the SDS, will, in turn, result in the protein traveling towards the anode when the electrophoresis begins. The samples are placed in wells filled with a liquid called electrophoresis buffer, which contains glycine and chlorine ions. The basic pH in the buffer will make the ions become negatively charged by the SDS and they too will move towards the anode. When glycine and chlorine ions reach the stacking gel with a pH of 6.8, the glycine will move slower than the proteins while the chlorine will move faster. This is because glycine will form a zwitterionic form when it reaches the stacking gel, while the smaller chlorine will still be negatively charged, which makes it migrate more easily. Since the ions are both in front and behind the proteins, it will cause the proteins to be concentrated and hit the separation gel in the shape of narrow bands.

The high pH in the separation gel causes the glycine to be negatively charged, thus speeding up and surpassing the proteins. The constant voltage in the separation gel causes the proteins to migrate at different rates based on their mass. The structure prevents large proteins to migrate as quickly as small proteins, although their large size would lead them to have a larger negative charge. The difference in size will result in the bands being divided into different depths, and by using a molecular weight marker, we can determine the weight of those protein bands. During this procedure, we use equipment made by Bio-Rad Laboratories Inc. [Hercules, CA, USA].

Procedure:

1. We assembly the Mini-PROTEAN® Tetra Cell casting frame and casting stand, then mount on clean glass plates in the frame.

2. The separation gel is mixed as listed in **Appendix 2**, with about 7 mL of the mixture being placed between the glass plates. It was topped with distilled water (dH_2O) until the upper end of the casting frame. After approximately 30 minutes, the polymerization is completed, and the residue dH_2O can be removed.
3. The stacking gel is mixed as listed in **Appendix 2**, where about 2 mL of the mixture is added on top of the polymerized separation gel. A comb is inserted into the mixture to make the wells needed for adding the samples. the polymerization is complete after approximately 30 minutes, and the comb is removed.
4. The glass plates are removed from the casting frame and stand, and gel residue is cleaned off. The Mini-PROTEAN® Tetra Cell electrophoresis module is assembled and the plates are inserted into it. Running buffer made as in **Appendix 2** is used to fill the inner chamber between the two glass plates, while the outer chamber is filled up to a specific point, depending on if we check 2 or 4 gels.
5. The samples are prepared for the gels by adding 10 % mercaptoethanol and bromophenyl blue. The bromophenyl blue is not necessary for the procedure but helps us visualize the samples and bands.
6. 2 µL of a loading marker indicating molecular weight was applied to the first well, with the subsequent wells being filled with 12 µL of the prepared samples. The well between the marker and the first sample was empty, to better visualize the ladder steps made by the marker.
7. The electrophoresis begins by starting the PowerPac™ HC Power Supply at 100 V. The ampere is noted and the electrophoresis is stopped. It is restarted using the ampere instead, as this makes the voltage increase continuously.
8. The electrophoresis is run at room temperature in the dark (since the loading marker is light sensitive) for about 1-1.5 hours and is complete when the marker has reached the bottom of the gel.

3.5.3 Transfer from gel to nitrocellulose membrane

The proteins on the gel are now separated, but there is no way to observe them by eye. It is, therefore, necessary to transfer them onto a membrane which then lets them be visualized by Ponceau S and incubated with antibodies. A nitrocellulose membrane (Amersham™ Protran®, GE Healthcare, Inc., [Chicago, IL, USA]) with pore sizes of 0.2 µm is used to capture the proteins. This happens through a process called electroblotting, where an electric current pulls the negative proteins from the gel onto the membrane. Since the membrane has an unspecific binding affinity, it

binds all proteins equally through hydrophobic interactions and charged protein-membrane interactions.

Procedure:

1. We make 1500 mL of transfer buffer using the recipe in **Appendix 2**. The membrane and filter paper are cut large enough to cover the entire gel and pads, but still small enough to fit the cassette.
2. The transfer cassette from the Mini Trans-Blot® Cell system was placed in a container (red side down) filled with transfer buffer. Pad, Filter paper, Membrane, Gel, Filter paper, and Pad, was placed in subsequent order in the cassette. A roller was used to remove any air bubbles between the filter paper and gel before sealing the cassette shut.
3. The transfer cassette is then inserted into the transfer chamber with a cooling element, then the chamber is filled with the rest of the transfer buffer. Make sure the red side of the cassette faces the red wall in the chamber or else the proteins will not transfer to the membrane.
4. The entire chamber is placed in an insulated box with additional colling elements covering it. There need to be enough cooling to last for 24 hours.
5. The transfer then begins by connecting the PowerPac™ HC Power Supply with a voltage of 35 V. The blot is then left to be until the next day (24 hours).

3.5.4 Ponceau S staining

To ascertain if the protein transfer was successful, the membranes are stained in a Ponceau S solution. It is a reversible staining method that can be washed off using Tris-buffered saline containing 0.1 % Tween® 20 (TBST) (**Appendix 2**). The anionic dye binds to the amino groups on every protein on the membrane, and color them red, giving a simple visual control of the proteins.

Procedure:

1. The membranes are washed in a container with dH_2O to remove any major gel residue.
2. Membranes are then put into a Ponceau S solution and placed in a rocking table for 1 minute until the bands are visible.
3. The membranes are transferred back to the dH_2O container, where any excess gel residue is removed. We make sure there is no gel over the area where our target proteins are located.
4. The membranes are left to dry in the dark at room temperature for 1 hour before they can be visually checked.

3.5.5 Immunodetection and protein detection

Western blotting is a method that uses antibodies to label specific proteins. The nitrocellulose membrane is incubated with antibodies specifically targeting the proteins of interest. Following that, the membranes are incubated with secondary antibodies, that only bind to the primary antibodies we placed before. The secondary antibody will be conjugated, which means they can be visualized when binding with the primary antibody, because of the secondary's fluorochrome. The signals strength will indicate how much the secondary antibody will bind to the primary. To prevent non-specific binding by the primary antibody, the membranes are blocked with proteins by using bovine serum albumin (BSA) before incubation. This blocks all other binding sites except the ones we want.

The data produced by western blot is not an absolute measure, as it only provides a relative comparison of protein levels. To normalize the data, we need to use all the samples for each experiment to serve as a basis for normalization (see section 3.6). We will also use a housekeeping gene to rule out any errors connecting to loading and protein amount.

Procedure:

1. The membranes are blocked by using a container with a lid, filled with Tris-buffered saline (TBS) with 3 % BSA added (**Appendix 2**). They are left to block at a rocking table for 30 minutes at room temperature.
2. Following the blocking, the membranes are incubated in a container with a lid, with 5 mL of TBST containing 1 % BSA (**Appendix 2**) and the specific primary antibody. The ratio for how much the antibody: TBST ratio should be used is listed in the manual that comes with the antibody. The membranes are left on a rocking table in a cold room (4 °C) until the following day (24 hours).
3. The membranes are washed three times for 5 minutes on a rocking table with TBST buffer. Following that, they are incubated with 1 % BSA TBST and secondary antibody at room temperature for 2-2.5 hours. This is also done on a rocking table.
4. After the incubation, the membranes are rewashed three times for 5 minutes with TBST on a rocking table. They are then left to dry in the dark, at room temperature for 1 hour.
5. The membranes can then be read in the infrared fluorescence system (Odyssey CLx, Li-Cor Biosciences, GmbH., [Bad Homburg, Germany]). The membranes are read at 800 nm.

3.5.6 Loading (α-Tubulin) control

α-Tubulin is a subunit of tubulin, a protein that is essential in the cytoskeleton and is important for eukaryotic cell structure and mobility. α-Tubulin is highly expressed in all cells because of this (a housekeeping gene), making it an optimal loading control for western blot. By comparing the levels of α-Tubulin in the samples, we can observe if there are similar amounts of protein. If there are similar amounts, it means that the samples are comparable and that a possible difference in protein expression is not simply caused by the amount of protein.

Procedure:

1. After the membranes have been scanned for proteins (section 3.5.5), the membranes are washed three times for 5 minutes with TBST.
2. They are then incubated with 1 % BSA and the direct conjugant anti-α-Tubulin antibody at a rocking table, in-room temperature, for 2-2.5 hours.
3. After incubation, the membranes are dried for 1 hour (in the dark, room temperature) before being read at the infrared fluorescence imaging system.
4. We read it at 700 nm, as the bonds may be too close to our target proteins if we read it at 800 nm. Reading at 700 nm will increase the background, but it removes any possible overlapping or effect that may happen on/cause our target protein bands.

3.6 Statistics

The resulting figures shown in the following section are a compilation of every experiment performed on that specific test. The values shown are mean $n \geq 3 \pm$ standard deviation (SD). Before we can use the data to make plots to better represent the data, we need to normalize it. For the MTT assay and the ROS data, we use a one-step normalization, where the samples in each experiment are normalized to the control, making the control 100% of the sample, and the other samples will then either increase or decrease from 100%. The GSH data is performed similarly, however, the data is not as accurate as MTT and ROS. The problem comes from the usage of mBBr as the indicator since there are many other things mBBr may bind to besides GSH. This then gives a far higher value than it is supposed to be. Hedley and Chow [Hedley and Chow, 1994] performed some experiments on this and came to the result that under optimal conditions, the background is 30 %. To take account for the conditions possibly not being optimal, we removed 40 % of the signal given from the control on every sample. This would give the most accurate data. After removing 40% of the background, the GSH data is normalized the same way as MTT assay and ROS.

The Western blotting data is however far less accurate than the others and needs a more precise normalization method. Here we instead use a two-step normalization, as described by Grytting and co. [Grytting *et al.*, 2019]. Each sample is scaled to the same level using this method. First, each value is divided by the mean of all data in the individual experiment. The values are then normalized to this mean, before the mean of all the control samples of the same-setup experiments is made. The samples are then again normalized to this control mean value, which should leave the controls as 100% while still retaining the variation of the samples.

During normalization, the control sample for HEMA was calculated using cells exposed to only medium, while for TEGDMA the control sample was exposed to 2 ‰ DMSO. For combined exposures, the controls were the average of samples exposed to medium and 2 ‰ DMSO separately. The difference between medium and 2 ‰ DMSO exposed controls were insignificant, so in reality either one could have been used, without changing the expression of the samples.

By normalizing all data, we also conduct a one-way analysis of variance (ANOVA). Statistical significance of the effects was calculated using one-way ANOVA, followed by the Tukey post hoc test for multiple comparisons. Dunnett's method was used to compare every average of the single exposures with a control. All normalizations were done with Microsoft Office Excel for Windows (Microsoft Corporation, Redmond, Washington, USA), while most graphs and statistical analyses were done with GraphPad Prism 9.3.1 (GraphPad Software, Inc., San Diego, CA, USA). Some figures and calculations connected to finding the Effective Concentration values where 50% of the population is affected (EC50), was performed in R (R Foundation for Statistical Design, Vienna, Austria). The formula used to calculate the toxic ratio between the substances is listed in **Appendix 3**. The data in the figures are presented as $n \geq 3 \pm SD$. ANOVA showing p-value < 0.05, is considered significant.

We can find out how plausible an additive interaction between HEMA and TEGDMA is by making an isobole. An isobole is a method to assess interactions between substances, that uses dose-response curves to derive what a dose combination is expected to be at certain levels. Say for example we find out that TEGDMA is twice as toxic as HEMA, then a mixture of 1 mM HEMA and 1 mM TEGDMA would be as toxic as 3 mM HEMA. We use the EC50 values received from the cell viability test (MTT assay) to determine this toxic ratio. A line connecting intercept on the x- and y-axis will show how the interaction is. The line itself is the isobole, and the method is generally used to distinguish between additive and nonadditive substances. A straight line will indicate that the substances are additive, while if the line bends either upwards or downwards it may indicate some interaction like synergism or antagonism between the substances. The line does not have to be perfectly straight to still be

linear, and while it may be slightly curved, it should not be mistaken for being either synergistic or antagonistic There are several factors beyond our understanding or control, such as drug optimizing, that can affect to the interaction of the substances, thus influencing the structure of the isobole [Tallarida, 2012]. This prediction can then act as a possible indicator of how the substances may inhibit, catalyst, add, or not affect each other at all. The isobole was created using R.

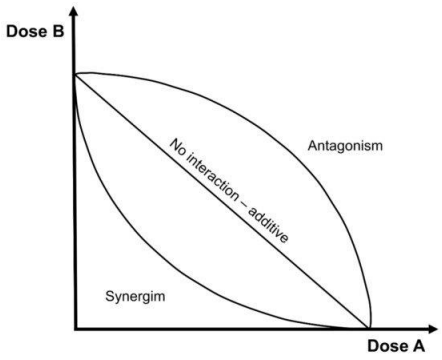

Figure 3.1: Representative figure of how an isobole may indicate the interaction between two substances. [Pezzani *et al.,* 2019]

(NOTE: The studies performed by Samuelsen and co. [Samuelsen *et al.,* 2019], and Nilsen and co. [Nilsen *et al.,* 2018] will be brough up often during the discussion. To improve the flow of the text, they will be referred to as “Samuelsen, 2019” and “Nilsen, 2018” respectively, for the rest of the paper. References will be given at least once on every section.)

4 Results

4.1 MTT assay

4.1.1 Individual tests

MTT assays were performed to estimate the effect on cell viability caused by different concentrations of HEMA and TEGDMA. There was a dose-dependent reduction in cell viability (**Figure 4.1 A-B**) when cells were exposed to HEMA (3-15 mM) and TEGDMA (1-5 mM). There was a significant reduction in cell viability when exposed to at least 6 mM HEMA. Likewise, there was a significant reduction at 2 mM TEGDMA exposure.

Between 24 and 48 hours of exposure time, there was a significant difference between HEMA exposed samples at 6 mM concentrations. For TEGDMA, there was a significant difference between the exposure times at 2 mM (not shown). In future experiments, only 24 hours exposure were used.

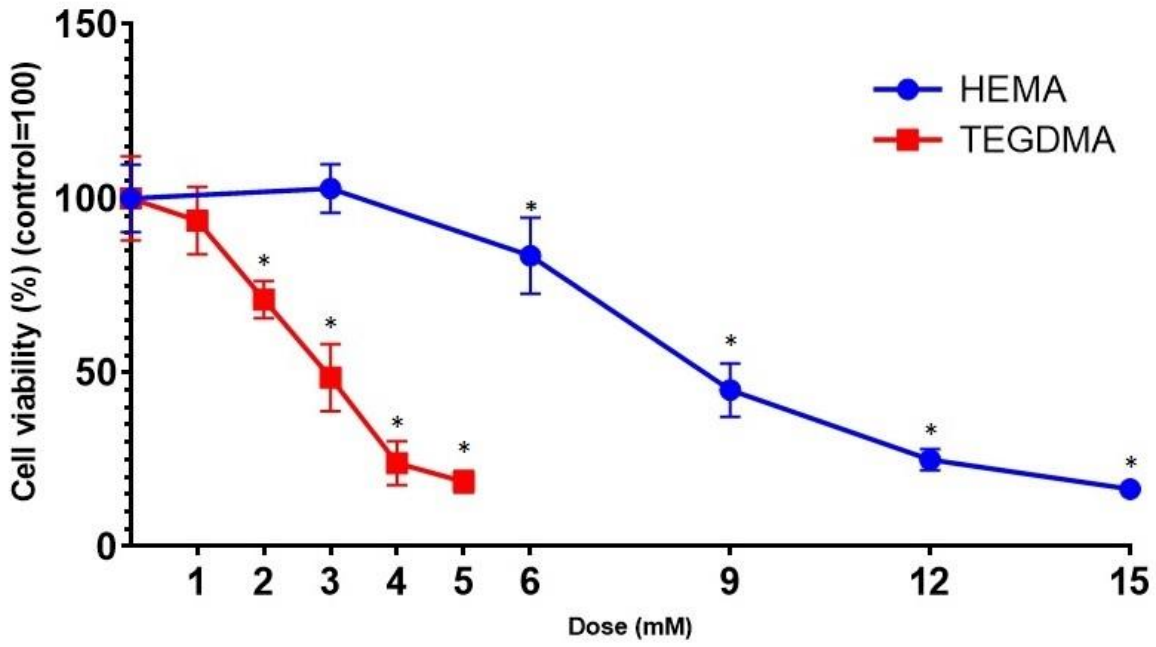

Figure 4.1 A: Cell viability of THP-1 cells after exposure to HEMA and TEGDMA for 24 hours, measured as SDH activity. Each sample was exposed to a different concentration of either HEMA or TEGDMA (n ≥ 3 ± SD). Asterisk (*) indicates a significant difference from the control ($p<0.05$).

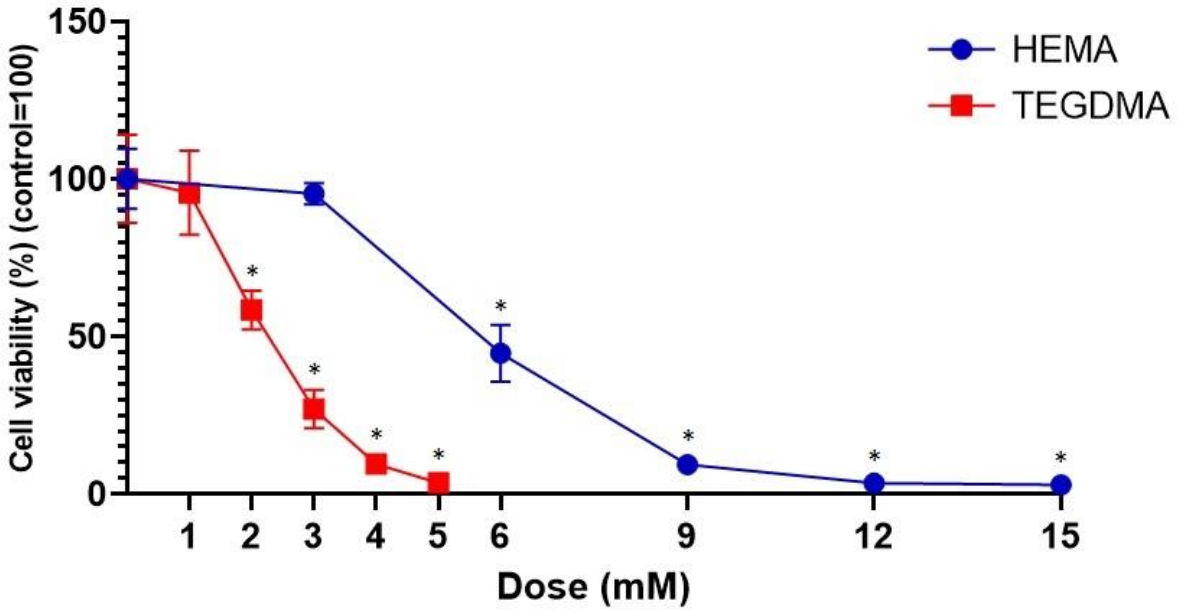

Figure 4.1 B: Cell viability of THP-1 cells after exposure to HEMA and TEGDMA for 48 hours, measured as SDH activity. Each sample was exposed to a different concentration of either HEMA or TEGDMA ($n \geq 3 \pm SD$). Asterisk (*) indicates that the sample is significantly different from the control ($p<0.05$).

To indicate the cytotoxic ratio between HEMA and TEGDMA, we estimated EC50 values from the individual MTT assay data (**Figure 4.1 A-B**). First, we made a rough estimation of the dataset, making a figure without standard deviations. We did this by averaging each exposure concentration's value, resulting in the figure shown in **Appendix 3**. The values shown should only be considered approximates, as the figure did not take into account for any standard deviation.

For the 24 hours data, the EC50 (MTT) for HEMA was 8.6, and TEGDMA was 2.9. This indicates that TEGDMA is about 3 times more potent than HEMA. For the 48 hours data, the EC50 (MTT) for HEMA was 5.6, while TEGDMA was 2.2. This indicates that TEGDMA is about 2.5 times more potent than HEMA.

The isobole created from the toxic ratio (**Figure 4.2**) resulted in a slightly curved line. This indicates an additive interaction, as the slight curve is not wide enough to be considered another type of interaction. A high p-value of 0.220 means it is a good fit for the data.

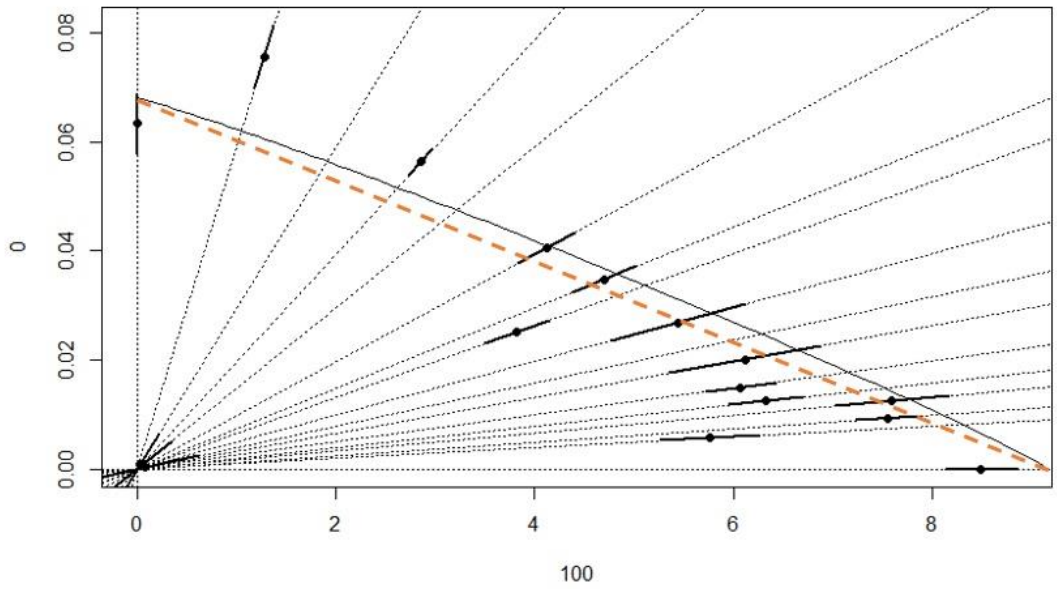

Figure 4.2: An isobole created using the 24-hour exposure data from the MTT assay, indicating an additive interaction between HEMA and TEGDMA. The toxic effect of TEGDMA was calculated to be expressed as HEMA with the ratio being: 1 mM TEGDMA = 3 mM HEMA. The straight orange dashed line is only a visual tool, as its straightness shows the curvature of the solid line isobole.

4.1.2 Combined tests

For the combination exposures, we used HEMA concentrations 3-9 mM and TEGDMA concentrations of 0.5-2 mM. The exposures led to decreased cell viability relative to increasing concentrations. The combinations are significantly more toxic compared to the individual results. An example of this is 3 mM HEMA and 1 mM TEGDMA. Individually they were not enough to cause a significant decrease in cell viability but combined, they were. There was a significant difference from the control at concentrations equal to or higher than 3 mM HEMA and 1 mM TEGDMA. **Figure 4.3 A** also shows how many samples significantly differed when only the TEGDMA dose was changed.

We calculated that TEGDMA is approximately 3 times more toxic than HEMA (4.1.1). We can check the plausibility of this ratio by comparing the combination samples that have the same toxic effect but with different mixtures of HEMA and TEGDMA. For example, assuming the ratio is correct, a sample exposed to 3 mM HEMA and 1 mM TEGDMA should have approximately the same toxic effect as a sample exposed to 6 mM HEMA and a sample exposed to 2 mM TEGDMA. **Figure 4.3 B** shows that only the 2 mM TEGDMA exposure is significantly different from the other exposures with an estimated equal toxic effect.

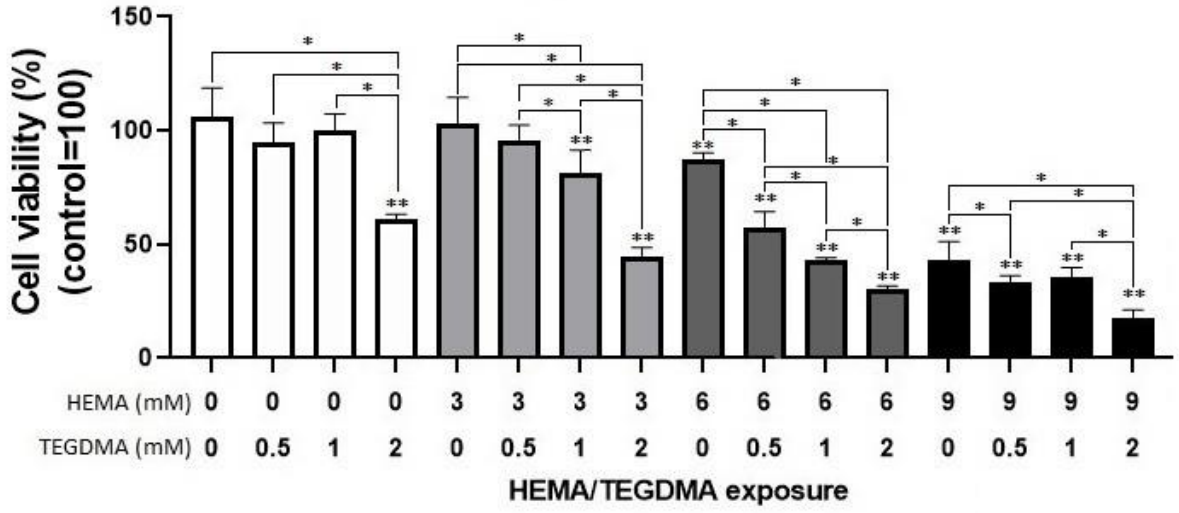

Figure 4.3 A: Cell viability of THP-1 cells after being exposed to mixtures of HEMA and TEGDMA, measured as SDH-activity. Samples were exposed to several mixtures of HEMA and TEGDMA (n ≥ 3 ± SD). Asterisk (*) indicates a significant difference between the two samples, while a double asterisk (**) indicates a significant difference from the control ($p<0.05$).

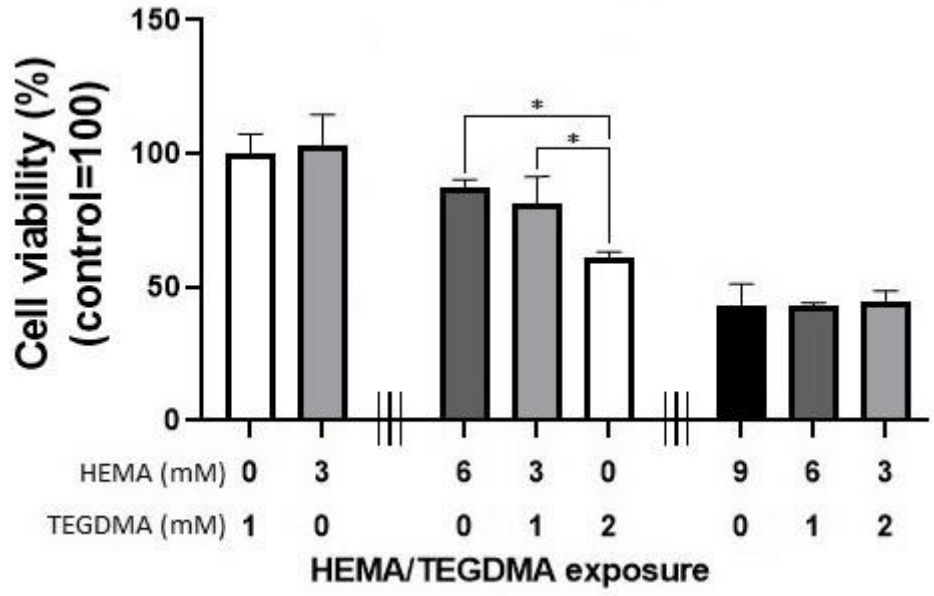

Figure 4.3 B: Cell viability of THP-1 cells exposed to mixtures of HEMA and TEGDMA, measured as SDH-activity. It is based on the same dataset used to make **Figure 4.3 A**; however, this compares the toxic ratio calculated from the MTT assay EC50 values. The lines on the x-axis separate the groups with the "same" toxic effect but different combinations (n ≥ 3 ± SD). Asterisk (*) indicates a significant difference between the two samples ($p<0.05$).

4.2 ROS levels

4.2.1 Individual tests

Reactive oxygen species levels can, directly and indirectly, affect cells' molecular mechanism. The antioxidant levels such as GSH and oxidative stress-reducing proteins connected to the Nrf2/ARE pathway like HO-1 may affect the oxidative stress in cells. ROS has been shown to induce autophagy, and if a substance causes increased ROS levels, autophagy may be a possible mechanism for cytotoxicity.

THP-1 cells were exposed to HEMA (0.5-9 mM), TEGDMA (0.5-4 mM), and positive control, H_2O_2. H_2O_2 was included to ensure that the method worked (not shown). There was an increase in ROS levels when the cells were exposed to HEMA or TEGDMA (**Figure 4.4**). However, TEGDMA kept increasing relative to the higher concentrations, but HEMA only caused the level to increase slightly, unaffected by the increasing concentrations. HEMA did not cause a significant increase in ROS. TEGDMA doses were significantly different at 3.5 mM and higher.

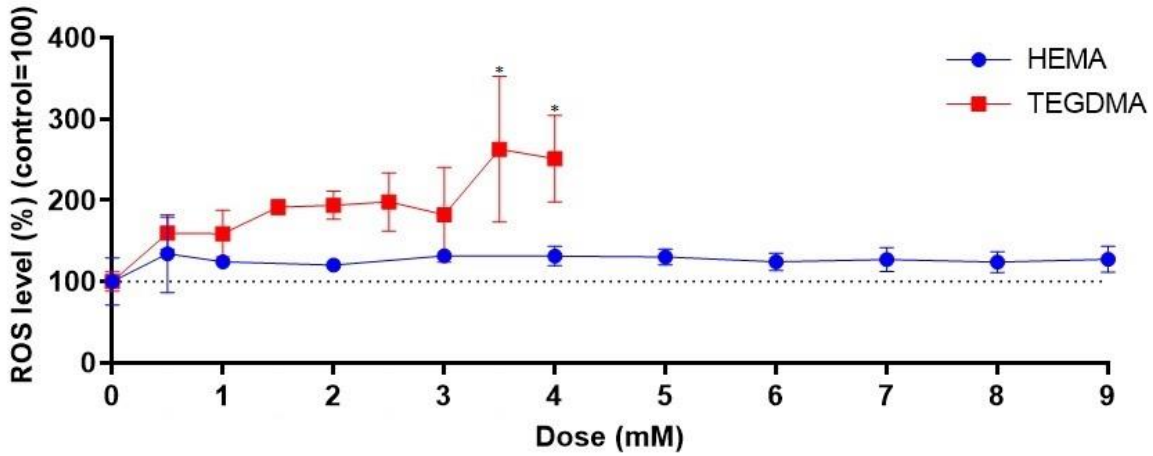

Figure 4.4: The level of reactive oxygen species in THP-1 cells when exposed to HEMA and TEGDMA individually. Samples were exposed to different concentrations of HEMA or TEGDMA ($n \geq 3 \pm SD$). Asterisk (*) indicates that a sample is significantly different from the control ($p<0.05$).

4.2.2 Combined tests

The cells were exposed to 3 or 6 mM HEMA, in combination with or without 1 or 2 mM TEGDMA concentrations. **Figure 4.5 A** shows that HEMA and TEGDMA combinations cause an increase in ROS levels compared to the controls. However, as with the individual results, the ROS increase depends not on HEMA concentration. Only TEGDMA shows a dose-dependent increase in ROS level. The effect of the combined exposure is at the same level as the individual. There is a significant difference to

the control at combinations with at least 3 mM HEMA and 1 mM TEGDMA. There is also a significant difference between mixtures with the same HEMA concentration but different TEGDMA concentrations.

We can check the plausibility of the toxic ratio calculated in the MTT assay using our ROS data. **Figure 4.5 B** shows that only the 6 mM HEMA exposure is significantly different from the other exposures with an estimated equal toxic effect.

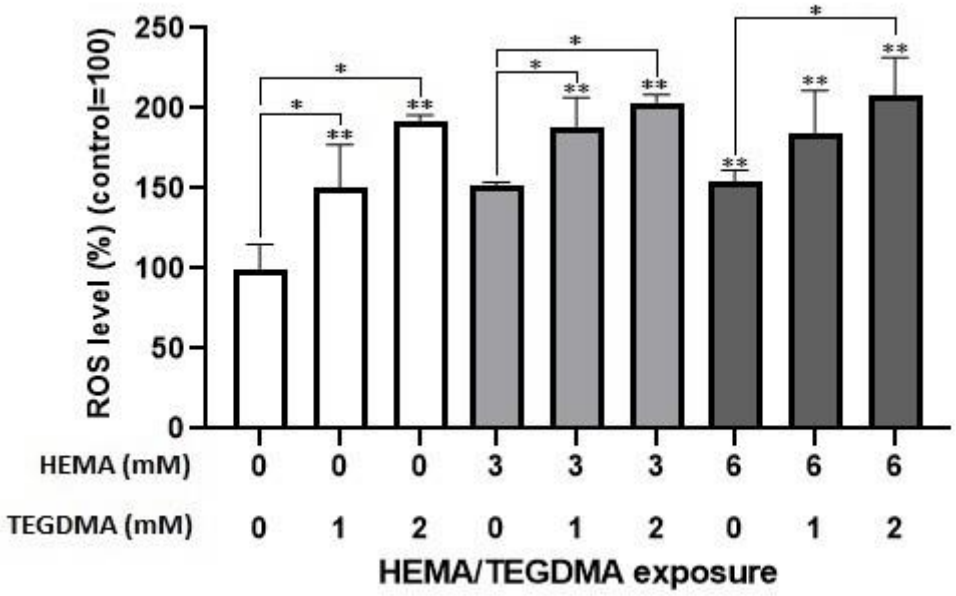

Figure 4.5 A: Reactive oxygen species level of THP-1 cells after exposure to different combinations of HEMA and TEGDMA. Samples were exposed to varying mixtures of HEMA and TEGDMA ($n \geq 3 \pm SD$). Asterisk (*) indicates a significant difference between the two samples, while a double asterisk (**) indicates a significant difference from the control ($p<0.05$).

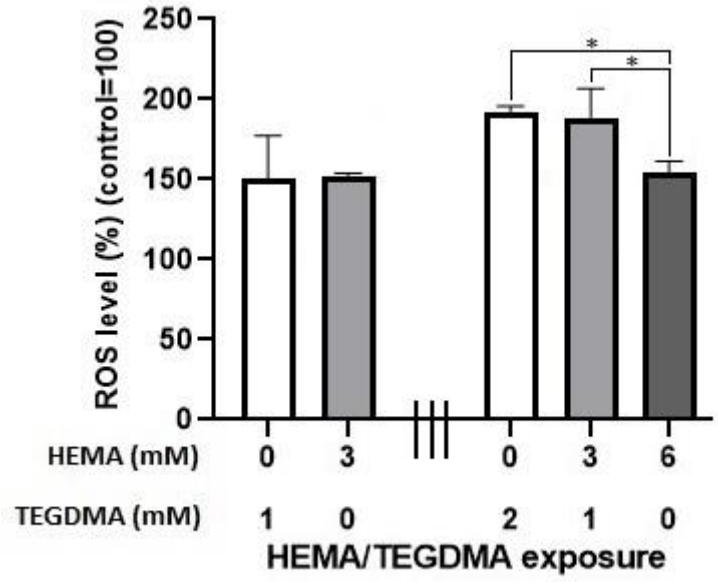

Figure 4.5 B: Reactive oxygen species level of THP-1 cells exposed to mixtures of HEMA and TEGDMA. It is based on the same dataset used to make **Figure 4.5 A**; however, this compares the toxic ratio calculated from the MTT assay EC50 values (1 mM TEGDMA = 3 mM HEMA). The lines on the x-axis separate the groups with the "same" toxic effect but different combinations ($n \geq 3 \pm SD$). Asterisk (*) indicates a significant difference between the two samples ($p<0.05$).

4.3 GSH levels

4.3.1 Individual tests

We also decided to measure glutathione, an important antioxidant in the body. A change in the GSH level may indicate which pathways HEMA and TEGDMA might affect.

THP-1 cells were exposed to HEMA (0.5-9 mM) and TEGDMA (0.5-4.5 mM). Both substances' concentrations led to a significant decrease in GSH levels (**Figure 4.6**). The decrease came in a dose-dependent manner.

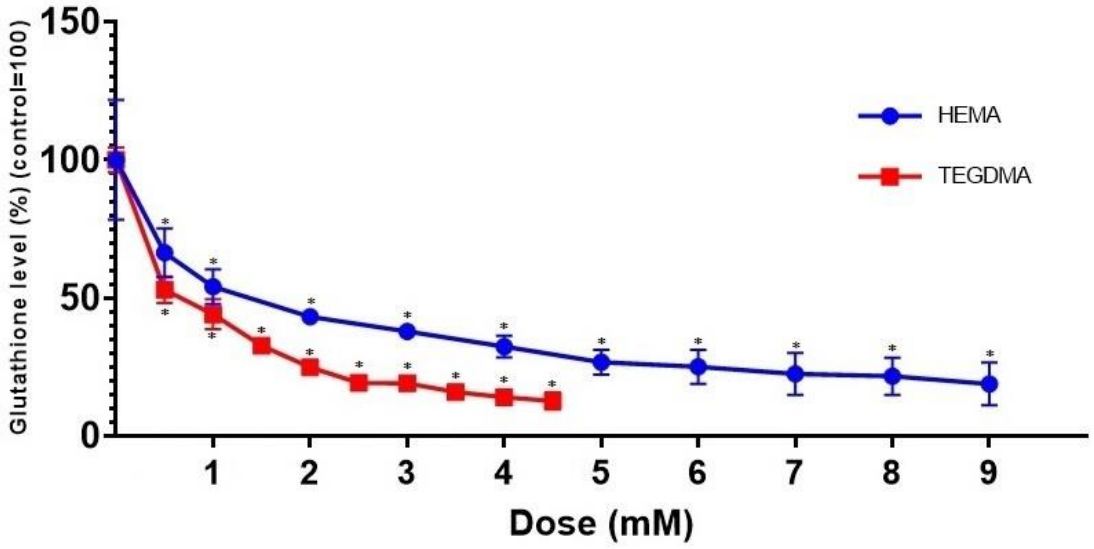

Figure 4.6: Glutathione levels of THP-1 cells when exposed to HEMA and TEGDMA separately. Samples were exposed to different concentrations of HEMA or TEGDMA ($n \geq 3 \pm SD$). Asterisk (*) indicates that a sample is significantly different from the control ($p<0.05$).

We made an estimation of the EC50 (GSH) values for both HEMA and TEGDMA, using figures (**Appendix 3**) made by averaging the values for each concentration. The toxic ratio can then be used to create an isobole. The EC50 (GSH) for HEMA was determined to be 1.4, while for TEGDMA, it was 0.7. That means TEGDMA is 2 times more toxic than HEMA on the glutathione level. Since the EC50 (GSH) values are made from an average, the ratio should be considered as approximate values.

The calculations resulted in an isobole (**Figure 4.7**) showing us a slightly curved line. It indicates an additive interaction, as the curvature of the line is not wide enough to be considered another type of interaction. A p-value of 0.5155 indicates that the figure is a good fit for the data.

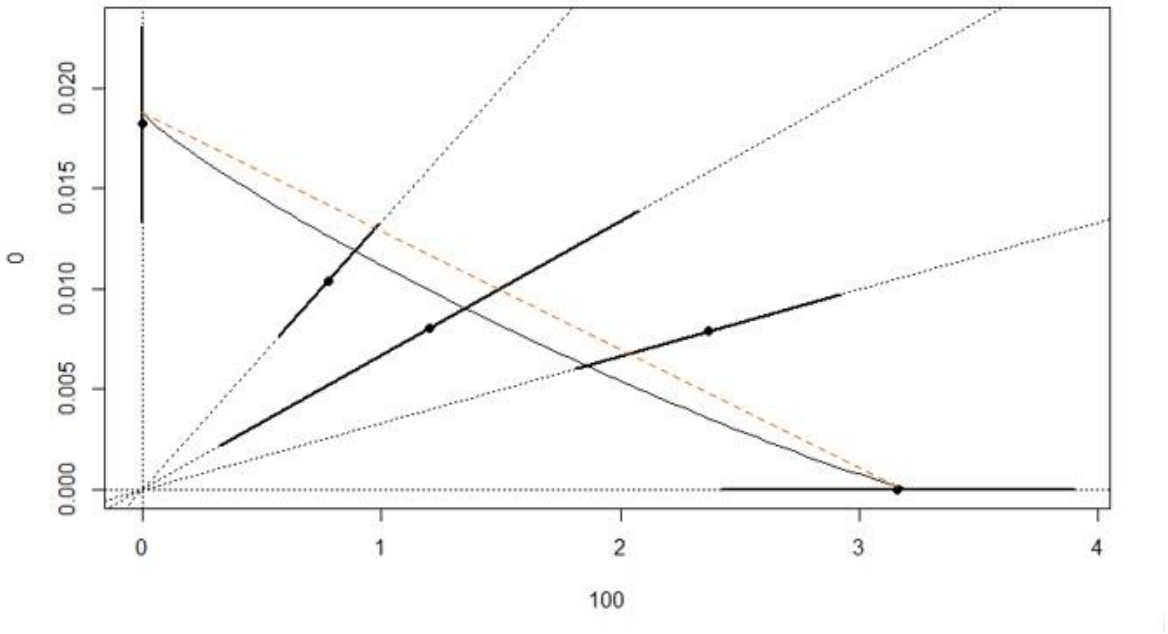

Figure 4.7: An isobole created using the exposure data from the GSH analysis, indicating an additive interaction between HEMA and TEGDMA. The effect of TEGDMA was calculated to be expressed as HEMA with the ratio being: 1 mM TEGDMA = 2.0 mM HEMA. The straight orange dashed line is only a visual tool, as its straightness shows the curvature of the solid line isobole.

4.3.2 Combined tests

During the combined exposure, cells were exposed to HEMA at 3 or 6 mM while mixed with or without TEGDMA at 1 or2 mM concentrations. **Figure 4.8 A** showed that the GSH level decreases with exposure relative to the concentration of the substances. The decrease is comparable with the individual results, and all combinations had a significant decrease in GSH level. There are also significant differences between different samples, with higher concentration mixture having a greater GSH level decrease.

Just as we did with the combined ROS data, we checked the plausibility of the toxic ratio calculated with the MTT assay. While we have calculated a different ratio for GSH since we had all the data needed. The concentrations chosen during combinations were determined by the toxic ratio determined by the MTT assay. Besides this, it would not have been possible to compare different mixtures with the GSH toxic ratio. **Figure 4.8 B** shows the comparisons, and the mixtures are very similar to each other.

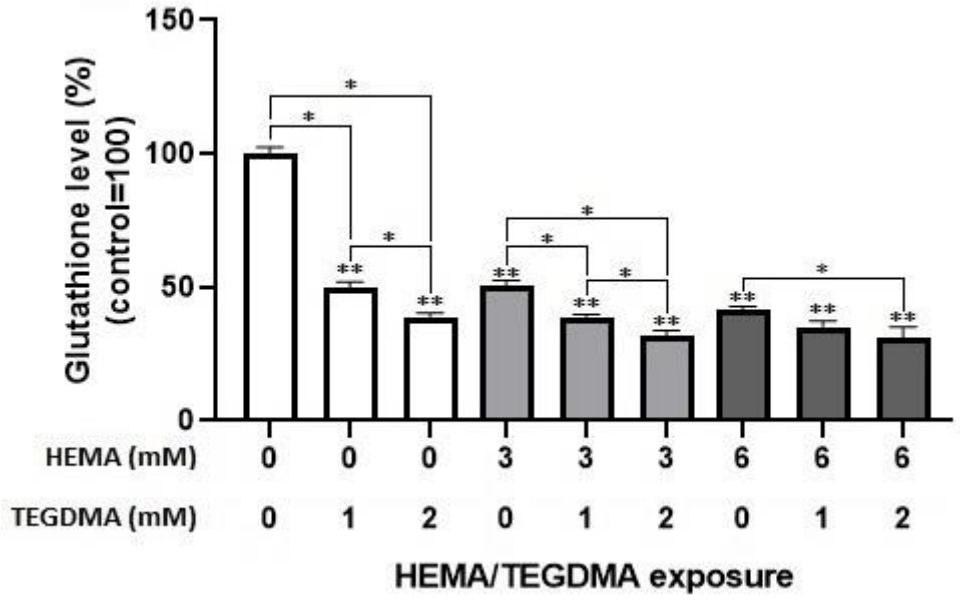

Figure 4.8 A: Glutathione levels of THP-1 cells when exposed to combinations of HEMA and TEGDMA. The cells were exposed to different mixtures of HEMA and TEGDMA (n ≥ 3 ± SD). Asterisk (*) indicates a significant difference between the two samples, while a double asterisk (**) indicates a significant difference from the control ($p<0.05$).

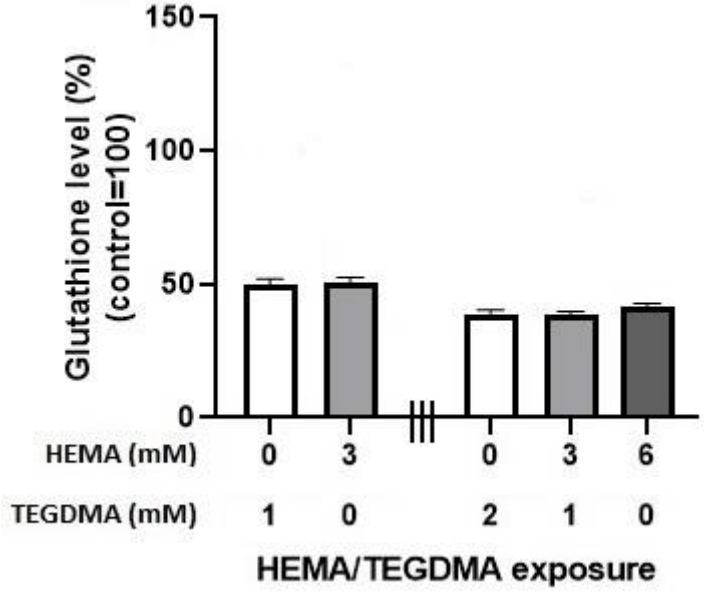

Figure 4.8 B: Glutathione level of THP-1 cells exposed to mixtures of HEMA and TEGDMA. It is based on the same dataset used to make **Figure 4.8 A**; however, this compares the toxic ratio calculated from the MTT assay EC50 values (1 mM TEGDMA = 3 mM HEMA). The lines on the x-axis separate the groups with the "same" toxic effect but different combinations (n ≥ 3 ± SD). Asterisk (*) indicates a significant difference between the two samples ($p<0.05$).

4.4 Western analysis

We used western blot to measure how the levels of selected proteins connected to the Nrf2/ARE pathway, found in Samuelsen, 2019 and Nilsen, 2018 [Samuelsen *et al.,* 2019; Nilsen *et al.,* 2018], were affected after exposure to HEMA and TEGDMA. These results may indicate something about the mechanisms our substances work through.

Individual exposures of HEMA were 1-4 mM, while for TEGDMA, they were 0.25-1 mM. The combined exposures were with HEMA 3 mM, with our without TEGDMA at 0.5 or 1 mM. To avoid dead cells affecting the analysis, we used lower concentrations than in previous studies.

Loading controls of α-Tubulin were included to verify equal protein loading (data not shown). However, there were not enough data to say if equal protein loading were maintained. While we did not get three replicates, the data suggest that most of the samples have an equal amount of protein. Therefore, we will continue with the assumption that the untested samples do as well.

4.4.1 p62

As shown in **Figure 4.9 A-B**, the individual tests gave increased p62 protein levels relative to the concentration of the substance. The increase was significant when the cells were exposed to at least 2 mM HEMA or 0.5 mM TEGDMA.

The combined experiments (**Figure 4.10**) showed a significant increase in p62 levels for all exposures compared to the control. The increase was comparable to the increase of the individual tests. There was also a significant increase between samples with no HEMA exposure and varying TEGDMA concentrations. There was, however, no significant difference between samples exposed to 3 mM HEMA and varying TEGDMA concentrations.

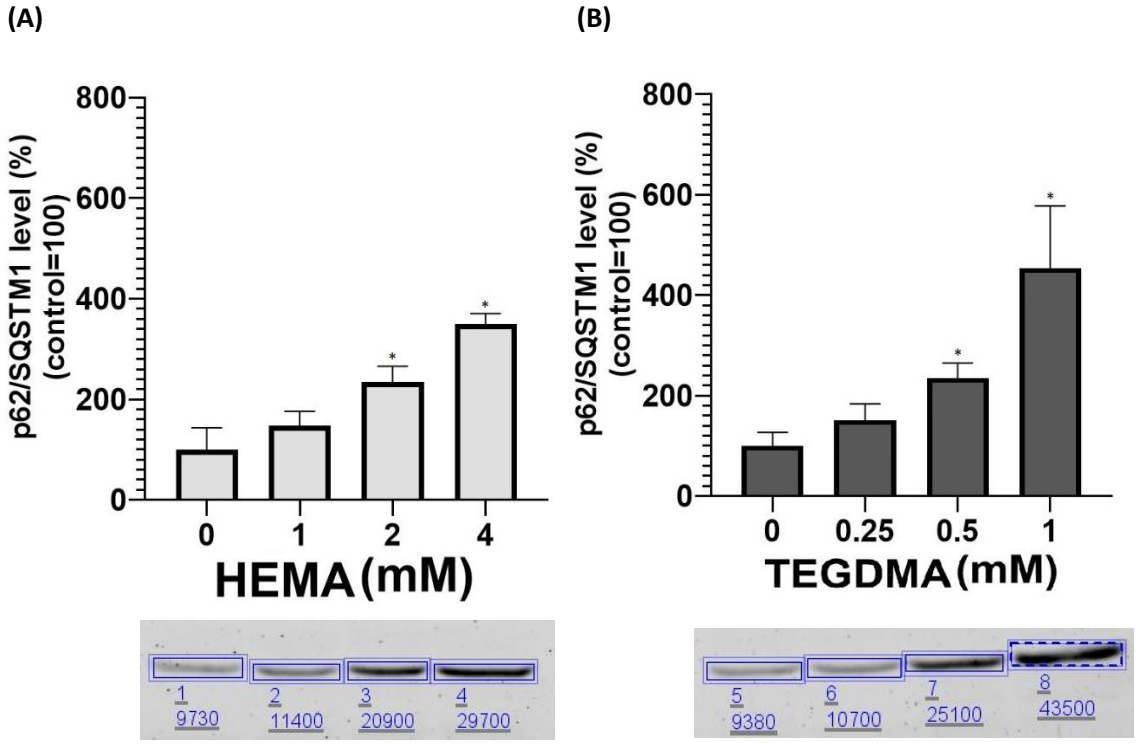

Figure 4.9 A-B: The expression of p62 level in THP-1 cells after exposure to HEMA (**A**) and TEGDMA (**B**) separately. Each sample was exposed to a different concentration of either HEMA or TEGDMA (n ≥ 3 ± SD). Asterisk (*) indicates that a sample is significantly different from the control ($p<0.05$). A representative blot from each experiment is shown below.

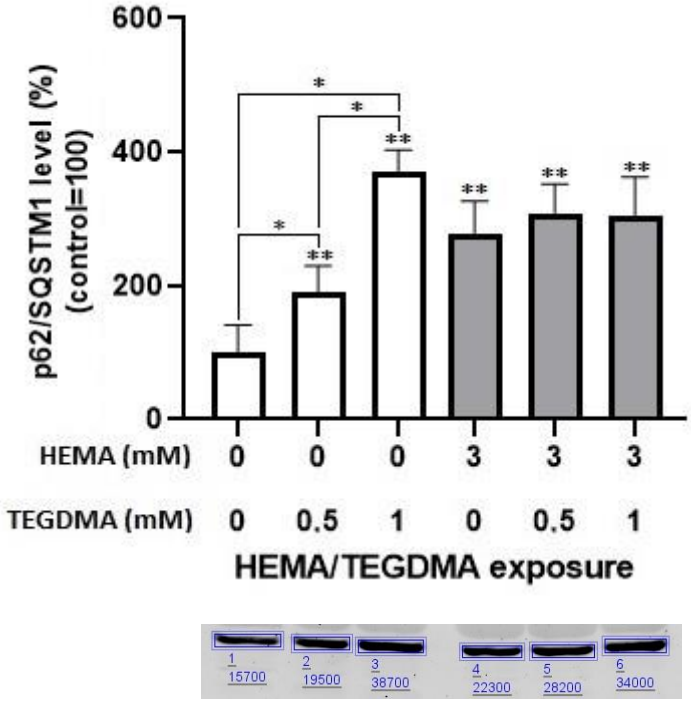

Figure 4.10: The expression of p62 levels in THP-1 cells when exposed to HEMA and TEGDMA. The cells were exposed to various mixtures containing HEMA and TEGDMA (n ≥ 3 ± SD). Asterisk (*) indicates a significant

difference between the two samples, while a double asterisk (**) indicates a significant difference from the control ($p<0.05$). A representative blot from each experiment is shown below.

4.4.2 HO-1

HO-1 levels had a significant increase with individual HEMA and TEGDMA exposures, as shown in **Figure 4.11 A-B**. There was a slight increase relative to the increasing concentrations, but the level increased significantly at 4 mM HEMA or 1 mM TEGDMA. For HEMA or TEGDMA exposure, this increase was about 10 or 20 times, respectively, compared to control.

The combined data (**Figure 4.12**) shows a significant increase in the HO-1 level, especially compared to the rise caused by the individual exposures. There was a significant difference for most of the mixtures compared to the control. But there were no significant differences between the 3 mM HEMA and 1 mM TEGDMA mixture compared to the other combinations.

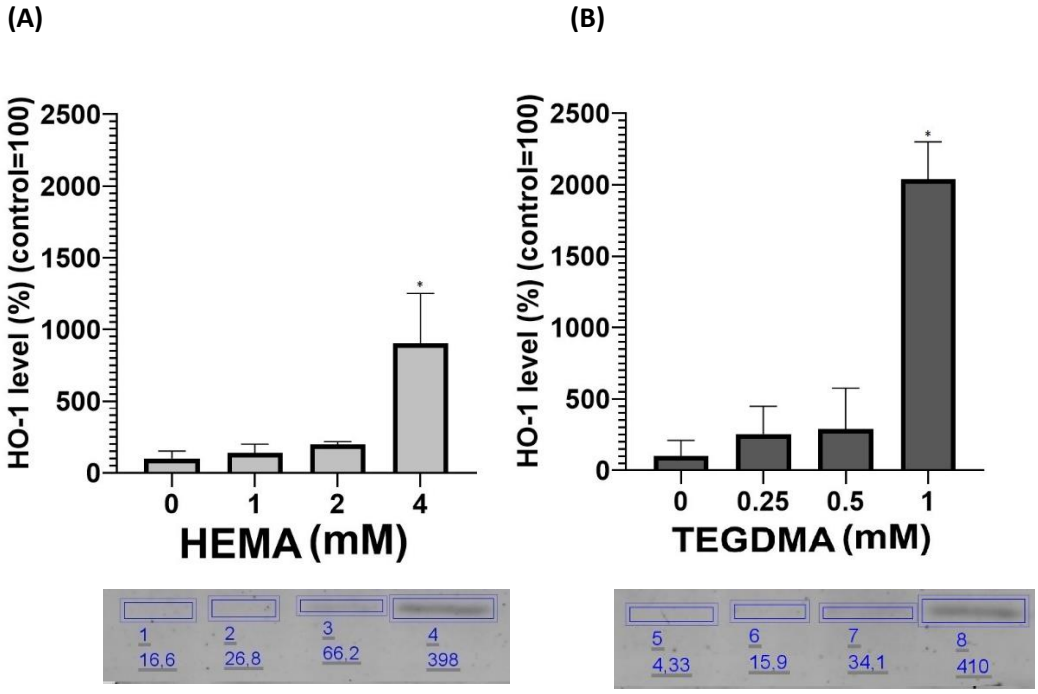

Figure 4.11 A-B: The expression of HO-1 levels in THP-1 cells when exposed to HEMA (**A**) and TEGDMA (**B**) separately. Each sample was exposed to a different concentration of either HEMA or TEGDMA ($n \geq 3 \pm SD$). Asterisk (*) indicates that a sample is significantly different from the control ($p<0.05$). A representative blot from each experiment is shown below.

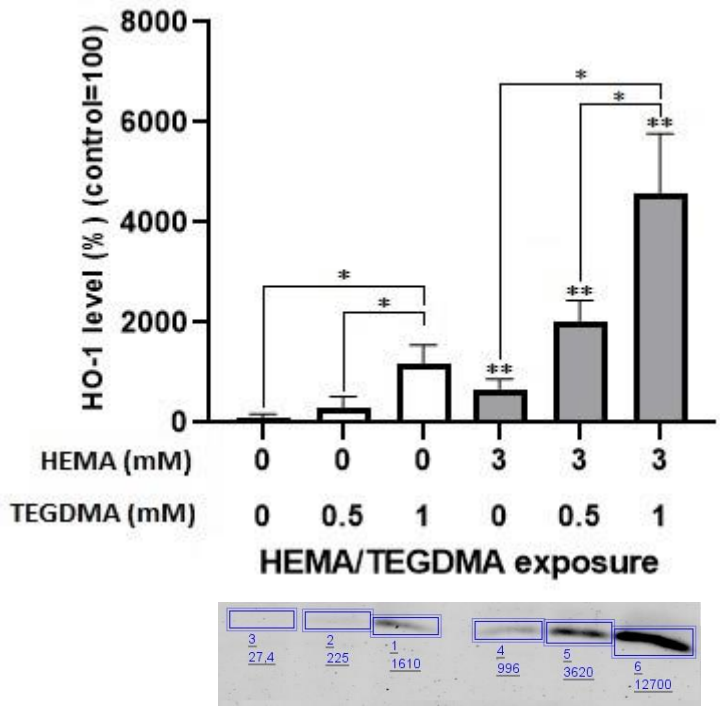

Figure 4.12: HO-1 levels in THP-1 cells when exposed to both HEMA and TEGDMA. The cells were exposed to various mixtures containing HEMA and TEGDMA (n ≥ 3 ± SD). Asterisk (*) indicates a significant difference between the two samples, while a double asterisk (**) indicates a significant difference from the control ($p<0.05$). A representative blot from each experiment is shown below.

4.4.3 Pirin

HEMA and TEGDMA exposure individually caused an increasing level of Pirin expression relative to the concentration of the substance (**Figure 4.13 A-B**). There was a significant difference to the control when the cells were exposed to at least 2 mM HEMA or 0.5 mM or higher TEGDMA concentrations.

The combined exposures to HEMA and TEGDMA (**Figure 4.14**) indicate that Pirin levels increase relative to concentration. However, the sample exposed to 3 mM HEMA and 1 mM TEGDMA shows a lower protein increase than the other samples. The mixtures also do not show any significant difference between them. The results are comparable with the individual exposures.

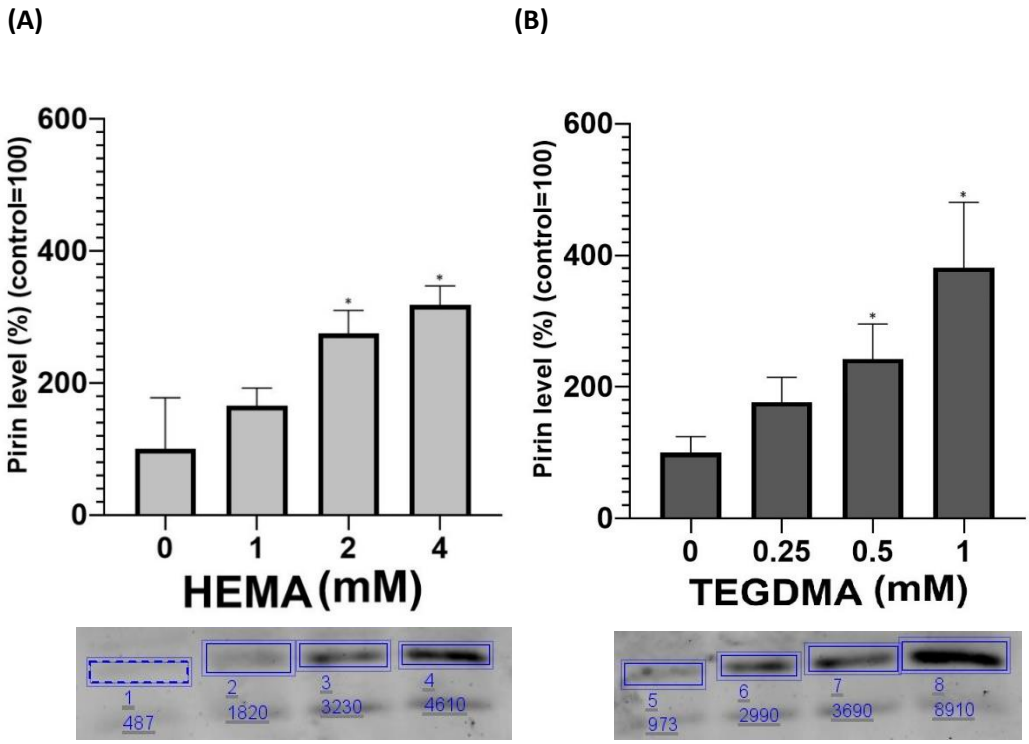

Figure 4.13 A-B: The expression of Pirin protein levels in THP-1 cells after being exposed to HEMA (**A**) and TEGDMA (**B**) separately. Each sample was exposed to a different concentration of either HEMA or TEGDMA (n ≥ 3 ± SD). Asterisk (*) indicates that a sample is significantly different from the control ($p<0.05$). A representative blot from each experiment is shown below.

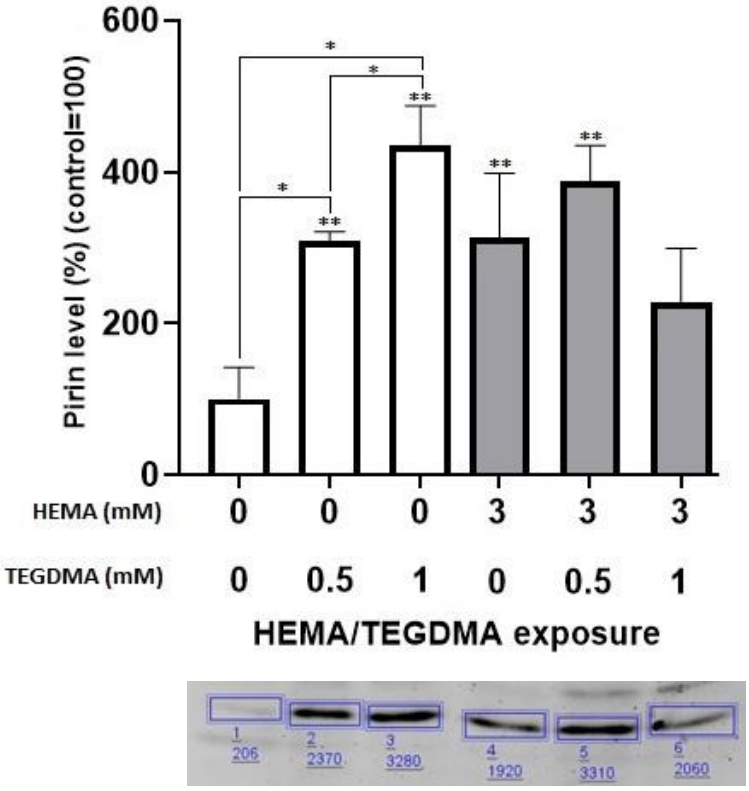

Figure 4.14: Pirin levels in THP-1 cells after exposure to HEMA and TEGDMA combined. The cells were exposed to mixtures containing HEMA and TEGDMA ($n \geq 3 \pm SD$). Asterisk (*) indicates a significant difference between the two samples, while a double asterisk (**) indicates a significant difference from the control ($p<0.05$). A representative blot from each experiment is shown below.

5 Discussion

This study aimed to further understand the toxic potential of HEMA and TEGDMA. Research has previously shown that patients and dental workers are exposed to resin composite substances during and after dental procedures. The two most common exposures are HEMA and TEGDMA. These monomers are major components of many commonly used materials. However, not all of the material will be polymerized during the procedure. Patients are exposed to the unpolymerized monomer directly, as the materials are in contact with their gums or other tissue in the oral cavity. Dentists are exposed to the unpolymerized monomer through inhalation or direct contact with skin during handling.

Independent studies have researched both substances and concluded that HEMA and TEGDMA likely cause an effect through a similar mechanism, like GSH depletion [Samuelsen *et al.*, 2005]. Since both are methacrylates, it is reasonable to assume they work through the same mechanisms. Studies such as those performed by Samuelsen, 2019 on HEMA [Samuelsen *et al.,* 2019], and Nilsen, 2018 on TEGDMA [Nilsen *et al.,* 2018] have compiled our understanding of the toxic effects by focusing on cytoprotective mechanisms in subcytotoxic cells. These studies, however, seem to differ. For HEMA, Samuelsen, 2019 suggest that the cytotoxic effect occurs through protein damage caused by electrophilic stress, while for TEGDMA Nilsen, 2018 indicate that DNA and mitochondrial damage caused by ROS are the cause. Both agree that exposure leads to increased oxidative stress, but its importance to the effect differs between the substances. As of writing this thesis, there has only been one separate experiment that have followed up on one of these hypothesis. Becher and co. [Becher *et al.,* 2019] followed up on the hypothesis that HEMA works through electrophile stress. And the study indicated that the hypothesis is correct.

We hypothesized that HEMA and TEGDMA work through different mechanisms based on the studies by Samuelsen, 2019 and Nilsen, 2018 [Samuelsen *et al.,* 2019; Nilsen *et al.,* 2018]. We performed tests on cell viability, ROS levels, GSH levels, and selected proteins essential to the Nrf2/ARE pathway's response to oxidative stress. While not as thorough as the two studies, the results would indicate if the hypothesis was plausible or not. Additionally, we also attempted to provide insight into any possible interaction between the substances, and that may suggest which mechanisms they affect. These results can then be used during more in-depth studies regarding their interactions as an indicator of the interaction type.

5.1 The research model and concentrations

We designed the experiments to best replicate the conditions used by Samuelsen, 2019, and Nilsen, 2018 [Samuelsen *et al.,* 2019; Nilsen *et al.,* 2018]. Like the previous studies, we used the human leukemia monocytic cell line called THP-1. This cell line is commonly used as a model for monocytic activities when performing *in vitro* testing. *In vitro* models are essential as they allow for continuous testing on cells, giving us a better opportunity to understanding of the molecular toxic effects. The issue with *in vitro* is an extrapolation to *in vivo* models, as *in vivo* are generally more complex. In addition, the cell lines used *in vitro* are often modified, such as making them immortal to make cultivation and preservation more efficient.

At first, the cells were exposed to various concentrations of HEMA or TEGDMA to determine at what concentrations there was a significant loss in viability. The concentrations chosen are considered as clinically relevant exposures, where studies like Noda and co. [Noda et al., 2002] have calculated (from *in vitro* studies) that about 1.5-8 mM HEMA and 4 mM TEGDMA reach the dental pulp. Concentrations below the threshold of significant viability loss were then used on experiments for other cellular events (such as proteins or ROS), as cell death would make it difficult to interpret the results.

Exposure times are chosen based on the exposure times used by Samuelsen, 2019 [Samuelsen *et al.,* 2019]. HEMA and TEGDMA shared the same exposure time across the same type of experiment. There are limited available data on the exposure time for patients and dentists. Michelsen and co-workers [Michelsen *et al.*, 2012] have shown that for a patient, most monomers have disappeared before 24 hours has passed. As for air exposure, Hagberg and co. [Hagberg *et al.*, 2005] have shown there are detectable levels of HEMA and TEGDMA (through a study by Henriks-Eckerman and co. [Henriks-Eckerman *et al.*, 2001]); however, how significant these levels were was difficult to determine. The ROS tests are a modification of Samuelsen, 2019, with the exposure time reduced.

5.2 HEMA and TEGDMA decreased cell viability

The MTT assay showed that increasing HEMA and TEGDMA concentrations decreased cell viability in a dose-dependent response. These results are similar to what research has previously shown. Based on EC50 values, it was determined that after 24 hours of exposure, TEGDMA was approximately 3 times more toxic than HEMA. After 48 hours of exposure, the ratio decreased, with TEGDMA being approximately 2.5 times more toxic than HEMA. Since TEGDMA contain two methacrylate-groups, it

makes sense that it is more toxic than HEMA, which only contain one such group. The difference between 24 and 48 hours toxic ratio is not a big issue, as the difference is not significantly different.

Cells managed to withstand the lower concentrations of HEMA and TEGDMA, but there was a significant decrease in viability at 6 mM HEMA and 2 mM TEGDMA when exposed for 24 and 48 hours. The explanation for why the cells survived in the beginning is likely that their defensive mechanism (either antioxidants, proteins, etc.) were good enough to supress the exposure. Since clinical data indicates that there is little exposure after 24 hours [Michelsen *et al.*, 2012], we will exclude further discussion on the 48 hours results. Our data is similar to the results shown in Samuelsen, 2019 regarding HEMA, and Nilsen, 2018 regarding TEGDMA [Samuelsen *et al.*, 2019; Nilsen *et al.*, 2018]. All results showed a dose-dependent response in cell viability. The only noticeable difference between our results and Nilsen, 2018, is that we never saw any increase in cell viability. However, since we did not test as low concentrations as Nilsen, 2018, we cannot confirm if there would have been a similarity.

The MTT assay is a standard method for evaluating cell viability *in vitro* (ISO 10993-5:2009(E)), used worldwide because of its low cost and easy-to-use performance [Rai *et al.*, 2018]. Furthermore, since it only measures mitochondrial activity in living cells, the measurements are also highly accurate. However, the MTT assay is not a direct measurement of viability. Firstly, what is measured is SDH-activity that transforms tetrazolium into formazan, measured as absorbance. Therefore, we do not directly read if the cells are alive or not. MTT assay also has a problem regarding false-positive or false-negative results due to the cell death mechanism [Weyermann *et al.*, 2005]. Alternative cell viability assays such as adenosine triphosphate (ATP) assay or resazurin reduction assay could be used in tandem with MTT to confirm the precision of the results. The MTT results confirmed that HEMA and TEGDMA cause cytotoxicity. The reason for this is still poorly understood, but one of the hypotheses was through ROS increase and GSH depletion.

5.3 HEMA and TEGDMA affects ROS and GSH levels

One of the possible factors causing cytotoxicity is reactive oxygen species (ROS). Research has shown that both HEMA [Morisbak *et al.*, 2015; Spagnuolo *et al.*, 2004] and TEGDMA [Yeh *et al.*, 2015] cause ROS levels to increase. Our results also showed an increase in ROS levels when exposed to both substances. TEGDMA showed an increasing ROS relative to the increase in concentration. But HEMA showed a level increase regardless of the concentration. That is particular, as it may indicate that ROS is not one of the primary mechanisms that cause cytotoxicity in HEMA exposed cells. Studies like the one performed by Morisbak and co. [Morisbak *et al.*, 2015] support this hypothesis, as it showed

that cell death was not always inhibited when external substances attenuated ROS induced by HEMA. The ROS increase from HEMA exposure is unnatural, as other articles (e.g. Spagnuolo *et al.*, 2004) has shown a dose-dependent increase (although the difference may be because of cell type).

Our positive control with H_2O_2 confirmed an increase in ROS level, showing that the method worked. The method itself, using H_2DCFDA as a probe to detect oxidative stress, is widely used because of its easy procedure and ability to react with multiple different ROS. However, the method has been questioned regarding its precision. Kalyanaraman and co-workers [Kalyanaraman *et al.*, 2012] collected the knowledge on disadvantages with the method and indicated that it is not suitable for measuring H_2O_2 or other oxidants. The method has an artificial amplification of the results because of a redox-cycling mechanism that involves DCF. Additionally, dichlorodihydrofluorescein (DCFH) does not directly react with H_2O_2, making it unsuitable for positive control. Furthermore, ROS levels are also cell-dependent, as different cell types can have different defences [Morisbak *et al.*, 2020]. The method should be further optimized before results can be discussed. Because of the unusual results from the HEMA exposure, and the possible error with the method, we will not take the ROS results into account during later discussions.

Glutathione (GSH) is an important molecule in phase II conjugation reaction. The phase II enzymes conjugate GSH to electrophilic compounds, which is the first step in eliminating toxic compounds [Townsend & Tew, 2003]. If either HEMA or TEGDMA caused cytotoxicity through oxidative stress, it would likely affect the GSH levels. The decrease in GSH and increase in ROS has often been partially attributed to why HEMA [Chang *et al.*, 2005] and TEGDMA [Stanislawski *et al.*, 2003] are cytotoxic. The hypothesis was that HEMA [Samuelsen *et al.*, 2011] and TEGDMA [Lefeuvre *et al.*, 2004] form a complex with GSH by binding with the -thiol group. Supporting this, there are studies like Nocca and co. [Nocca *et al.*, 2014] performed, have indicated that total glutathione levels (i.e., GSH + GSSG) decrease when exposed to HEMA and TEGDMA, even at subcytotoxic concentrations.

Our results showed that GSH levels decrease with the increasing concentrations of HEMA or TEGDMA. The results also support the indication made by Nocca and co. [Nocca *et al.*, 2014], as there was a significant decrease in the GSH levels even at subcytotoxic concentrations of HEMA or TEGDMA. We are also aware that these measurements were performed after only 4 hours of exposure. If it had been the same 24 hours as used in the MTT assay, the GSH levels would likely have been even lower. This indicates that GSH depletion is not a major contributor to cytotoxicity caused by HEMA or TEGDMA. The results may also indicate that GSH does protect the cells well enough during lower concentrations exposure, but after a certain point it is too much.

Our results are similar to the GSH results from Samuelsen, 2019 [Samuelsen *et al.,* 2019]. There was a significant decrease in GSH levels even at subcytotoxic concentrations of HEMA. While our ROS data on its own is not reliable, it is similar to the data shown in Samuelsen, 2019. ROS increase and GSH decrease reaches maximum effect at a subcytotoxic concentration. Therefore, both our and Samuelsen, 2019 data indicate that ROS and GSH levels are, at the very least, not major components of HEMA exposed cytotoxicity.

While Nilsen, 2018 [Nilsen *et al.,* 2018] did not include a GSH measurement, the study has indicated from measurements of the glutathione-disulfide reductase (GSR) protein that GSH levels are depleted. However, since the study lacks data that indicates at what concentrations of TEGDMA led to a significant GSH depletion, it is difficult to connect a possible GSH depletion to the cytotoxic mechanism. The same can be said for ROS levels, as upregulated or downregulated proteins connected to ROS cannot be used as indicators of ROS level.

The precision of the GSH measurements needs to be considered. The use of monobromobimane (mBBr) as a probe to detect GSH is a common method [Anderson *et al.*, 1999]. Because it can penetrate the cell membrane and react directly with cellular thiols, it gives them an advantage over alternative methods [Čapek *et al.*, 2017]. However, a disadvantage is that mBBr also reacts to other agents with a -thiol group, which leads to overestimating the results. Another issue is that it does not measure GSSG [Anderson *et al.*, 1999]. While GSSG amount is little compared to GSH, it is crucial to indicate if the effect may be caused by increased ROS or decreased GSH.

5.4 HEMA and TEGDMA alter protein levels

Central cellular defence mechanisms against oxidative or electrophile stress are regulated by the Nrf2/ARE pathway [Nguyen *et al.*, 2009]. Nuclear factor erythroid 2-related factor 2 (Nrf2) controls many genes that code for proteins with antioxidant properties. These genes are connected to an element called the antioxidant responsive element (ARE). Many have referred to Nrf2 as “the master regulator of antioxidative responses” [Vomund *et al.*, 2017]. Some of these genes include SQSTM1, which codes for SQSTM1/p62 protein; PIR, which codes for Pirin protein; and HMOX1, which codes for HO-1 protein

Sequestosome 1 (p62/SQSTM1)

The SQSTM1/p62 protein is a multifunctional protein mainly associated with autophagy. It may be altered as a result of oxidative stress response, and metabolic reprogramming [Sanchez-Martin & Komatsu *et al.*, 2018].

Our data indicated a dose-dependent increase in level based on the concentrations of HEMA or TEGDMA. The level increase was comparable between the two substances. The increase was expected, as p62 role as a multifunctional protein means it could be induced through multiple mechanisms [Ning & Wang, 2019]. The results from Samuelsen, 2019 [Samuelsen *et al.*, 2019] were similar to ours, showing a significant increase in p62 level. A study by Liu and co. [Liu *et al.*, 2016] suggested that p62 is induced since it interacts with ubiquitin chains on damaged proteins and then transports them to the proteasome. Samuelsen, 2019 also suggested that the increased p62 came from the Nrf2 pathway being activated by HEMA. However, we lack a proper method to determine if p62 interacts with ubiquitin chains, and we lack the Nrf2 measurements to confirm if p62 is increased by activated Nrf2.

Our data differ from Nilsen, 2018 [Nilsen *et al.*, 2018], as their data does not show a significant increase in p62 levels at higher exposures. There may be an error in our data or it is because we did not use Stable isotope labelling by amino acids in cell culture (SILAC). The most likely explanation, however, is that Nilsen, 2018 used a too high TEGDMA concentration based on their cell viability data (significant cell death at 2.5 mM). Our data and Nilsen, 2018 show a significant increase in p62 at 0.25 and 0.33 mM TEGDMA, respectively. p62 is involved with oxidative stress response and autophagy, two of the mechanisms that cause cytotoxicity, as hypothesized by Nilsen, 2018. However, Nilsen, 2018 performed a gene ontology enrichment analysis that indicates that p62 acts through stress, not necessarily meaning oxidative stress. For example, it may increase because of electrophilic stress, as suggested by Samuelsen, 2019 [Samuelsen *et al.*, 2019].

It has been indicated that p62 inhibit the recruitment of factors necessary to DNA repair [Wang *et al.*, 2017]. As such, it would make sense if there were higher levels of p62 in higher TEGDMA exposures if the cytotoxicity occurred through DNA damage. However, there are reports that p62 effect on autophagy may be cell-dependent [Liu *et al.*, 2016], the results seem to be the same, hinting that the dependence is not so strong.

Heme oxygenase 1 (HO-1)

HO-1 is an important protein and key enzyme in cellular response to oxidative stress, as it mediates the first step on catabolising heme. It does this by cleaving heme into iron (II) ion, carbon monoxide (CO), and biliverdin [Gozzelino *et al.*, 2010; Maines, 1997]. The cytoprotective properties are believed to come from reducing heme, in addition to production of CO (a cell-signalling molecule [Kim *et al.*, 2006]) and biliverdin (that converts into antioxidant bilirubin) [Barañano *et al.*, 2002]. CO has also been shown to modulate activation of MAPK in response to stress and inflammation [Kim *et al.*,

2006], which is connected to what was mentioned earlier with how MAPK can notice GSH depletion and induce autophagy [Filomeni *et al.*, 2003]. Perhaps there is a connection there.

The data we collected shows a large increase in HO-1 levels, with the increase being measured up to 10x for HEMA and 20x for TEGDMA. What is interesting is that there was a massive increase in HO-1 between the lower concentrations and 4 mM HEMA and 1 mM TEGDMA. It could be explained by there being a "threshold" of some kind that when passed, the level of protein increases significantly. The increase may also be a bias from having few samples, or uneven protein loading could explain the increase. The increase in HO-1 level was as expected, as articles beforehand has shown an increased HO-1 level to HEMA [Jiao *et al.,* 2019] and TEGDMA exposure [Murakami *et al.*, 2019].

While Samuelsen, 2019 [Samuelsen *et al.,* 2019] did not include any data on the HO-1 level, there are other articles that may indicate the results it may have. A study by Becher and co. [Becher *et al.,* 2019] have followed up on the hypothesis by Samuelsen, 2019 regarding electrophilic stress, and has included a measurement on the HO-1 level. Becher and co. used BEAS-2B cells to show an increase in HO-1 levels after exposure to HEMA. The study suggested that HO-1 increase was independent of the Nrf2 and backed it up with data showing that N-acetylcysteine did not lower the ARE activity. Since the toxic effect was independent of the GSH/ROS changes but still showed an activation in Nrf2/ARE, it indicated that the effect may be induced through electrophilic stress. these results are similar to our own. There is a dose-dependent increase (albeit not so noticeable in our figures), with there being a large increase on the highest concentration. Becher and co. does not go into any reason for this increase, but it does indicate that there may be some "threshold" regarding protein production. However, since Becher and co. used BEAS-2B cells and not THP-1 cell like us or Samuelsen, 2019, there may be an extrapolation in results between the cell types.

Our TEGDMA results are very similar to the HO-1 levels measured by Nilsen, 2018 [Nilsen *et al.,* 2018]. A gene ontology enrichment analysis performed by Nilsen, 2018 indicated that the gene responsible for inducing HO-1, HMOX1, were expressed in multiple pathways. The interesting part was how TEGDMA may affect immune functions. A cascade of HMOX1/Nrf2 has been reported to mediate anti-inflammatory effects induced in THP-1 cells [An *et al.*, 2016; Ren *et al.*, 2016]. This indicates that TEGDMA may cause an inflammatory effect, perhaps through mitochondrial damage caused by ROS, which Nilsen, 2018 hypothesized. However, autophagy avoids inflammation, and as such any inflammation has to come from another source, for example mitophagy, or another mechanism. Based on the results from Nilsen, 2018, there does not seem to be any "threshold" for protein production to TEGDMA exposure.

For our “threshold” hypothesis, there are not much data that give an indication for its cause, mostly because there is not enough variance in concentrations to observe the sudden increase. The mechanism behind it is also unknown. A study by Torun and co. [Torun *et al.*, 2016] hypothesized that it might be because of oxygen tension. To see if oxygen tension is a possible factor might an aim during a future study.

Pirin

Compared to p62 and HO-1, there have not been many studies about Pirin’s function in the cell. Because of this, our understanding of the protein is still limited. One functions that is often mentioned is its co-regulation of NF-κB together with iron [Liu *et al.*, 2013]. NF-κB is a transcription factor that regulates several mechanisms in adaptive and innate immune functions. It also serves as an important mediator for inflammatory responses [Oeckinghaus & Ghosh *et al.*, 2009]. There are studies that have suggested that the gene expressing Pirin, PIR, is connected to apoptosis [Orzaez *et al.*, 2001] and stress response [Hihara *et al.*, 2004]. The studies have also suggested that the functions may be cell-type and species-specific activity.

The results from our study showed a significant dose-dependent increase in Pirin levels. Since the understanding about Pirin function is not well studied, it is difficult to form a proper hypothesis of what the increase can be a response to. Additionally, since the response may be cell-type dependent, what little is known may not properly explain the mechanisms that happens in our cell type. If we assume that the mechanisms regarding NF-κB, apoptosis and stress response is correct, there are indications that Pirin may be involved in these processes. Our GSH and ROS experiments indicate that the redox balance has been destroyed. This would lead to Pirin being induced, which regulates NF-κB to help against inflammation, and also to induce apoptosis to avoid more inflammation.

These functions are mentioned in Samuelsen, 2019 [Samuelsen *et al.*, 2019] as well, with them showing elevated Pirin levels as well. They also hypothesize an activation of NF-κB, however, there was no observation to support this. Both our results and Samuelsen, 2019 shows a significant increase in Pirin at subcytotoxic concentrations. This further indicates that the Pirin increase is likely unrelated to apoptosis. As for the electrophilic stress mechanisms, the Pirin increase cannot show if the hypothesis is true or not. The NF-κB pathway has been shown to be regulated by oxidative stress, but its connection to electrophilic compounds is less known [Fagiani *et al.*, 2020].

Nilsen, 2018 [Nilsen *et al.*, 2018] result is also difficult to explain. Since NF-κB is affect by the ROS, it may be a main component for the cause of cytotoxicity. We also know that induced Pirin has been associated with apoptosis in certain cell types [Gelbman *et al.*, 2007]. Pirin has been shown to form complexes between anti-apoptotic NF-κB and its DNA target sequence [Orzaez *et al.*, 2001]. This can

indicate that the Pirin increases to lower to autophagy cause by ROS damaging mitochondria, however, there is nothing that indicate that this is what happens. There is just too little information regarding Pirin to properly connect it to any mechanism, let alone attempt to explain any difference between HEMA and TEGDMA's mechanisms.

5.5 HEMA and TEGDMA interactions

Besides evaluating the hypothesis of HEMA and TEGDMA acting through different mechanisms, we also wanted to begin giving an indication for how the substances may interact. By checking the same events, we were able to compare them to the individual results and observe if there were any difference from when they were exposed individually.

Cell viability

A study performed by Ratanasathien and co. [Ratanasathien *et al.*, 1995] tested the interaction between different monomers commonly used during dental filling procedures. The results implied that just knowing how they affect cell viability individually is not adequate to determine the toxic potential of the monomers.

For cytotoxicity, the combinations also showed a decreased cell viability. However, the decrease was more significant than the individual tests. The result indicates either an additive or synergistic interaction between HEMA and TEGDMA. The interaction is further supported by the isobole our data created, as it implies that there is an additive interaction between HEMA and TEGDMA. The toxic ratio (1 mM TEGDMA = 3 mM HEMA) used to make the isobole can also be seen in our combined MTT tests. Combinations with different doses have no significant difference between different mixtures with the same toxic effect. There is one significantly different sample (2 mM TEGDMA). However, this may come from a bias caused by not having many results or that the effect caused by the specific sample was more significant. It can also be that the cells were particularly vulnerable to that dose, as it was around those doses that the cell viability began to decrease more rapidly.

The isobole may, however, contain some inaccuracies regarding the results. The curvature of the isobole compared to an additive straight line may indicate other types of interactions. One way to assess the difference is to use the confidence lines on different points and see if they overlap with the additive isobole. Our MTT isobole has more points overlapping, meaning that we cannot exclude that the isobole is additive. Additionally, drug optimizing (use of specific concentrations) can affect

the structure of an isobole [Tallarida, 2012]. If the results are accurate, the additive interaction further imply that HEMA and TEGDMA uses the same mechanism.

GSH and ROS

The combined data does make our ROS data more plausible. We can see a clear redox-response between the data, with GSH levels decreasing while ROS levels increase. However, since the individual results was discarded, the same will be done with the combined results. If the result was used, it would have indicated that the combinations add to each other, but since HEMA do not affect ROS much, it is difficult to determine a concrete interaction.

The GSH combinations showed similar results as the individual tests, with a dose-dependent decrease in GSH levels. Similar to the MTT data, the mixtures are indicated to have an additive effect with each other. Different from the MTT data, however, is that the toxic ratio is different, with the EC50 values indicating that TEGDMA is 2 times more effective than HEMA. From the MTT data, we determined that TEGDMA was about 3 times more toxic. The difference may be explained by us not considering for standard deviation during the calculation. Because the EC50 values for HEMA and TEGDMA are low, slight difference in these values can greatly affect the toxic ratio. If we use the 3 times toxic ratio, we can see that GSH and ROS combinations shows similar results compared to each other. There are similarities between mixtures of different HEMA or TEGDMA concentrations, but with similar toxic effect.

The isobole created from the GSH toxic ratio further implies an additive interaction between the substances. The accuracy of the MTT toxic ratio on the GSH and ROS data indicates that the effect of both substances are constant across all three components. The additive interaction indicates that HEMA and TEGDMA affects through similar mechanisms.

Proteins

The combinations for proteins are a little more difficult to measure. Because proteins are (compared to the other measured components) complex, there may be several mechanisms that can induce or reduce their expression. Since the western blot only gives a measurement of one moment in time, it is difficult to properly quantify the protein level [Aymoz *et al.,* 2016]. We see this with the first protein, p62, where the combined increase is similar to the increase caused with individual exposure. Unlike the other components, there does not seem to be any additive effect. Actually, the interaction seems to be contrary to an additive effect, as the substances likely hinder each other's from gaining the optimal effect. If they had acted as additives or synergistic, the p62 level should have been higher than it was during individual exposure. But since the increase is comparable to the individual

exposures, it may indicate that they are somehow blocking each other in some for. This in turn, may give some indication that they are working through the same mechanism.

The combinations are different for HO-1 and Pirin levels. The mixtures clearly shows that the level increases when concentrations increase. The only outlier is the highest concentrations of Pirin, where the level of the highest concentration decreases. However, since the increase is lower than samples exposed to only 0.5 mM TEGDMA, the reason is likely that there was excessive cell death in the samples, which led to the level decreasing. The combination results from HO-1 and Pirin therefore indicate that HEMA and TEGDMA either have an additive or synergistic effect on each other. This, however, may also indicate that the HO-1 and Pirin levels are regulated through different mechanisms.

6 Conclusion

Our data cannot verify the hypothesis that HEMA and TEGDMA affects the cells through different mechanisms. Our results indicate that HEMA and TEGDMA affects cells similarly. The experiments showed:

- There was a dose-dependent decrease in cell viability by both HEMA and TEGDMA. TEGDMA was approximately 3 times more cytotoxic than HEMA. Combination exposures indicates an additive interaction.
- Both substances showed a decrease in GSH levels, but only TEGDMA showed a significant increase in ROS. GSH combination exposures indicates and additive interaction. Because of the imprecision of our ROS method, it is difficult to conclude based on the ROS results.
- All three proteins showed an increase in level, regardless of substance. The increase was comparable between HEMA and TEGDMA.

Our results show no indications of other interactions than additive between HEMA and TEGDMA. The combination experiments indicated that the substance had an additive effect on each other. This assumption is backed up by additional calculations in the form of isoboles, that further indicate an additive interaction. The combinations thus indicate that HEMA and TEGDMA work through the same mechanisms. Further studies are needed to strengthen this new hypothesis, however.

7 Future considerations

The study concluded in that there were no results that could support the hypothesis that HEMA and TEGDMA works through different mechanisms. By performing each experiment more in depth than what was performed here, it may be possible to give a much clearer indication on the substances mechanisms. Future experiments should be performed with the same cell type used in this study, THP-1. This is because several cellular events that the substances may affect have been suggested to be cell-type dependent. Cells should be tested at various timeframes to several different exposures. This will also test if there are any oxygen tension, in regard to protein levels, to observe is there are a "threshold" of some kind. Some samples should be treated with antioxidants, to observe if ROS has a significant effect on cytotoxicity. Additional testing outside those performed in this study is also suggested, such as measurements of Nrf2, NF-κB, as well as types of cytotoxicity (if it was apoptosis or necrosis). This will better indicate the mechanisms in which the protein levels are induced.

It is highly suggested to get better understanding of the mechanisms of the Pirin protein (as well as the PIR gene in general). There is very little information about Pirin, which can cause misinformation through wrong assumptions about its effect. If we get a better understanding of the protein, it may help better explain the effect of HEMA and TEGDMA, at least regarding the regulation of NF-κB.

For tests regarding the interactions, future experiments should set up the combinations so that different mixtures of calculated "equal" toxic effect may be easier to compare. Using several mixtures is recommended, as the effectiveness may differ between measurements performed on events like GSH, ROS and cytotoxicity, may differ from measurements of proteins. It is advised to create an isobole on the data, as it can be a precise indication of the interaction.

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9 Appendix

Appendix 1 Chemicals, antibodies, and kits

Table 1. Chemicals, substances, and reagents used in the study.

Product	Producer
2-Hydroxyethyl methacrylate (HEMA), CAS: 868-77-9	Sigma-Aldrich, [Saint-Louis, MO, USA]
Triethylene glycol dimethacrylate (TEGDMA), CAS: 109-16-0	Sigma-Aldrich, [Saint-Louis, MO, USA]
THP-1 cell line, Catalogue No.: 88081201	Sigma-Aldrich, [Saint-Louis, MO, USA]
RPMI-1640 Medium, 500 ml MDL Number: MFCD00217820	Sigma-Aldrich, [Saint-Louis, MO, USA]
Gentamicin (10 mg/ml), GIBCO, Invitrogen	Thermo Fisher Scientific, [Waltham, MA, USA]
Sodium Pyruvate, 100 mM, 100 ml	Lonza, [Basel, Switzerland]
1 M Hepes $_{\text{in 0.85\% NaCl}}$, 100 ml	Lonza, [Basel, Switzerland]
Fetal Bovine Serum (FBS)	Sigma-Aldrich, [Saint-Louis, MO, USA]
Thiazolyl Blue Tetrazolium Bromide CAS: 298-93-1	Sigma-Aldrich, [Saint-Louis, MO, USA]
Dimethyl sulfoxide (DMSO) CAS: 67-68-5	Sigma-Aldrich, [Saint-Louis, MO, USA]
Phosphate-buffered saline (PBS)	Lonza Group AG, [Basel, Switzerland]
CM-H2DCFDA, Invitrogen	Thermo Fisher Scientific, [Waltham, MA, USA]
30% Acrylamide/Bis Solution 29:1 (3.3% Cross.)	Bio-Rad, [Hercules, CA, USA]
30% Acrylamide/Bis Solution 37.5:1 (2.6% Cross.)	Bio-Rad, [Hercules, CA, USA]
Bovine Serum Albumin CAS: 9048-46-8	Sigma-Aldrich, [Saint-Louis, MO, USA]
10x Tris buffered saline (TBS)	Bio-Rad, [Hercules, CA, USA]

10x Tris/Glycine Buffer (Transferbuffer)	Bio-Rad, [Hercules, CA, USA]
N,N,N',N'-Tetramethyl-ethylenediamine (TEMED) CAS: 110-18-9	Sigma-Aldrich, [Saint-Louis, MO, USA]
2-Mercaptoethanol CAS: 60-24-2	Sigma-Aldrich, [Saint-Louis, MO, USA]
Bromphenol Blue sodium salt CAS: 62625-28-9	Sigma-Aldrich, [Saint-Louis, MO, USA]
Amersham Protran® Supported NC Nitrocellulose Membranes: Sheets	Cytiva, [Marlborough, MA, USA]
THIOLYTE® Monobromobimane Reagent CAS: 71418-44-5	Sigma-Aldrich, [Saint-Louis, MO, USA]
Ponceau S solution CAS: 6226-79-5	Sigma-Aldrich, [Saint-Louis, MO, USA]
Tween® 20 CAS: 9005-64-5	Sigma-Aldrich, [Saint-Louis, MO, USA]
Hydrogen peroxide (30% w/w in H_2O) CAS: 7722-84-1	Sigma-Aldrich, [Saint-Louis, MO, USA]
Methanol CAS: 67-56-1	Merck, [Darmstadt, Germany]

Table 2: Antibodies used in the study

Antibody	**Catalog nr.**	**Producer**
Alexa Fluor® 680 Anti-alpha Tubulin antibody [DM1A] – Loading control	ab184093	Abcam, [Cambridge, United Kingdom]
Anti-Heme Oxygenase 1 antibody	ab13243	Abcam, [Cambridge, United Kingdom]
Anti-Pirin/PIR antibody	ab227280	Abcam, [Cambridge, United Kingdom]
IRDye® 800CW Goat anti-Rabbit lgG Secondary Antibody	926-32211	LI-COR, [Lincoln, NE, USA]

IRDye® 800CW Donkey anti-Guinea Pig lgG Secondary Antibody	926-32411	LI-COR, [Lincoln, NE, USA]
Anti-p62/ SQSTM1 (C-terminus) guinea pig polyclonal, serum	GP62-C	PROGEN, [Heidelberg, Germany]

Table 3: Kit used in the study

Kit	Product number	Producer
MycoAlert™ Mycoplasma Detection Kit	LT07-118	Lonza Group AG, [Basel, Switzerland]
MycoAlert™ Assay Control Set	LT07-518	Lonza Group AG, [Basel, Switzerland]

Appendix 2 Solutions and buffers

Glycine-SDS-PAGE separation gel

dH_2O	11.9 mL
30 % Acrylamide/Bis Solution 37.5:1	5 mL
30 % Acrylamide/Bis Solution 29:1	5 mL
1.5 M Tris buffer (pH 8.8)	7.5 mL
10 % SDS	300 µL
10 % APS	300 µL
TEMED	12 µL

Glycine-SDS-PAGE stacking gel

dH_2O	6.1 mL
30 % Acrylamide/Bis Solution 37.5:1	1.3 mL
0.5 M Tris buffer (pH 6.8)	2.5 mL
10 % SDS	100 µL
10 % APS	50 µL

TEMED	10 µL

Complete THP-1 cell culture medium

2.75 mL of Gentamicin is added to an entire bottle (500 mL) of RPMI-1640 medium

RPMI-1640 (+ Gentamicin)	44 mL
Sodium pyruvate	0.5 mL
1 M Hepes	0.5 mL
Fetal Bovine Serum	5 mL

H_2O_2 stock (50 mM)

H_2O_2 concentrated (30 %)	5 µL
PBS	1 mL

Monobromobimane stock

PBS	10 mL
FBS	110 µL
40 mM mBBr (in methanol)	10 µL

Running buffer

10x Tris/Glycine buffer	100 mL
10 % SDS	10 mL
dH_2O	890 mL

Transfer buffer

10x Tris/Glycine buffer	150 mL
Methanol	225 mL
dH_2O	1125 mL

Sample buffer

10 % SDS	12 mL
dH_2O	50 mL
Trizma® base	1.817 g
Glycerol	30 g

Adjusted to pH 7.0 with HCl at room temp.
and then heated at 37 °C for 24 hours.

TBST buffer

Tween® 20	1 mL
10x TBS	100 mL
dH_2O	900 mL

TBS + 3 % BSA stock

1x TBS	50 mL
BSA	1.5 g

TBST + 1 % BSA

1x TBST	50 mL
BSA	0.5 g

0.5 M Tris buffer		**1.5 M Tris buffer**	
Trizma® base	15.125 g	Trizma® base	45.375 g
dH_2O	250 mL	dH_2O	250 mL
HEMA stock (1000 mM)		**H_2DCFDA stock (3 mM)**	
HEMA concentrated	15.2 µL	H_2DCFDA powder	50 µg
Medium (RPMI-1640)	109.8 µL	DMSO	29 µL

TEGDMA dilution series

Stock 1 (2000 mM)		Stock 2 (1000 mM)		Stock 3 (500 mM)	
DMSO	238 µL	DMSO	100 µL	DMSO	50 µL
TEGDMA	262 µL	Stock 1	100 µL	Stock 2	50 µL
Stock 4 (2500 mM)		**Stock 5 (2250 mM)**		**Stock 6 (1500 mM)**	
DMSO	238 µL	Stock 1	50 µL	Stock 1	50 µL
TEGDMA	327.5 µL	Stock 4	50 µL	Stock 2	50 µL
Stock 7 (1750 mM)		**Stock 8 (1250 mM)**		**Stock 9 (250 mM)**	
Stock 1	25 µL	Stock 2	25 µL	DMSO	25 µL
Stock 6	25 µL	Stock 6	25 µL	Stock 3	25 µL

Stock 10 (750 mM)	
Stock 2	25 µL
Stock 3	25 µL

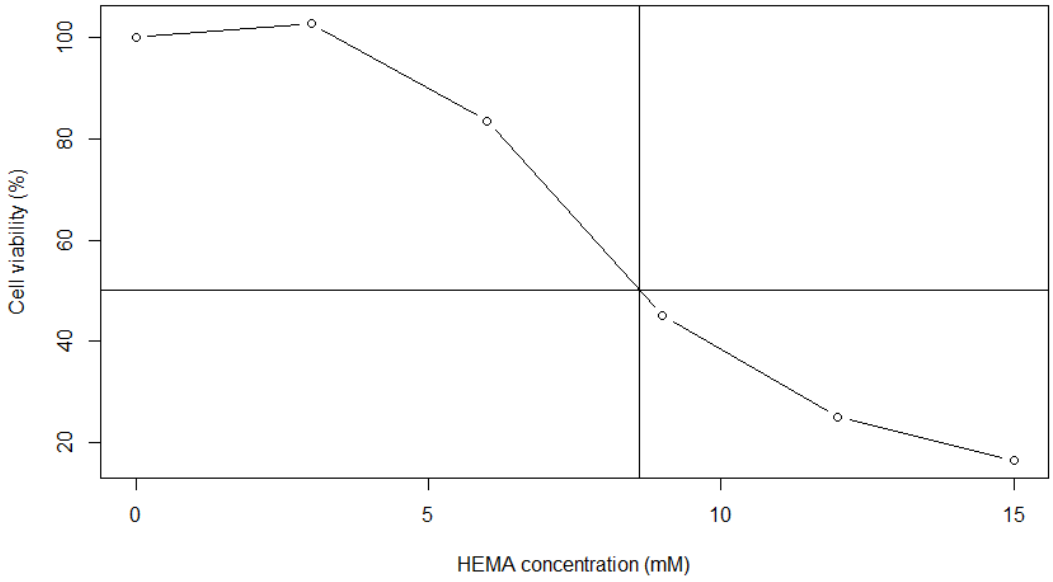
Appendix 3 EC50 figures

The formula for calculating toxic ratio:

$$\boldsymbol{EC50_{HEMA} \,/\, EC50_{TEGDMA} = Toxic\ ratio}$$

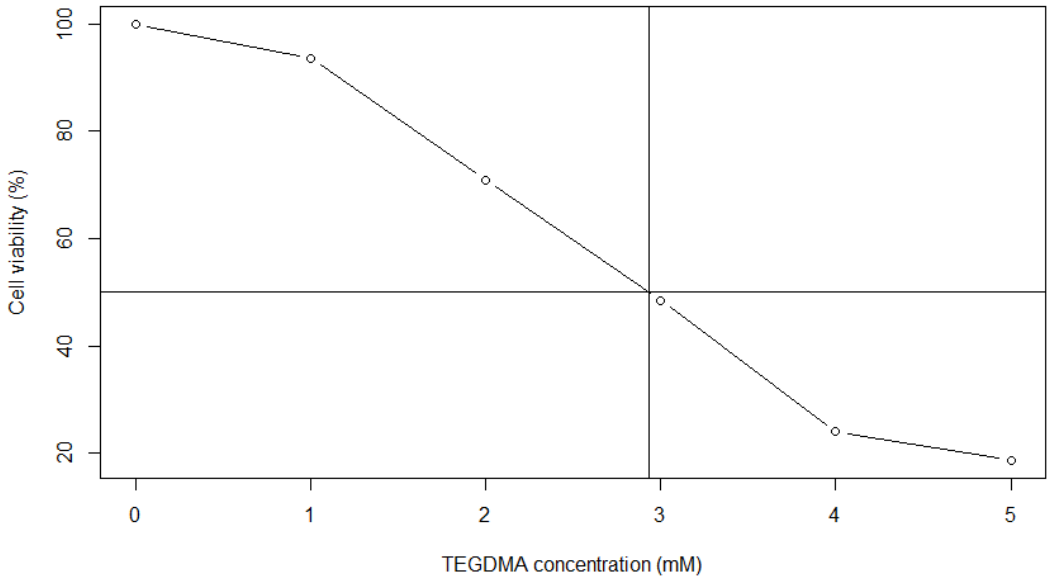
MTT HEMA (24 hours)

EC50 value = 8.61034



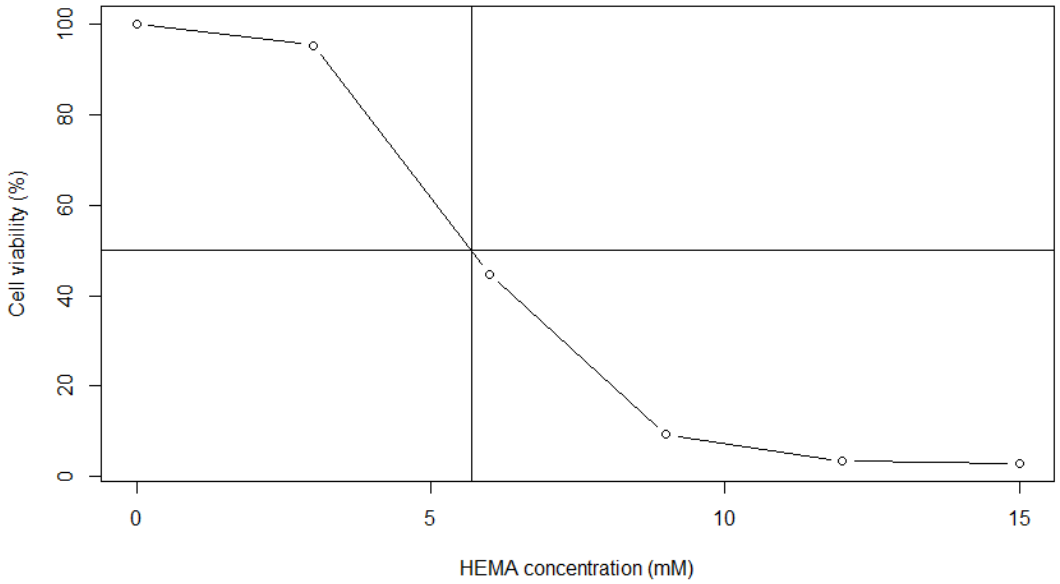
MTT TEGDMA (24 hours)

EC50 value = 2.933943



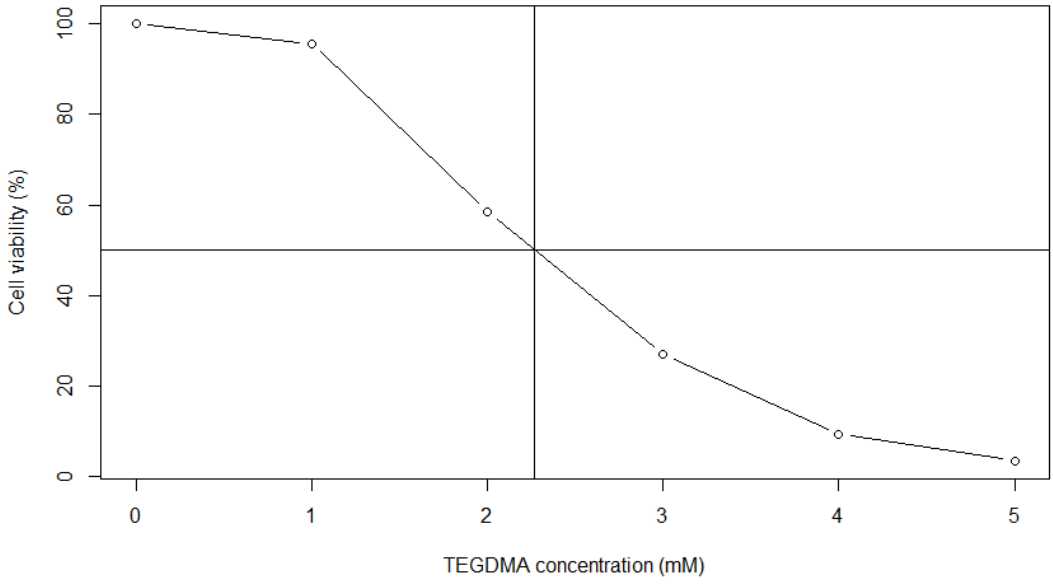
MTT HEMA (48 hours)

EC50 value = 5.68613



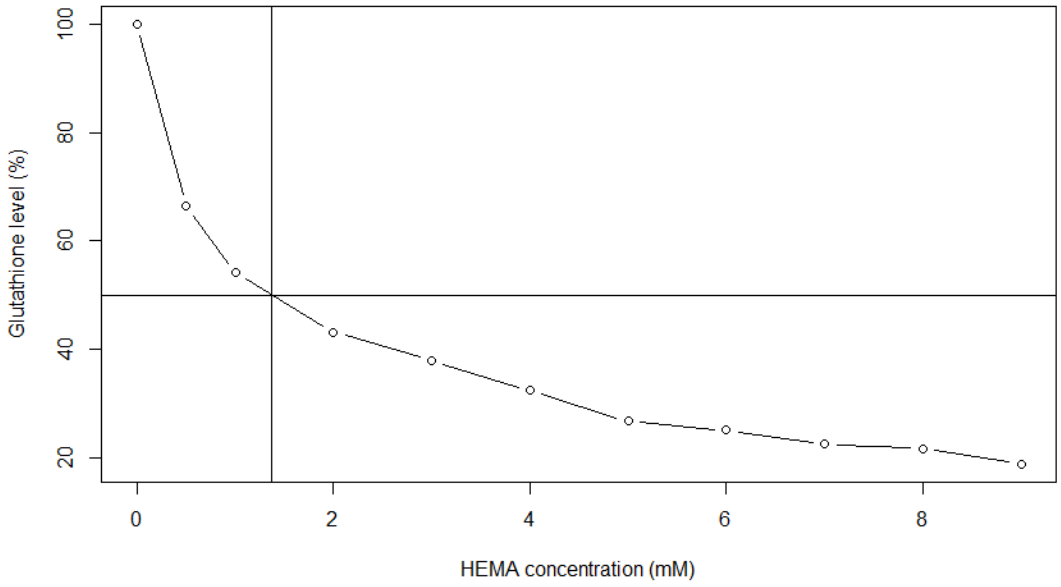
MTT TEGDMA (48 hours)

EC50 value = 2.269178



GSH HEMA

EC50 value = 1.373731



GSH TEGDMA

EC50 value = 0.6673265