2013

# Semi-Interpenetrating Network Gelatin Fiber Sca old for Oral Mucosal Delivery of Insulin

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# Semi-Interpenetrating Network Gelatin Fiber Scaffold for Oral Mucosal Delivery of Insulin

by

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Thesis submitted to the Faculty of the
Virginia Commonwealth University
in partial fulfillment of the requirements for the degree of

**Master of Science**

in

**Biomedical Engineering**

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August 2013
Richmond, Virginia

Keywords: Drug delivery, insulin, photoinitiator, scaffold

# Acknowledgments

I would like to express my deep gratitude to my academic advisor, Dr. Hu Yang, for his extraordinary patience, mentoring, support and encouragement. I would also like to thank Dr. Andrew Yeudall and Dr. Gary Bowlin for allowing me to use the instruments, providing numerous advice, and serving as committee members.

I would like to thank all of my colleagues, current and past for making the lab a home away from home. I would like to particularly thank Dr. Olga Zolotarskaya and Dr. Quan Yuan as well as my student colleagues, Donald Aduba, Jingfei Tian, Christopher Holden, Gunjan Saxena and Natasha Sheybani for their help.

I would also like to acknowledge my former research advisors, Dr. Shunlin Ren, Dr. William Pandak, Dr. Daniel Rodriguez-Agudo, Dr. Phillip Hylemon and Dr. Huiping Zhou for their training, support and guidance at Hunter Holmes McGuire VA Medical Center, as well as my former research colleagues, Dr. Qianming Bai, Dr. Xin Zhang, Dr. Genta Kakiyama, Kaye Redford, Dalila Marques and Jin Koung Kim for their previous excellent technical support.

Lastly but not the least, I would like to thank my friends and family for supporting me throughout this journey. Specially, I want to thank my wonderful girlfriend, Hao, for your sharing, caring and support. To my dear mom and dad, thank you for your unconditional love, care, and prayers. You have made every possible opportunity to my life and I love you mom and dad. I will keep making you all proud.

Leyuan Xu

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*August 2013*

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# Abbreviations

| | |
|---|---|
| AKT | Protein kinase B |
| ANOVA | Analysis of variance |
| DMEM | Dulbecco's Modification of Eagle's Medium |
| DMPA | Dimethoxyphenyl acetophenone |
| DMSO | Dimethyl sulfoxide |
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Endoplasmic reticulum |
| FBS | Fetal bovine serum |
| GF | Gelatin fiber scaffold |
| GIF | Gelatin-co-insulin fiber scaffold |
| GK | Glucokinase |
| GSK | Glycogen synthase kinase |
| HFP | 1,1,1,3,3,3-Hexafluoro-2-propanol |
| Irgacure 2959 | 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2methyl-1-propanone |
| IRS-1 | Insulin receptor substrate-1 |
| NA | No addition |
| NVP | 1-vinyl-2 pyrrolidinone |
| p-AKT | Phosphorylated AKT |
| PBS | Phosphate buffer solution |
| PCL | Poly($\varepsilon$-caprolactone) |
| PEG-DA | Polyethylene glycol diacrylate |
| PI3K | Phosphoinositide 3-kinase |

| | |
|---|---|
| PLA | Poly(lactide) |
| PLGA | Poly(lactide-co-glycolide) |
| PVDF | Polyvinylidene difluoride |
| ROS | Reactive oxygen species |
| SD | Standard deviation |
| sIPN | Semi-interpenetrating network |
| sIPN GF | Semi-interpenetrating network gelatin fiber scaffold |
| sIPN GIF | Semi-interpenetrating network gelatin-co-insulin fiber scaffold |
| T1DM | Type 1 diabetes mellitus |
| T2DM | Type 2 diabetes mellitus |
| TBS | Tris-buffered saline |
| TEOA | Triethanolamine |

# Semi-Interpenetrating Network Gelatin Fiber Scaffold for Oral Mucosal Delivery of Insulin

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## Abstract

Common therapy for diabetes mellitus is subcutaneous administration of insulin that is subject to serious disadvantages, such as patient noncompliance and occasional hypoglycemia. Hence, oral administration of insulin could be more convenient and serve as a desired route. However, oral administration of insulin is severely limited by the low bioavailability of insulin through the gastrointestinal tract. In this study, a semi-interpenetrating network gelatin fiber scaffold (sIPN GF) was fabricated for oral mucosal delivery of insulin as an alternative route. This sIPN GF was engineered from an electrospun gelatin fiber scaffold (GF), which was further crosslinked with polyethylene glycol diacrylate (PEG-DA) to enhance its stability. Within the crosslinking process, eosin Y served as a photoinitiator, and the ratio of PEG-DA to eosin Y was optimized with respect to cytocompatibility and degradation rate. The results showed that the fabricated scaffold morphology, mechanical properties, and degradation rate were significantly enhanced after the crosslinking process. This optimized formulation was used to fabricate sIPN gelatin-co-insulin fiber scaffold (sIPN GIF). Enzyme-linked immunosorbent assay (ELISA) was used to monitor the insulin releasing kinetics of sIPN GIF. Western blot analysis showed that sIPN GIF activated intracellular AKT phosphorylation in a releasing time-dependant manner. Oil red O staining confirmed the released insulin was able to induce 3T3-L1 preadipocyte differentiation. The permeability of insulin from sIPN GIF was determined on the

order of $10^{-7}$ cm/s using a vertical Franz diffusion cell system mounted with porcine buccal mucosa. These findings suggest that sIPN GIF holds a great potential for oral mucosal delivery of insulin.

# Chapter 1

# Introduction

## 1.1 Background

Diabetes mellitus, or simply diabetes, is defined as a group of metabolic diseases characterized by hyperglycemia that results from lack of insulin production or insulin response [28]. Diabetes is a major cause of heart disease and stroke, and it can also lead to vision loss, kidney failure, and amputations of legs and feet. It is reported as the seventh leading cause of death in the U.S. according to the National Diabetes Fact Sheet 2011 and Diabetes Report Card 2012 from the Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/diabetes/pubs/reportcard.htm). The reports show that about 8.3% of the U.S. population, including children and adults, has diabetes. The prevalence of diabetes is projected that one of three U.S. adults could have diabetes by 2050 if the current trend continues.

Generally, there are three common types of diabetes, referred to as type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM) and gestational diabetes. T1DM accounts for about 5% of all diagnosed cases of diabetes in the U.S., and it is commonly diagnosed in children and young adults. T1DM is characterized by the destruction of the pancreatic beta cells and insulin deficiency. The risk factors of T1DM can be autoimmune, genetic or environmental. To date, there is no therapy available to prevent T1DM. T1DM patients require daily insulin administration from an injection or a pump in order to survive. T2DM accounts for about 95% of all

diagnosed cases of diabetes in the U.S. T2DM is characterized by insulin resistance and impaired insulin secretion by the beta cells in the pancreas. As T2DM progresses, patients require more insulin, which leads to the pancreas eventually to lose its ability to produce insulin. Current reports indicate that healthy diet, regular exercise and prescribed medication can help to prevent, control health complications, and delay the onset of T2DM. Last, gestational diabetes is diagnosed as a result of pregnancy in 2-10% of pregnant women. Gestation diabetes can cause health problems for both mother and child. Women who have gestational diabetes may have a higher risk of developing T2DM in the future; while their children may have an increased risk of developing T2DM and obesity [28].

Insulin is a peptide hormone that is the key to regulate carbohydrate and fat metabolism in the body. Proinsulin is a prohormone and the precursor of insulin that is produced by beta cells of the islets of Langerhans. Proinsulin is synthesized and folded in the endoplasmic reticulum (ER) where its disulfide bonds are oxidized. After it is transported to the Golgi apparatus and packaged into secretory vesicles, it undergoes a series of digestion by proteases to form mature insulin. As an illustration shown in Figure 1.1, mature insulin (insulin) has 35 fewer amino acids, in which 4 amino acids are cleaved altogether and the remaining 31 amino acids form the C-peptide. After abstraction of C-peptide from the center of the proinsulin, insulin becomes a dimer of an A-chain (30 amino acids) and a B-chain (21 amino acids) connected by disulfide bonds.

Under physiological circumstances, when blood glucose concentration is below 90 mg/dl, it does not cause any insulin release. When carbohydrates are consumed, carbohydrates are digested and broken down to glucose molecules in the gut. These glucose molecules are subsequently absorbed into the bloodstream and elevate blood glucose concentration. The pancreas senses the rise of blood glucose concentration and in turn stimulates the secretion of insulin from the beta cells. Insulin is required for most cells to be able to uptake of glucose from the blood stream. The excess glucose can be stored in the liver in the form of glycogen for future use. The liver is

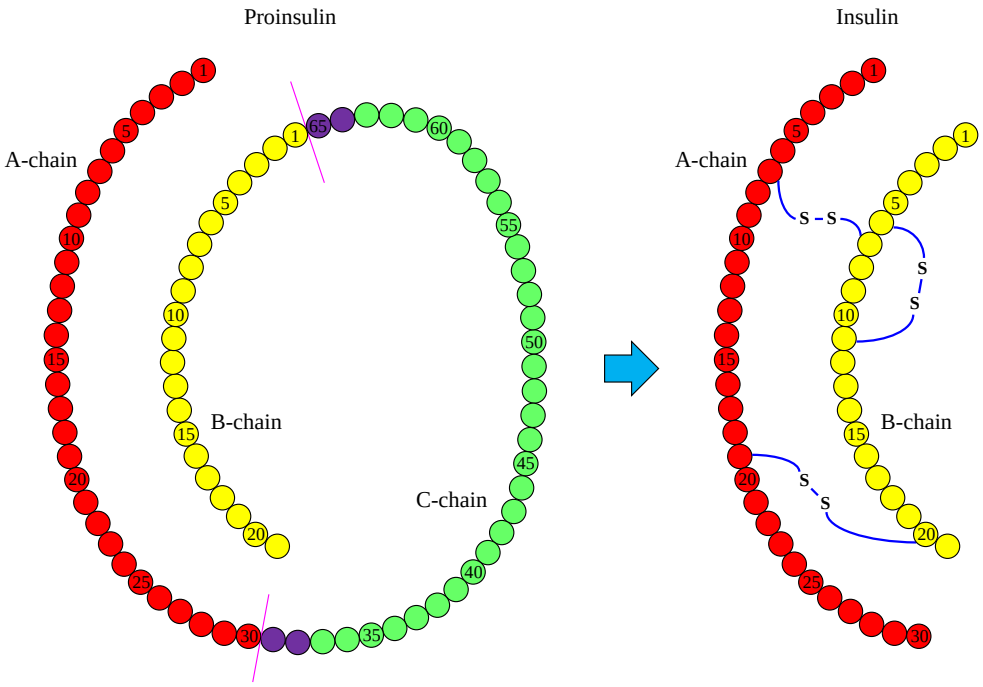


**Figure 1.1: Structures of proinsulin and insulin [74].**

able to convert glucose from fatty acids and amino acids to fat and protein respectively via gluconeogenesis. As a result, the increased secretion of insulin from the pancreas lowers blood glucose level, which in turn decreases insulin secretion.

Under pathophysiological circumstances, the insulin production and secretion are altered, which results the blood glucose uptake also changes. The limitation of the insulin production causes the decrease of glucose uptake by the cells, resulting in hyperglycemia. Because the pancreas cannot produce insulin in T1DM patients, glucose cannot enter cells, and it remains in the bloodstream. Subsequently, the fat is broken down to glycerol and free fatty acids via lipolysis in order to provide energy. Glycerol is converted to glucose as the energy source; while fatty acids are converted to ketone bodies. The elevated ketone bodies in body fluids cause a decrease of pH and loss of electrolytes, resulting diabetic ketoacidosis. The untreated diabetic ketoacidosis will cause coma or death. In T2DM, it is characterized by three disorders as peripheral insulin resistance, especially in muscle cells, increased glucose production from the liver, and eventually impaired insulin secretion pancreas.

The insulin signaling pathway plays an important role in homeostasis at the cellular level. This signaling pathway is regulated by feeding/fasting states, stress levels, and a variety of other hormones. Insulin does not directly enter the cell in its original form, but follows a sequential process as shown in Figure 1.2.

In detail, (1) Insulin binds to the extracellular portion of the $\alpha$ subunits of the insulin receptor. (2) It causes a conformational change in the insulin receptor that activates the kinase domain in the intracellular portion of the $\beta$ subunits. (3) The activated kinase domain in turn autophosphorylates tyrosine residues on the C-terminus of the receptor and tyrosine residues in the insulin receptor substrate-1 (IRS-1) protein. (4) The phosphorylated IRS-1 binds and activates phosphoinositol 3 kinase (PI3K). (5) The activated PI3K catalyzes the reaction of PIP2 + ATP $\rightarrow$ PIP3 + ADP. (6) PIP3 phosphorylates and activates protein kinase B, also as known as AKT. (7) The phosphorylated AKT (p-AKT) further phosphorylates and inactivates glycogen synthase kinase (GSK). (8) The phosphorylated GSK in turn cannot phosphorylate glycogen synthase (GS). (9) The unphosphorylated GS becomes active form and produce glycogen. (10) The p-AKT can also facilitate glucose transporter type 4 into the plasma membrane.

Insulin serves as a common protein therapy to diabetes mellitus, and it is conventionally administered to patients via subcutaneous injection. Two common subcutaneous injection methods are recommended in order to mimic physiological release of insulin. One is multiple daily injections via a syringe or a pen; the other one is an external continuous subcutaneous insulin infusion via a pump [28, 17]. However, these subcutaneous injection methods have serious disadvantages, including patient noncompliance and occasional hypoglycemia. Thus, oral administration of insulin becomes a more convenient and desired route if available [17, 71]. Nonetheless, oral administration of insulin is severely limited by the low bioavailability of insulin, including inherent instability and low permeability across biological membranes through the gastrointestinal tract [71]. A variety of formulations for oral insulin delivery have been developed in the last decade. Nanoparticle-based insulin

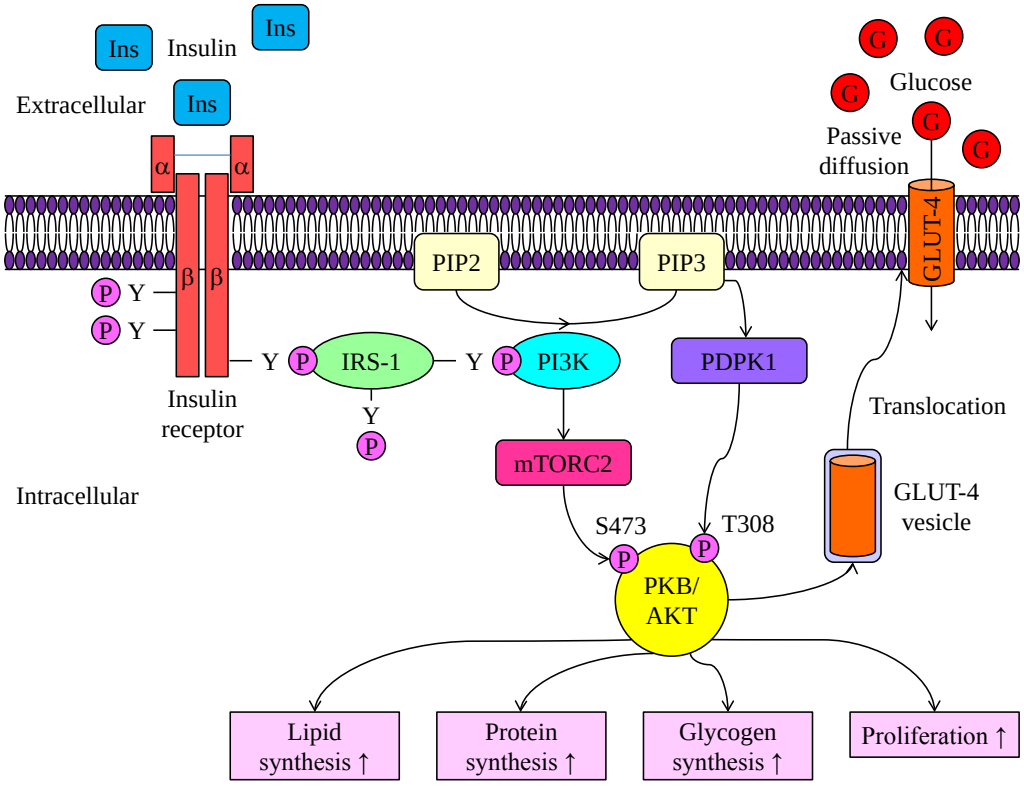


**Figure 1.2: A schematic diagram of insulin signaling transduction pathway [78].**

delivery systems were reported to be able to overcome these barriers, and these systems have been received considerable attention. The nanoparticles can be made from natural or synthetic polymers. Natural polymers are shown great interest due to their high biocompatibility and biodegradability. Chitosan and its derivatives, as one family of these natural polymers, have been widely explored for oral insulin delivery owing to their excellent mucoadhesive properties and permeability [54, 76, 39]. Synthetic polymers are attracting growing attention due to the high possibility of new dosage formulations and the potential applications in cell-specific drug targeting [3]. Poly(lactide-co-glycolide) (PLGA) [19, 20], poly(lactide) (PLA) [86], and poly($\varepsilon$-caprolactone) (PCL) [21] have been used to fabricate nanoparticles for oral insulin delivery owing to their well-established safety records. As the summary shown in Table 1.1, the maximum bioavailability of insulin through oral administration is around 30% among these nanoparticle-based delivery systems. At this point, maximization of the absorptive cellular intestinal uptake and stabilization of insulin within the gastrointestinal tract remains as a challenge for nanoparticle oral delivery of insulin. Efficacy and economic reasons will impact a market launch for these nanoparticles as well.

## 1.2 Hypothesis and research goals

A gelatin-based electrospun fiber scaffold was developed to locally deliver nystatin for candidiasis [2]. Electrospun gelatin fiber scaffolds (GFs) possess high biocompatibility and mucoadhesive property that allow local sustained release of drug at the absorption site [2, 7]. GF can be stabilized using a crosslinker of photocurable polyethylene glycol diacrylate (PEG-DA) and form a semi-interpenetrating network fiber scaffold (sIPN). The fiber morphology, mechanical and physical properties as well as drug release kinetics can be controlled by modulating the concentration of crosslinker [2]. In this work, a semi-interpenetrating network gelatin-co-insulin fiber scaffold (sIPN GIF) was hypothesized for transbuccal mucosal delivery of insulin. The cytocompatibility

**Table 1.1: Summary of in vivo bioavailability of insulin-loaded nanoparticles on oral administration [17, 12].**

| NP systems | BA | References |
|---|---|---|
| Chitosan NPs | 14.9% | [57] |
| Chitosan-polyglutamic acid NPs | 15.1% | [51] |
| Dextran sulfate-chitosan NPs | 5.6% | [62] |
| VB12-dextran sulfate-chitosan NPs | 26.5% | [14] |
| Alginate crosslinked dextran | 13.0% | [83] |
| Aminoalkyl Vitamin B12 dextran sulfate chitosan | 29.4% | [15] |
| Chitosan-TBA | 1.7% | [38] |
| PLGA-Hp55 NPs | 6.3% | [19] |
| Insulin-phospholipid complex loaded NPs | 7.7% | [20] |
| Aspart-insulin loaded CS/$\gamma$-PGA NPs | 15.0% | [69] |

Abbreviations: PLGA, poly(lactide-co-glycolide); Hp55, hypromellose phthalate, HPMCP-55; VB12, vitamin B12; SLNs, solid lipid nanoparticles; $\gamma$-PGA, poly(g-glutamic acid); TBA, thiobutylamidine; NPs, nanoparticles; BA, relative bioavailability; Bioavailability of insulin systems was determined based on the serum insulin level relative to that achieved with subcutaneous insulin injection.

of each components of sIPN GF was evaluated, and the formulation of sIPN GF was optimized in respect to cytocompatibility and degradation rate. This optimized formulation was used to fabricate sIPN GIF. Enzyme-linked immunosorbent assay (ELISA) was used to monitor the insulin release kinetics of sIPN GIF. Western blot analysis and 3T3-L1 preadipocyte differentiation analysis were used to determine the bioactivity of insulin released from sIPN GIF. The permeability of the insulin released from sIPN GIF was evaluated through a porcine buccal mucosal membrane, which possesses analogous thickness, structure, morphology, and composition to normal human buccal epithelium [91].

## 1.3 Thesis structure

The goal of this work is to explore the potential of sIPN GF in transbuccal mucosal delivery of insulin. Photopolymerization was used to crosslink gelatin fiber in

order to modulate the scaffold stability and insulin release kinetics. Chapter 2 investigates the cytocompatibility of different photoinitiator systems, including eosin Y, dimethoxyphenyl acetophenone (DMPA) and 2-hydroxy-1-[4-(hydroxyethoxy)phenyl]-2methyl-1-propanone (Irgacure 2959) systems. This chapter illustrates the correlation of intracellular AKT signaling transduction to cytocompatibility of photoinitiator systems and their free radicals on HN4 cells. Chapter 3 investigates sIPN GF as the primary material platform for transbuccal mucosal delivery of insulin. This chapter provides insights into: (1) the optimization of sIPN GF formulation in respect to cytocompatibility and degradation rate; (2) the structure stability and mechanical properties of sIPN GF; (3) the release kinetics and the bioactivity of insulin from sIPN GIF; and (4) the permeability of insulin released from sIPN GIF through a porcine buccal mucosal membrane. Chapter 4 summarizes the conclusions drawn from the presented investigations and proposes possible future research directions based on the results of this work.

# Chapter 2

# Correlating intracellular AKT signaling transduction to cytocompatibility of photoinitiators

## 2.1 Introduction

Photopolymerization has the ability to initiate rapid formation of a polymer network from a liquid monomer or macromer under physiological conditions [9]. It has been shown in great advantages of well spatial and temporal control of reaction kinetics, minimal heat production, ability to uniformly encapsulate cells, and significant adaptability for in situ polymerization by adapting light sources to fit biomedical applications [81]. Photopolymerizations have been used in a broad range of biological applications, such as dentistry [8], optical materials [72], encapsulating pancreatic islet cells [42], blood vessel adhesives [46], controlled release applications [13], drug delivery [29], and bone restorations [22]. For instance, a novel photopolymerized dendrimer hydrogel was reported for delivery of two antiglaucoma drugs, brimonidine and timolol maleate [29]. This dendrimer hydrogel was mucoadhesive to mucin par-

ticles and nontoxic to human corneal epithelial cells. Subsequently, this dendrimer hydrogel could increase the solubility of brimonidine and sustain the in vitro release of both drugs over 56-72 hours. This dendrimer hydrogel was also shown a significant increase of bovine corneal transport and human corneal epithelial cell uptake for both drugs compared to PBS-based eye drop formulations [29].

The mechanism for photopolymerization is to dissociate initiator molecules into radicals by exposure to specific wavelengths of light [9]. During irradiation, a photon from a light source excites and dissociates the photoinitiator molecule into a high-energy radical that can induce the polymerization of a macromer solution [81]. To date, a number of photoinitiators have been developed with their unique absorption spectrum. For instance, dimethoxyphenyl acetophone (DMPA) has a peak maximum wavelength of 310 nm [44], eosin Y has a peak maximum wavelength of 510 nm [6], 1-Hydroxy-cyclohexyl-phenyl-ketone (Irgacure 184) has a peak maximum wavelength of 244 [48], 2-Methyl-1-[4-(methylthio)phenyl]-2-(4-morpholinyl)-1-propanone (Irgacure 907) has a peak maximum peak wavelength of 309 nm [48], and 2-Hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone Irgacure 2959 has a peak maximum wavelength of 276 nm [48]. Despite their unique absorption spectrums, these photoinitiators could be dissociated into free redicals at the wavelength of 365 nm and convert liquid monomers or macromers into hydrogels [9, 29].

The major limitation of photopolymerization techniques for tissue engineered scaffolds or hydrogels is that the high-energy free radicals can create the potential for oxidative damage to the photoencapsulated cell populations [9]. Unlike surgical adhesives and adhesion barriers, many photopolymerization applications involve cell encapsulation that leads direct contact between cells and free radicals. Free radicals can directly react with different cellular macromolecules (cell membranes, proteins and DNA) or indirectly induce formation of reactive oxygen species (ROS), thereby causing unwanted cellular damage [72]. Although the use of antioxidant reagents such as uric acid and melatonin could help prevent oxidative damage and quench intracellular ROS, exposure to ultraviolet A radiation can still induce ROS production, potentially leading to malignant transformation of photo-exposed cells [72]. Such

free radical-induced oxidative stress or UV-induced lesions may cause mutagenicity, but relatively few studies have investigated intracellular signaling transduction on photo-exposed cells.

Cells utilize numbers of signaling pathways to regulate their biological activities and transmit information from environment. AKT, also known as protein kinase B, is a serine/threonine-specific protein kinase that regulates cell proliferation, survival and oncogenesis [4]. It has been well documented that AKT plays a central role in promoting downstream of activated growth factor receptor signaling [47]. In cell survival signaling pathways, AKT enhances cell survival by blocking the function and processes of proapoptotic proteins. AKT suppresses the function and expression of several B-cell lymphoma 2 (Bcl-2) homology domains-only proteins, which binds and inactivates prosurvival Bcl-2 family members [47, 23]. AKT activates nuclear factor kappa-light-chain-enhancer of activated B cells (NFB) survival signaling or inhibits c-Jun N-terminal kinase/mitogen-activated protein kinase (JNK/p38) apoptotic signaling under certain circumstances [47, 37]. In cell growth signaling pathways, AKT promotes cell growth by activating mammalian target of rapamycin complex 1 (mTORC1) that is a critical regulator of translation initiation and ribosome biogenesis [47, 85]. In cell proliferation signaling pathways, AKT stimulates cell proliferation through multiple downstream targets regulating cell cycling such as glycogen synthase kinase 3 (GSK3), tuberous sclerosis 2 (TSC2) and proline-rich AKT substrate of 40 kDa (PRAS40) [47]. GSK3 plays central role in the G1 to S phase cell cycling transition [90]; while TSC2 and PRAS40 promotes cell growth by activating mTORC1 [66]. In angiogenesis signaling pathways, vascular endothelial growth factor (VEGF) robustly activates AKT in endothelial cells and subsequently promotes endothelial cell survival, growth, proliferation and migration [47, 56]. Understanding the fundamentals above, it is of interest to determine intracellular AKT signaling transduction in photo-exposed cells during photopolymerization process.

In this chapter, the intracellular AKT signaling and the cell viability were evaluated in response to the photoinitiators. HN4 cells were treated with DMPA, eosin Y and Irgacure 2959 in a broad range of concentrations with and without UV radiation.

The intracellular AKT activity were determined by Western blot analysis, and the cell viability was measured using WST-1 assay. The stability of free radicals induced by UV irradiation was also evaluated in HN4 cells in respect to the intracellular AKT activity and the cell viability.

## 2.2 Materials and Methods

### 2.2.1 Materials

1-vinyl-2 pyrrolidinone (NVP), dimethoxyphenyl acetophone (DMPA), dimethyl sulfoxide (DMSO), eosin Y, ethanol, phosphate buffer solution (PBS), and triethanolamine (TEOA) were purchased from Sigma-Aldrich (St. Louis, MO). Irgacure 2959 was provided by Ciba Corporation (Newport, DE). Cell proliferation reagent WST-1 was purchased from Roche Applied Science (Indianapolis, IN). Phospho-Akt (Ser473) (p-AKT) antibody was purchased from Cell Signaling Technology (Danvers, MA). AKT1 (559028) antibody was purchased from BD Biosciences Pharmingen (Mississauga, ON, Canada). $\beta$-actin (ACTBD11B7) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit antibody conjugated to horseradish peroxidase and goat anti-mouse antibody conjugated to horseradish peroxidase were purchased from Bio-Rad (Hercules, CA).

### 2.2.2 Photoinitiator preparation

Three ultraviolet photoinitiating systems were examined in this work. The photoinitiator solutions were prepared based on weight per volume calculations per industry standard. Briefly, 0, 25, 50, 100 and 250 mg of DMPA was dissolved in 1 ml of ethanol, resulting 0, 25, 50, 100 and 250 mg/ml of DMPA stock solutions. 0, 50, 100, 250 and 500 mg of DMPA was dissolved in 1 ml of ethanol, resulting 0, 50, 100, 250 and 500 mg/ml of Irgacure 2959 stock solutions. Eosin Y photoinitiator stock solution was prepared by 0.1% eosin Y, 4% NVP, and 40% TEOA in PBS according to the previous reports [29, 24]. All resulting photoinitiator solutions were protected

from light and stored at room temperature until use.

### 2.2.3 Cell culture

HN4 cells were derived from a primary SCC of the head and neck. The cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with high glucose, L-glutamine, 10% (v/v) fetal bovine serum (Fisher Scientific, Pittsburgh, PA), 100 units/ml of penicillin and 100 $\mu$g/ml of streptomycin (Thermo Fisher Scientific, Ashville, NC) at 37°C in a humid environment with 5% $CO_2$ before any biocompatibility study was performed.

### 2.2.4 Comparison of photoinitiators

40 $\mu$l of each DMPA or Irgacure 2959 stock solution was added to 2 ml of fresh culture media to reach a final concentration of 0, 0.5, 1, 2 and 5 mg/ml of DMPA and 0, 1, 2, 5 and 10 mg/ml of Irgacure 2959. 0, 5, 10, 20 and 50 $\mu$l of eosin Y stock solution was added to 2 ml of fresh culture media to reach a final concentration of 0, 2.5, 5, 10 and 25 $\mu$l/ml of eosin Y. For cell viability analysis, Pre-seeded HN4 cells on a 96-well plate were treated with above condition media for 1-4 days. The spent media were replaced by the same fresh condition media every day during the treatments in order to maintain a constant concentration of each photoinitiator. For protein expression analysis, Pre-seeded HN4 cells on a 6-well plate were treated with above condition media for 30 min, and total protein lysates were harvested by total cell lysate buffer with protease and phosphatase inhibitors (Roche Applied Science, Indianapolis, IN).

### 2.2.5 Comparison of photoinitiators after UV irradiation

The fresh cell culture media were mixed with different concentrations of each photoinitiator stock solution to reach a final concentration at 0, 0.5, 1 and 2 mg/ml of DMPA and 1, 2 and 5 mg/ml of Irgacure 2959 as well as 2.5, 5, 10 $\mu$l/ml of eosin Y. These mixtures were equally exposed to UV radiation at 365 nm with an intensity of 100 watts for 30 min in order to decompose the photoinitiator into free radicals. Pre-

seeded HN4 cells were then subjected to the media right after UV irradiation. After 1 and 2 days treatments, the cellular proliferation rate was determined by WST-1 assay. After 30 min treatment, total cellular p-AKT and AKT1 expression was evaluated by Western blot analysis.

### 2.2.6 Cytotoxicity and cellular response of free radicals

The fresh cell culture media were mixed with the eosin Y stock solution to reach a final concentration at 20 $\mu$l/ml of and then exposed to UV radiation at 365 nm with an intensity of 100 watts for 30 min to decompose into free radicals. Pre-seeded HN4 cells the conditioned media in 0, 6, 24, 48 hours after UV irradiation. After 2 days treatments, the cellular proliferation rate was determined by WST-1 assay. After 30 min treatment, total cellular p-AKT and AKT1 expression was evaluated by Western blot analysis.

### 2.2.7 Cellular proliferation rate

HN4 cells were seeded on a 96-well plate with a density of 10,000 cells/well and cultured for 2 days to allow the cells adhesion. At the end of each treatment, the cellular proliferation rate of surviving cells was determined by addition of 10% (v/v) of WST-1 reagent according to the manufacturers protocol. After 30 min incubation, the absorbance of the samples was measured at 450 nm, against a background control as a blank, and this value was then subtracted by the absorbance at 650 nm as a reference wavelength.

### 2.2.8 Western blot analysis

Western blot analysis of total cellular protein was carried out by standard procedures, as described previously [89, 88]. Briefly, total cell lysates (30 $\mu$g) were separated on a 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride (PVDF; Bio-Rad, Hercules, CA) membrane using Bio-Rad Mini-Blot transfer apparatus. The membrane was blocked for 2 hours in Tris-buffered solution (TBS) containing 5%

of non-fat dried milk. The specific proteins on the membrane were determined by incubating with the primary antibodies overnight at 4 °C with shaking. The membrane was washed three times and then incubated in a 1:3,000 dilution of a secondary antibody at room temperature for 1 hour in the washing buffer (Tris-buffered solution containing 0.5% Tween 20). The specific antigen-antibody interactions were detected using enhanced chemiluminescence (Pierce ECL Western Blotting Substrate; Thermo Scientific, Rockford, IL). The total cellular expression of $\beta$-actin was used as the loading control.

### 2.2.9 Statistical analysis

All the data were expressed as means ± standard deviation (SD) and subjected to one way analysis of variance (ANOVA) followed by Holm-Sidak method for significance using SigmaPlot 12. A value of $p < 0.05$ was considered as statistically significant. Statistical analysis data is provided in Appendix A (p85-119).

## 2.3 Results and discussion

### 2.3.1 The effect of photoinitiators on HN4 cell viability

The effects of photoinitiators including eosin Y, Irgacure 2959 and DMPA on the viability of HN4 cells were first evaluated. As shown in Figures 2.1, 2.2 and 2.3, eosin Y exhibited less cytotoxicity than Irgacure 2959 and DMPA, as the evidence showed that HN4 cells were tolerant to a wider concentration range of eosin Y than Irgacure 2959 and DMPA. Up to 4-day treatment of 2.5 $\mu$l/ml of eosin Y or 1 mg/ml Irgacure 2959, HN4 cells were able to maintain nearly 100% cell viability (Figures 2.1 and 2.2). Eosin Y reduced HN4 cell viability in a concentration-dependent manner at each time period treatment; whereas nearly no viable HN4 cell was observed after 1-day treatment of Irgacure 2959 at the concentration greater than 2 mg/ml. In contrast,

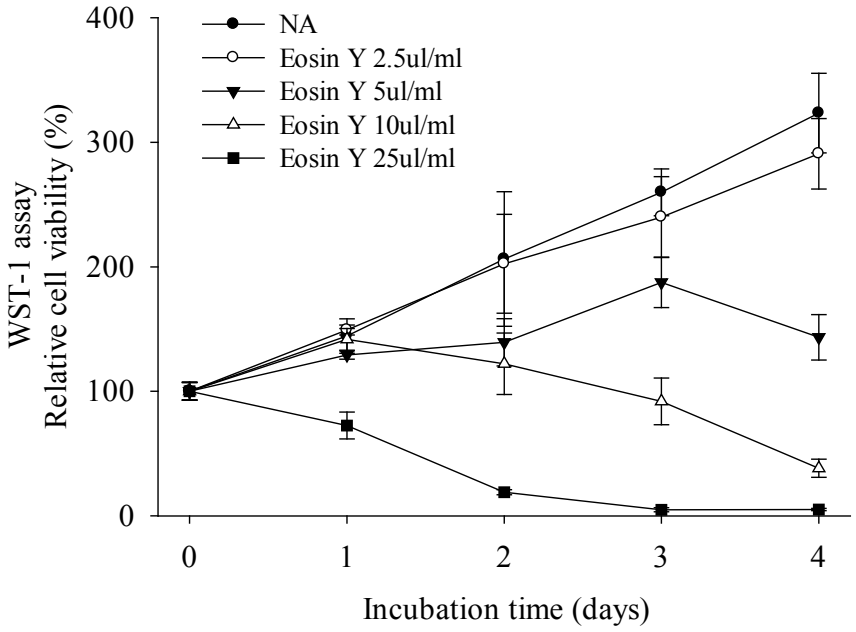


**Figure 2.1: The effect of eosin Y on HN4 cell viability.**
HN4 cells were plated at a density of $1\times10^4$ cells/well into 96-well plates and treated with different concentrations of eosin Y as indicated for 0, 1, 2, 3 and 4 days. The cell viabilities were measured by WST-1 assay at the end of the treatment. The bars are mean ± standard deviation.

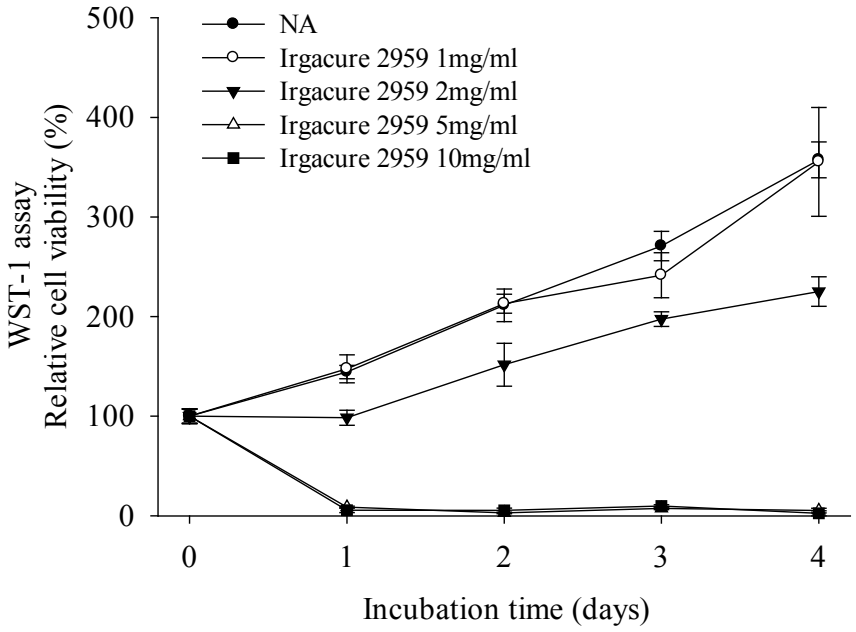


**Figure 2.2: The effect of Irgacure 2959 on HN4 cell viability.**
HN4 cells were plated at a density of $1\times10^4$ cells/well into 96-well plates and treated with different concentrations of Irgacure 2959 as indicated for 0, 1, 2, 3 and 4 days. The cell viabilities were measured by WST-1 assay at the end of the treatment. The bars are mean ± standard deviation.

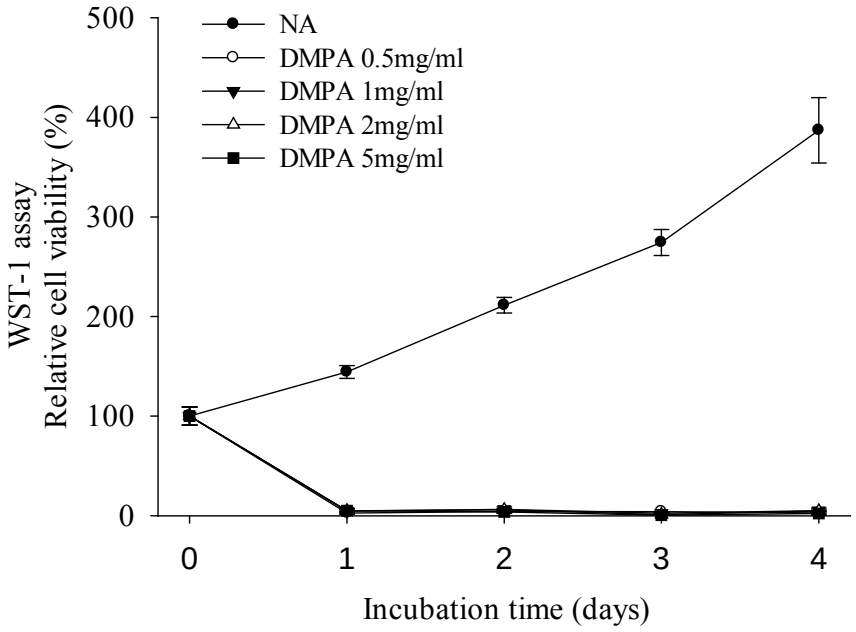


**Figure 2.3: The effect of DMPA on HN4 cell viability.**
HN4 cells were plated at a density of $1\times10^4$ cells/well into 96-well plates and treated with different concentrations of DMPA as indicated for 0, 1, 2, 3 and 4 days. The cell viabilities were measured by WST-1 assay at the end of the treatment. The bars are mean ± standard deviation.

DMPA exhibited a significant cytotoxic effect to cells, as the evidence showed that no viable HN4 cell was observed at each concentration of DMPA in each time period treatment (Figure 2.3).

The cytotoxicity profile of Irgacure 2959 was consistent with the previous report, which showed there was nearly no cytotoxicity of Irgacure 2959 up to 1 mg/ml in both bovine chondrocytes and human fetal osteoblasts [9, 81]. Up to date, there is very few reports investigating a wide concentration range of photoinitators on the cell viability. This is the first time to show that an approximate maximum concentration of eosin Y and Irgacure 2959 can be used in cell culture with a desirable cytotoxicity. However, in vivo circumstances are different as in vitro 2-dimensional cell culture. The use of photoinitiators in in vivo biomedical application needs to be further evaluated based on these in vitro cell culture results.

### 2.3.2 The effects of photoinitiators on intracellular AKT signaling transduction in HN4 cells

AKT is one of the key regulators in cell proliferation and survival that transmits information from environment and regulates cell viability. To further evaluate the effects of photoinitiators on cellular response, the intracellular signaling transduction was determined in response to eosin Y, Irgacure 2959 and DMPA. HN4 cells were used as our model cell line to determine the intracellular AKT activity in acute response to the three photoinitiators because HN4 cells keep a constitutive high AKT activation [79]. After 30 min treatment, eosin Y had no significant effect on the phosphorylation of AKT up to 25 $\mu$l/ml in HN4 cells as shown in Figure 2.4. In contrast, both Irgacure 2959 and DMPA diminished the phosphorylation of AKT in a concentration-dependent manner as shown in Figure 2.5 and Figure 2.6, respectively.

These results suggest that the inhibition of AKT activation by Irgacure 2959 and DMPA could lead to several downstream effects, including reducing cell viability and motility, decreasing angiogenesis, and inducing apoptosis, which are consistent

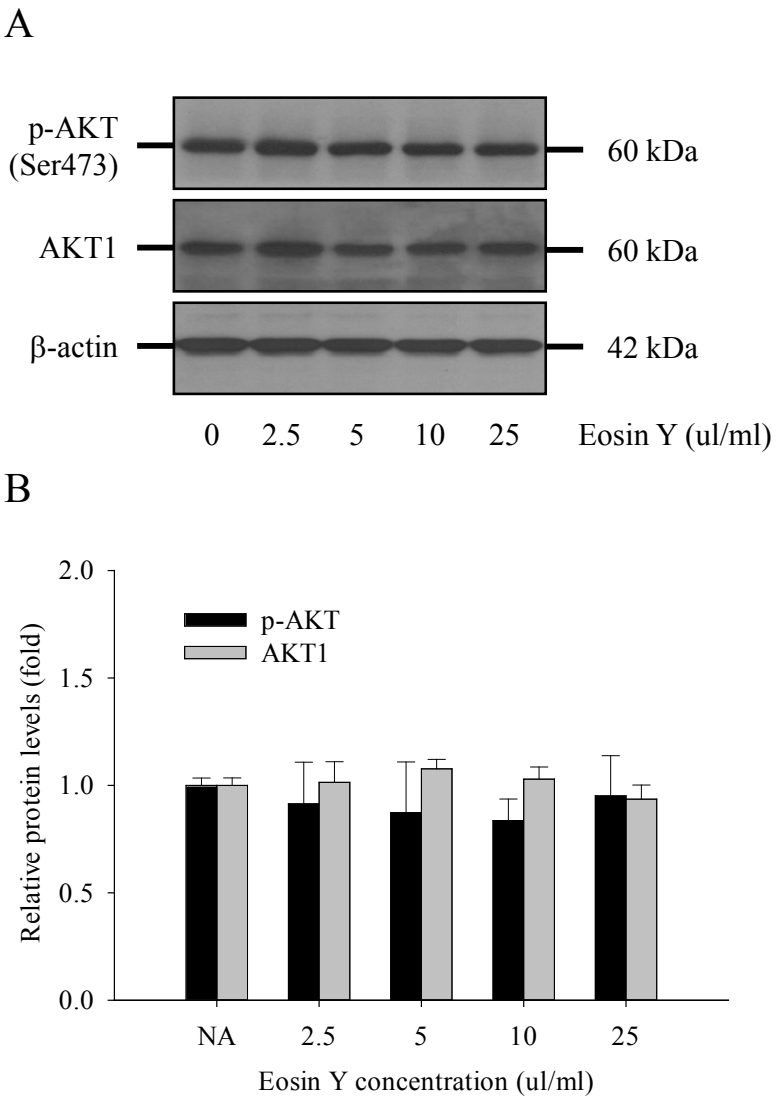


**Figure 2.4: The effect of eosin Y on intracellular AKT activity in HN4 cells.**

HN4 cells were HN4 cells were plated at a density of $5\times10^4$ cells/well into 6-well plates and treated with different concentrations of eosin Y as indicated for 30 min. At the end of treatment, the total cell lysates were harvested and the intracellular phospho-AKT (p-AKT) and total AKT levels were analyzed by Western blot analysis (A). Each positive band was normalized to $\beta$-actin and was quantified by NIH ImageJ (B). The data represent typical one of three experiments. Each value represents mean $\pm$ standard deviation.

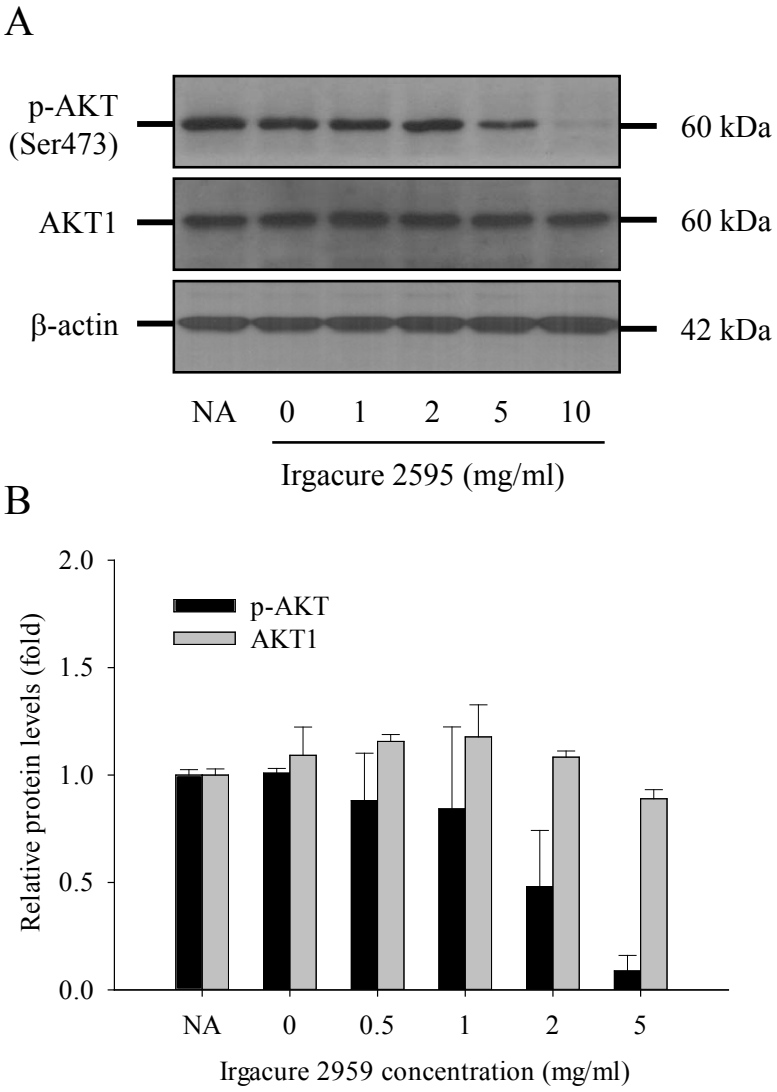


**Figure 2.5: The effect of Irgacure 2959 on intracellular AKT activity in HN4 cells.**

HN4 cells were HN4 cells were plated at a density of $5\times10^4$ cells/well into 6-well plates and treated with different concentrations of eosin Y as indicated for 30 min. At the end of treatment, the total cell lysates were harvested and the intracellular phospho-AKT (p-AKT) and total AKT levels were analyzed by Western blot analysis (A). Each positive band was normalized to $\beta$-actin and was quantified by NIH ImageJ (B). The data represent typical one of three experiments. Each value represents mean $\pm$ standard deviation.

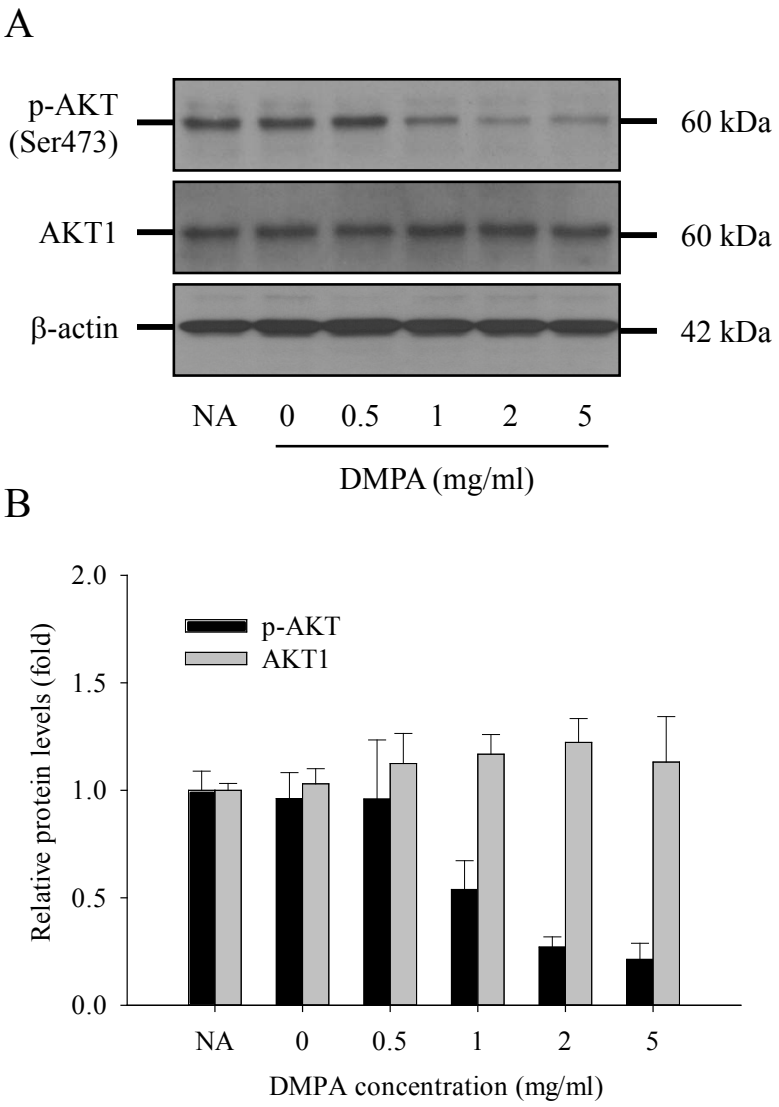


**Figure 2.6: The effect of DMPA on intracellular AKT activity in HN4 cells.**

HN4 cells were HN4 cells were plated at a density of $5\times10^4$ cells/well into 6-well plates and treated with different concentrations of eosin Y as indicated for 30 min. At the end of treatment, the total cell lysates were harvested and the intracellular phospho-AKT (p-AKT) and total AKT levels were analyzed by Western blot analysis (A). Each positive band was normalized to $\beta$-actin and was quantified by NIH ImageJ (B). The data represent typical one of three experiments. Each value represents mean $\pm$ standard deviation.

with the cell viabilities shown in Figures 2.1, 2.2 and 2.3. Cell invasion, migration and proliferation as well as angiogenesis are crucial for the success of photocrosslinking polymer scaffold in biomedical applications, especially tissue engineering. This is the first time to show the interaction of photoinitiators on the intracellular signaling pathway. The results suggest that eosin Y could be a much safer photoinitiator for photopolymerization in biomedical applications than Irgacure 2959 and DMPA.

### 2.3.3 The effect of UV-exposed photoinitiators on viability and intracellular AKT signaling transduction in HN4 cells

Besides the precursor of photoinitiators, the free radicals generated during the photocrosslinking process could be cytotoxic as well. As shown in Figure 2.7, eosin Y, Irgacure 2959 and DMPA can be decomposed into high-energy radicals. The cell viability and intracellular AKT activity were evaluated in HN4 cells in response to these free radicals. Eosin Y, Irgacure 2959 and DMPA were mixed with cell culture media first and subjected to UV irradiation for 30 min to allow free radical generation. HN4 cells were then immediately treated with these photoinitiator solutions for 1 and 2 days. HN4 cells failed to maintain 100% viability during the 2 days incubation with 2.5 $\mu$l/ml of eosin Y and 0.5 mg/ml of Irgacure 2959 after UV irradiation (Figures 2.8 and 2.9), comparing to the non-UV irradiation treatment (Figures 2.1 and 2.2). After UV irradiation, 2.5 $\mu$l/ml of eosin Y and 0.5 mg/ml of Irgacure 2959 reduced cell viability about 30% and 50%, respectively; while 10 $\mu$l/ml of eosin Y and 2 mg/ml of Irgacure 2959 reduced cell viability about 70% and 95%, respectively. DMPA was consistently cytotoxic to HN4 cells as its precursor (Figures 2.3 and 2.10), and both precursor and free radicals could cause a significant cytotoxicity effect. These results suggest that eosin Y is more biocompatible than Irgacure 2959 and DMPA after UV irradiation, which is consistent with Figures 2.1, 2.2 and 2.3. It was noticed that the free radicals generated from UV irradiation were more cytotoxic to cells than their precursor. These results were further confirmed by intracellular AKT signaling

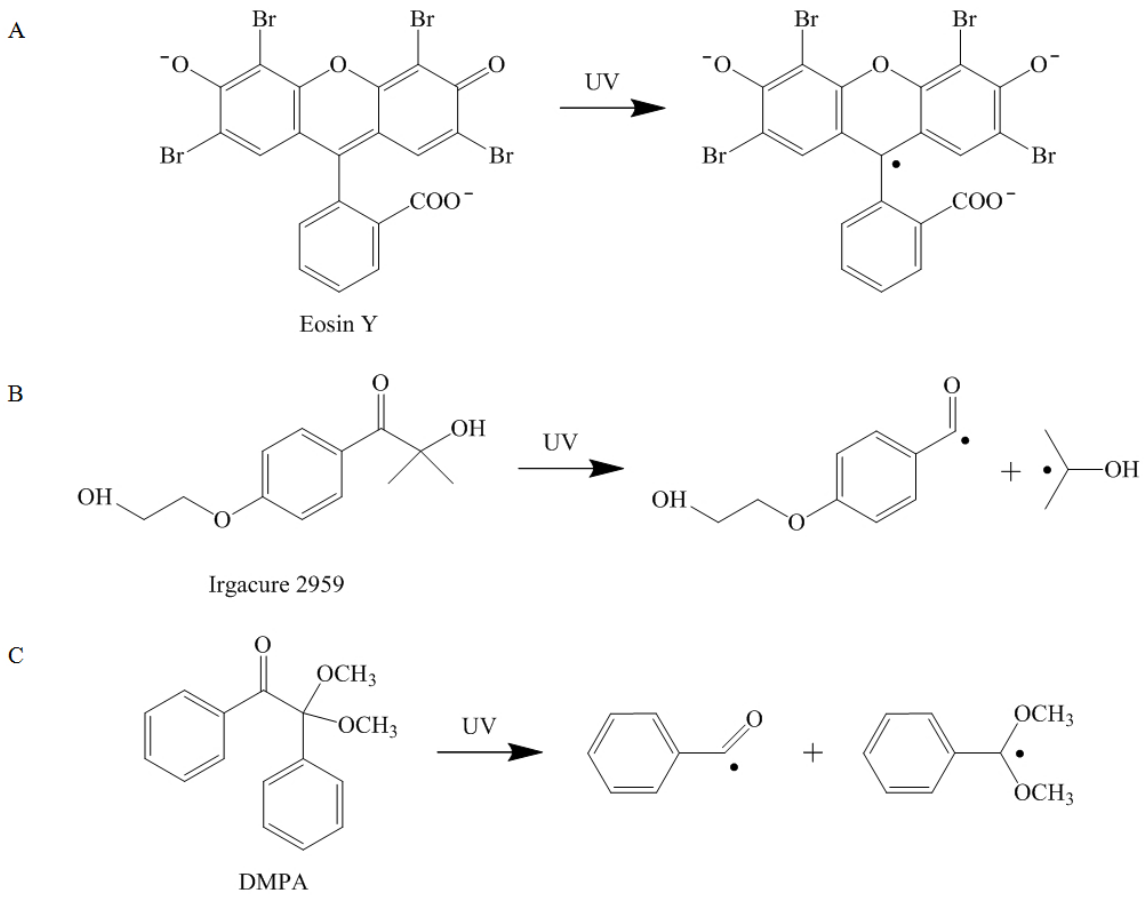


**Figure 2.7: A schematic diagram of photoinitiator decomposition to free radicals [1, 52, 59].**

A, eosin Y; B, Irgacure 2959; and C, DMPA.

transduction in HN4 cells in response to these free radicals. All three UV exposed photoinitiators diminished the phosphorylation of AKT in HN4 cells as shown in Figure 2.11.

These results only represent the worst case scenario, in which all the free radicals generated from photopolymerization process have the highest likelihood to interact with cells. However, these results indicate the way in which cells response to free radicals at the early stage. The free radicals induce fast AKT dephosphorylation, which in turn alters numerous intracellular signaling transductions, such as inhibiting

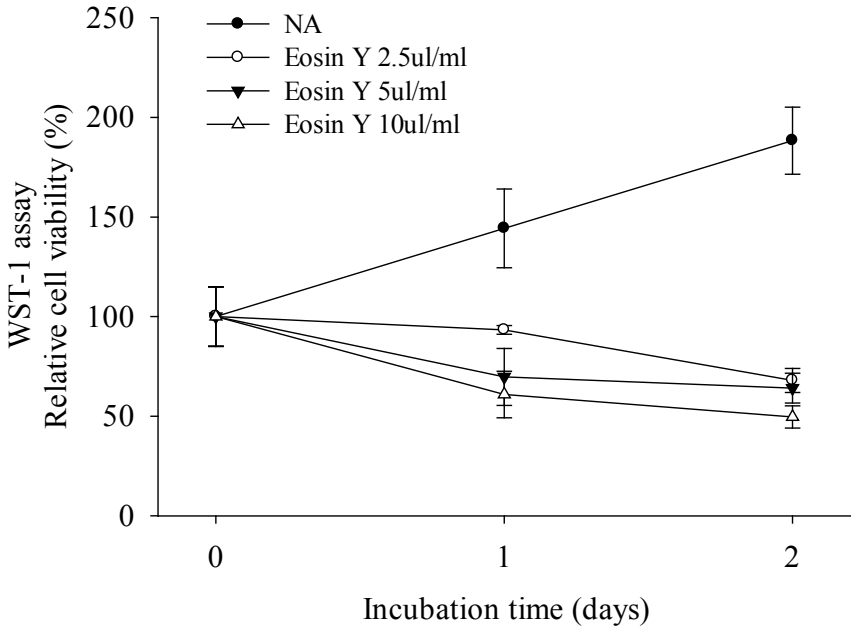


**Figure 2.8: The effect of UV-exposed eosin Y on HN4 cell viability.** The cell culture media were first mixed with different concentrations of eosin Y, and then the mixtures were exposed to the longwave UV at 365 nm with an intensity of 100 watts for 30 min. After the UV irradiation, the mixtures were immediately to treat HN4 cells for 0, 1 and 2 days. The cell viabilities were measured by WST-1 assay at the end of the treatment. The bars are mean ± standard deviation.

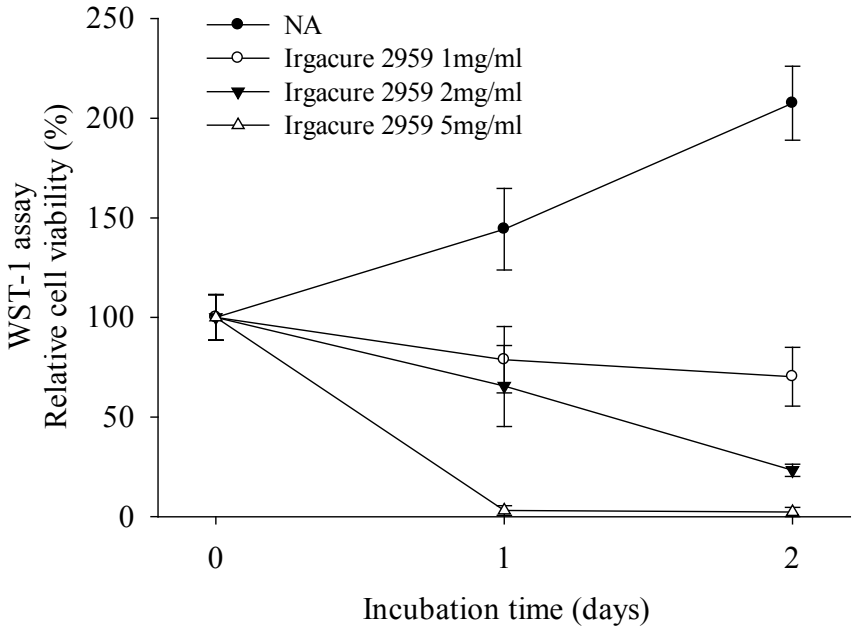


**Figure 2.9: The effect of UV-exposed Irgacure 2959 on HN4 cell viability.** The cell culture media were first mixed with different concentrations of Irgacure 2959, and then the mixtures were exposed to the longwave UV at 365 nm with an intensity of 100 watts for 30 min. After the UV irradiation, the mixtures were immediately to treat HN4 cells for 0, 1 and 2 days. The cell viabilities were measured by WST-1 assay at the end of the treatment. The bars are mean ± standard deviation.

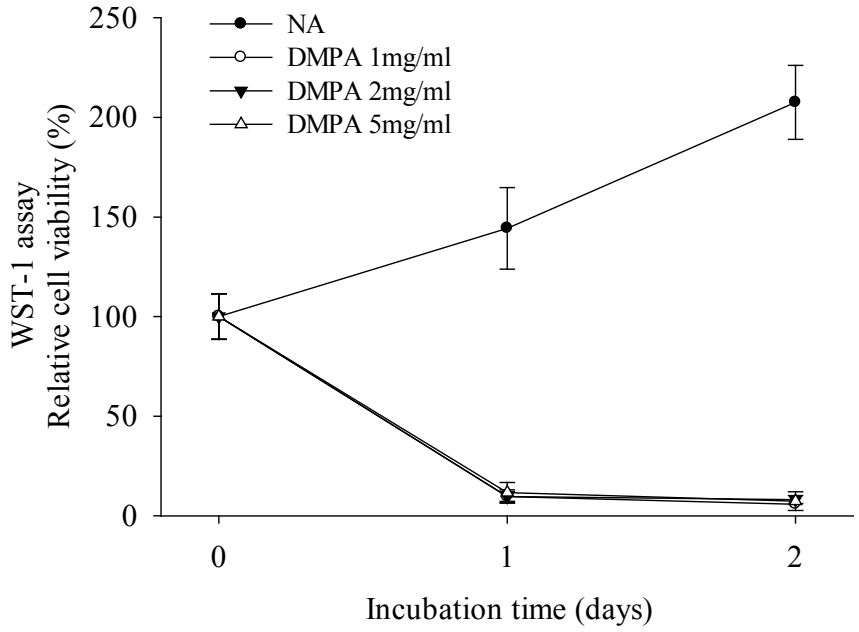


**Figure 2.10: The effect of UV-exposed DMPA on HN4 cell viability.** The cell culture media were first mixed with different concentrations of DMPA, and then the mixtures were exposed to the longwave UV at 365 nm with an intensity of 100 watts for 30 min. After the UV irradiation, the mixtures were immediately to treat HN4 cells for 0, 1 and 2 days. The cell viabilities were measured by WST-1 assay at the end of the treatment. The bars are mean ± standard deviation.

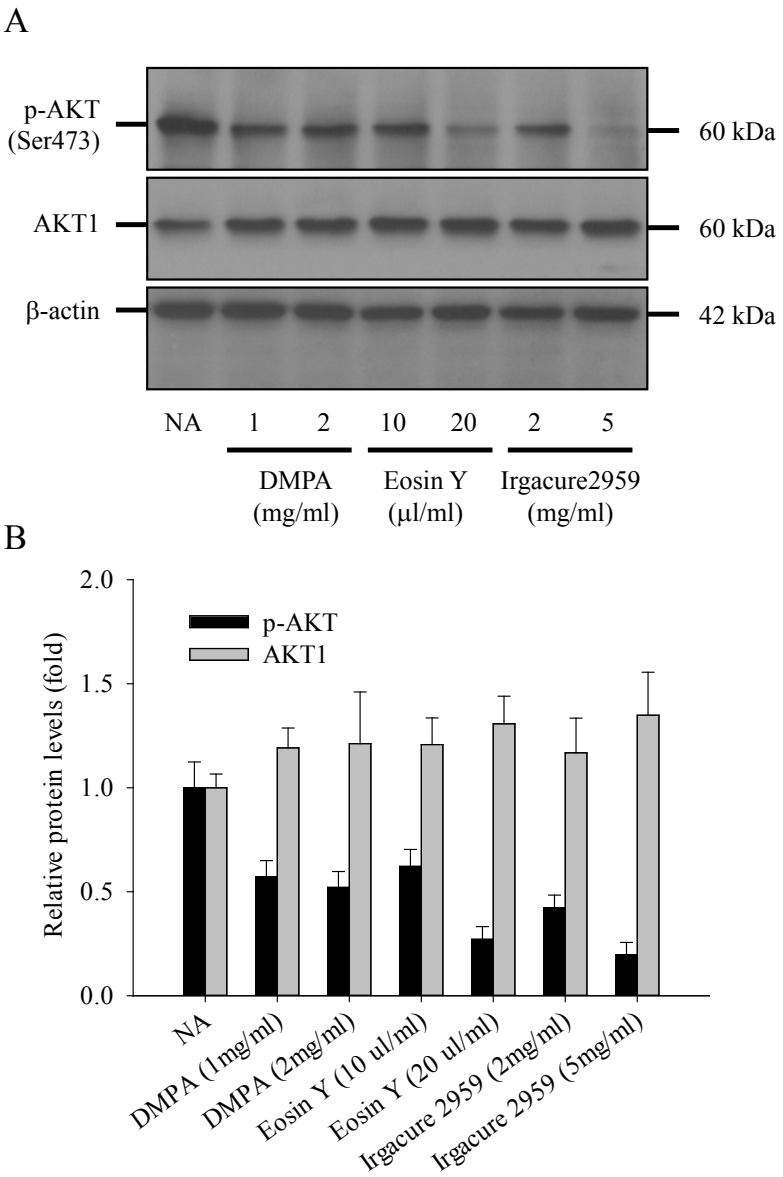


**Figure 2.11: The effect of UV-exposed photoinitiators on intracellular AKT activity in HN4 cells.**

The cell culture media were first mixed with different concentrations of eosin Y, Irgacure 2959 or DMPA, and then the mixtures were exposed to the longwave UV at 365 nm with an intensity of 100 watts for 30 min. After the UV irradiation, the mixtures were immediately to treat HN4 cells for 30 min and the total cell lysates were harvested. The intracellular phospho-AKT (p-AKT) and total AKT levels were analyzed by Western blot analysis (A). Each positive band was normalized to $\beta$-actin and was quantified by NIH ImageJ (B). The data represent typical one of three experiments. Each value represents mean $\pm$ standard deviation.

mTOR signaling pathway in cell proliferation and p53 signaling pathway in cell survival, activating ROS signaling pathway in ER stress and caspase 9 signaling pathway in cell apoptosis [64]. For the potential use of photoinitiators to crosslink polymers in biomedical applications, it becomes crucial to optimize the concentration of photoinitiators and keep the minimal free radical residues after photopolymerization process.

### 2.3.4 The evaluation of free radicals stability on viability and intracellular AKT signaling transduction in HN4 cells

To further evaluate the stability of free radicals, the cell viability and intracellular AKT activity were determined in HN4 cells in response to the quenched eosin Y condition media. As shown in Figure 2.12, the quenched UV-exposed eosin Y exhibited more cytotoxicity than unquenched UV-exposed eosin Y, indicating the free radicals generated from UV irradiation of eosin Y were not diminished up to 48 hours of quencher. The reason is yet unclear, but it could be due to the interaction between free radicals and cell culture media. Such reaction then affects growth factors in the culture media which in turn inhibits cell signaling transductions. The evidence showed that the phosphorylation of AKT was significantly diminished in the HN4 cells treated with the quenched eosin Y condition media (Figure 2.13). These results suggest that free radicals may not only interact with cells but extracellular components as well. The actual cell survival in the 3-dimensional polymer scaffold might be higher than this simplistic 2-dimensional cell culture model in in vivo biomedical applications [81]. It depends on the ability of polymer scaffold to scavenge the free radicals generated from photopolymerization process.

Up to date, a few of papers have been published to demonstrate the biocompatibility of photoinitiators in different cell lines and conclude that Irgacure 2959 is the promising photoinitiator for cytocompatibility among Irgacure 184, Irgacure 907, Irgacure 651 and Irgacure 2959 [9, 81]. The biocompatible concentration range of Irgacure 2959 is within 0.5% (w/w) as reported [9, 81]. This conclusion is consistent

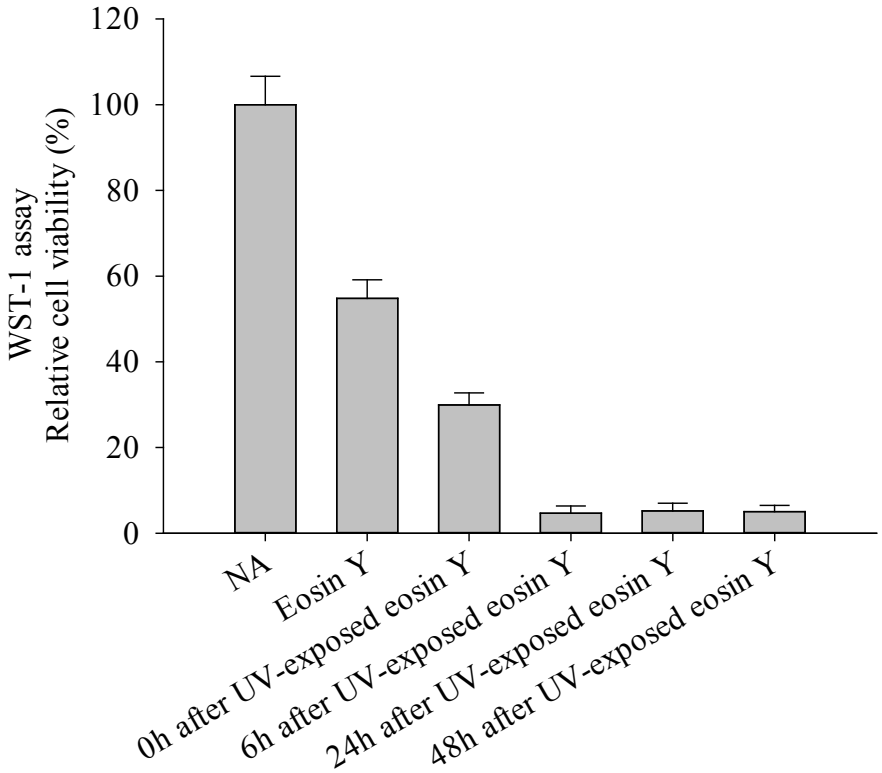


**Figure 2.12: The effect of the free radicals stability on HN4 cell viability.** The cell culture media were first mixed with eosin Y to reach a final concentration of 20 $\mu$l/ml, and the mixtures were exposed to the longwave UV at 365 nm with an intensity of 100 watts for 30 min. After the UV irradiation, the mixtures were stored in dark for 0, 6, 24 and 48 hours before the cell treatment. HN4 cells were then treated with no addition (NA), 20 $\mu$l/ml of eosin Y without UV irradiation or the UV exposed mixtures for 2 days. The cell viabilities were measured by WST-1 assay at the end of the treatment. The bars are mean $\pm$ standard deviation.

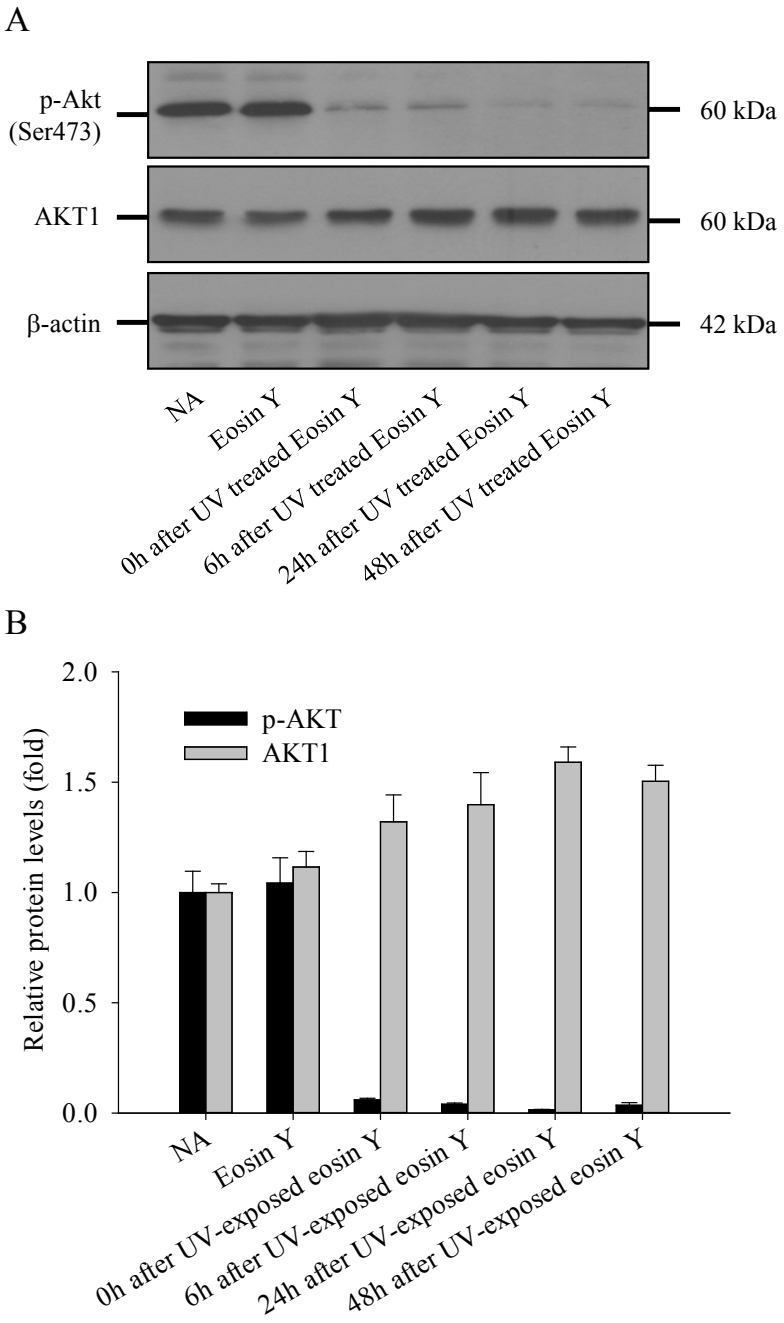


**Figure 2.13: The effect of the free radicals stability on intracellular AKT activity in HN4 cells.**

The preparation of the condition media was described in Figure 2.12. HN4 cells were then treated with no addition (NA), 20 $\mu$l/ml of eosin Y without UV irradiation or the UV exposed mixtures for 30 min and the total cell lysates were harvested. The intracellular phospho-AKT (p-AKT) and total AKT levels were analyzed by Western blot analysis (A). Each positive band was normalized to $\beta$-actin and was quantified by NIH ImageJ (B). The data represent typical one of three experiments. Each value represents mean $\pm$ standard deviation.

with our observations (Figures 2.2 and 2.5). However, the biocompatibility also depends on the materials of polymer and UV intensity during the photopolymerization [9]. Our finding agrees well with photoinitiator system of eosin Y with TEOA has little effect on cell viability [25]. In this work, it is the first time to illustrate the immediate response of cells regarding to the precursor and free radicals of photoinitiators. It was observed that free radicals significantly inhibited intracellular AKT signaling transduction (Figures 2.11 and 2.13), which was closely correlated to cytocompatibility in HN4 cells. Inhibition of AKT signaling transduction can further induce ROS formation and lead oxidative stress [72]. In order to diminish oxidative stress within the cells, antioxidants are commonly introduced to quench the activation of ROS signaling during the photopolymerization process. Nonetheless, it is of interest to test the intracellular AKT signaling transduction in the cells encapsulated within the photoinitiated crosslinking polymers along with the antioxidants. It is of importance to keep cells not only viable but migrating, proliferating and functioning in the photoinitiated crosslinking polymers as a function unit.

## 2.4 Conclusions

In this chapter, the effects of three photoinitiators and their free radicals on cytocompatibility and intracellular AKT signaling transduction were determined in HN4 cells. It was shown that the cytocompatibility of the photoinitiators was closely correlated to intracellular AKT signaling transduction within the cells. The most promising cytocompatible photoinitiator in this work was eosin Y at concentrations below 10 $\mu$l/ml. Eosin Y had minimal effect on intracellular AKT signaling transduction up to 25 $\mu$l/ml. Irgacure 2959 was also a promising cytocompatible photoinitiator only at concentration below 1 mg/ml. It was also shown that free radicals had significant effects on HN4 cells, including reducing cell viability and inhibiting intracellular AKT signaling transduction. These free radicals may interact with cell directly or indirectly via interaction with cell culture media. These findings could further enhance our understanding of the biocompatibility in the photoinitiator systems and provide

a novel way to look into the interaction between cells and photoinitiators or their free radicals.

# Chapter 3

# Fabrication of semi-interpenetrating network scaffolds for transbuccal mucosal delivery of insulin

## 3.1 Introduction

Insulin has been delivered to diabetic patients exclusively through the subcutaneous route since it was discovered. Due to the relatively short duration of action of 4 to 8 hours, patients in severe diabetic condition are required to take two to four daily injections. The stress and noncompliance of multiple daily injections attract numerous investigations to develop a safe and effective noninvasive route for insulin delivery. Oral administration of insulin is the first viable alternative to multiple daily injections. Oral administration of insulin can rapidly elevate insulin level in the portal blood, which simulates the physiological secretion pattern of the pancreas [71]. However, insulin has to travel through stomach, small intestine and the colon intact before reaching the blood stream. Subsequently, insulin has to be protected from the gastric enzymes, such as pepsin, trypsin and chymotrypsin in the duodenum as well as

exopeptidases when it reaches the brush border. Nanoparticle-based insulin delivery systems were reported to be able to overcome these barriers [71], and these systems have been received considerable attention. To date, there are three mechanisms for intestinal uptake of insulin encapsulated nanoparticles: (1) uptake by paracellular pathway, (2) transcytosis or receptor-mediated transcytosis through the epithelial cells of the intestinal mucosa, and (3) lymphatic uptake through the M cells of the Peyers patches mostly abundant in the ileum [71].

Therefore, the properties of a successful drug carrier for insulin have been summarized as follows: (1) resistant against gastrointestinal enzymes and pH gradients, (2) stable and biocompatible to ensure that the main fraction of insulin remains biologically active from encapsulation to release, (3) highly permeable along with the intestinal membrane, (4) absorbable through the epithelial cell layer or the mucosal epithelium to enhance the absorption of drug, (6) fast release in the circulation to control the glucose concentration in blood, and (7) safe after oral administration [71].

Due to the large size, hydrophilicity, susceptibility to enzymatic degradation and poor intestinal absorption, oral delivery of free insulin has low bioavailability. A variety of formulations for oral insulin delivery have been developed in the last decade. Most strategies have shown promising results, such as pH sensitivity, enzymatic inhibition among others, but the bioavailability is still low as shown in Table 1.1.

Transbuccal mucosal administration could be a secondary alternative route to deliver insulin. It has been reported that co-administration of soybean-lecithin could enhance transbuccal mucosal delivery of insulin in rabbits and rats [77, 87]. In addition, poly(methacrylic acid)-grafted-poly(ethylene glycol) (P(MAA-g-EG)) hydrogel holds a great potential for transmucosal and intracellular delivery of insulin [41]. Both adhesion property and release kinetics can be modulated by tuning the polymer composition [41]. All of these interesting findings inspire our great interest to explore the transbuccal mucosal route for insulin delivery.

The anatomical and physiological properties of the oral mucosa have been generally reviewed as follows. The oral cavity contains lips, cheek, tongue, hard

palate, soft palate and floor of the mouth. The lining of the oral cavity is the oral mucosa that includes the buccal, sublingual, gingival, palatal and labial mucosa [58]. Among these mucosa, the buccal and sublingual mucosa have the largest surface area, which accounts for about 60% of the total oral mucosal surface area. The top layer of the oral mucosa comprises closely compacted epithelial cells, whose function is to protect the underlying tissue against potential harmful agents in the oral environment and from fluid loss [58]. Lamina propia and submucosa are under the epithelium, referred to as the basement membrane.

There are some significant challenges for systemic drug delivery through the oral cavity. (1) The drug has to be released from the carrier to the delivery site, such as buccal or sublingual mucosa, and the drug has to pass through the mucosal layers to enter the systemic circulation. (2) The drug has to remain bioactive in this process, including pH, fluid volume, enzyme activity and the permeability of oral mucosa in the oral cavity [58]. (3) The turnover of the mucosal surface is a determinant of this delivery route. The surface area of oral cavity is estimated at about 100 $cm^2$, with a local pH from 5.8 to 7.6. The relative drug absorption capacity is moderate in oral cavity comparing to stomach, small intestine, large intestine and rectum [58]. (4) The physiological environment of the oral cavity, including pH, fluid volume and composition, is determined by the secretion of saliva. Saliva provides a water rich environment of the oral cavity, which can be favorable for drug release from delivery systems, especially those based on hydrophilic polymers.

Biological polymers, such as collagen, gelatin, hyaluronic acid, and thrombin are widely employed as backbones in the construction of hydrogels or scaffolds for various biomedical applications [73]. Gelatin, a hydrophilic protein fragment derived from collagen, has been extensively investigated in drug delivery system, owing its biodegradability, its low level of immunogenicity and cytotoxicity, and its FDA approval as a clotting agent and exudate-absorbing construct. Several investigations indicate that gelatin can be chemically crosslinked with polyethylene glycol diacrylate (PEG-DA) to form a semi-interpenetrating network (sIPN) [73, 87, 27, 92]. This crosslinking process is driven by photopolymerization, which can physically enhance

the entanglement of polymer chains in three-dimensional structure. The application of photopolymerization techniques allows the ease of loading a desired drug dosage and control over crosslinking density [73]. It has been reported that (1) the degradation and drug release rate can be modulated by turning the weight percent of PEG-DA during the photopolymerization [92]; (2) increasing the gelatin content can significantly increase Youngs modulus [27]; (3) the gelatin sIPN exhibits stability and adhesive strength to fresh tissue [87, 92, 10]; and (4) the adhesion values of gelatin IPN is up to 5.7 N [92]. Indeed, gelatin sIPN displays moisture absorbance, high tensile strength and elasticity, tissue integration and adhesion, and favorable tissue response, which can be for transbuccal mucosal drug delivery.

Electrospinning is a materials-processing method that is used to fabricate continuous ultrafine fibers with a diameter range from 50 nm to 10 $\mu$m. Electrospinning is reported to be versatile, inexpensive, scalable, and reliable. Electrospun fibers are produced from a solution of natural or synthetic polymers, including collagens, gelatin, elastin, fibrinogen, hemoglobin and myoglobin and silk fibroin, or poly(dioxanone) (PDO), poly(ethylene oxide) (PEO), poly(ethylene terephthalate), poly(glyconate), PLGA, PLLA, PCL, poly(styrene), poly(vinyl alcohol) (PVA), chitosan, and hyaluronic acid (HA) [35, 63]. These electrospun fibers have been widely explored in biomedical applications such as tissue engineering, wound healing, orthopedics, biosensors and drug delivery [35, 32, 43, 82]. Electrospun fiber scaffold exhibits a high surface to volume ratio and direct local adhesion ability and holds a great potential for transbuccal mucosal drug delivery.

An electrospun gelatin fiber scaffold (GF) was developed to locally deliver nystatin for candidiasis [2]. GFs possess high biocompatibility and mucoadhesive property that allow local sustained release of drug at the absorption site [2, 7]. GF can be stabilized using a crosslinker of photocurable PEG-DA and form sIPN. The fiber morphology, mechanical and physical properties as well as drug release kinetics can be controlled by modulating the concentration of crosslinker [2]. However, the major limitation of photopolymerization techniques for these sIPN scaffolds or hydrogels is that the high-energy free radicals can create the potential of oxidative damage to the

photoencapsulated cell populations [9, 81]. The cytocompatibility of photoinitiators was investigated in Chapter 2. The results suggest that eosin Y is more cytocompatible than Irgacure 2959 and DMPA, with minimal effect on intracellular AKT signaling transduction in HN4 cells (Chapter 2).

In this chapter, a sIPN gelatin-co-insulin fiber scaffold (sIPN GIF) was fabricated by applying an optimized formulation of sIPN GF in respect to cytocompatibility and degradation rate. Enzyme-linked immunosorbent assay (ELISA) was used to monitor the insulin release kinetics of sIPN GIF. Western blot analysis and 3T3-L1 preadipocyte differentiation analysis were used to determine the bioactivity of insulin from sIPN GIF. The permeability of the insulin from sIPN GIF was evaluated through a porcine buccal mucosal membrane, which possesses analogous thickness, structure, morphology, and composition to normal human buccal epithelium [91]. Our results suggest that sIPN GIF holds a great potential for transbuccal mucosal delivery of insulin.

## 3.2 Materials and Methods

### 3.2.1 Materials

1-vinyl-2 pyrrolidinone (NVP), eosin Y, ethanol, human insulin, oil red O, phosphate buffer solution (PBS), Polyethylene glycol diacrylate (PEG-DA575, Mn = 575 g/mol), porcine type-A gelatin, and triethanolamine (TEOA) were purchased from Sigma-Aldrich (St. Louis, MO). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFP) was purchased from TCI America (Portland, OR). Cell proliferation reagent WST-1 was purchased from Roche Applied Science (Indianapolis, IN). Insulin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Calbiotech (Spring Valley, CA). Phospho-Akt (Ser473) (p-AKT) antibody was purchased from Cell Signaling Technology (Danvers, MA). AKT1 (559028) antibody was purchased from BD Biosciences Pharmingen (Mississauga, ON, Canada). $\beta$-actin (ACTBD11B7) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit antibody

conjugated to horseradish peroxidase and goat anti-mouse antibody conjugated to horseradish peroxidase were purchased from Bio-Rad (Hercules, CA).

### 3.2.2 Cell culture

3T3-L1 preadipocytes were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with high glucose, L-glutamine, 10% (v/v) fetal bovine serum (FBS) (Fisher Scientific, Pittsburgh, PA), 100 units/ml of penicillin (Thermo Fisher Scientific, Ashville, NC) and 100 $\mu$g/ml of streptomycin (Thermo Fisher Scientific, Ashville, NC) at 37°C in a humid environment with 5% $CO_2$.

### 3.2.3 Scaffold preparation

The preparation of gelatin fiber scaffold required two steps, referred to as electrospinning and cross-linking, as previously reported [2]. Briefly, 1 g of gelatin was first added into 10 ml of HFP solution, and the mixture was shaken continuously overnight to obtain a homogeneous transparent 10% (w/v) gelatin/HFP solution. To prepare gelatin-co-insulin solutions, 5 mg of insulin was added to 10 ml of the gelatin/HFP solution, and the mixture was shaken continuously overnight to obtain a homogenous 1:200 (w/w) insulin/gelatin solution.

### 3.2.4 Preparation of GF and GIF

To fabricate GF and GIF, a conventional electrospinning of gelatin solution was performed as shown in Figure 3.1. Briefly, a syringe of the gelatin solution or gelatin-co-insulin solution described above was placed in an electrostatic field with the applied voltage of 25 kV to a blunt needle (18 gauge 1.5"). A grounded aluminum collecting mandrel was placed 20 cm away from the tip of the needle; while a flat stainless steel collecting mandrel (7.5 × 2.5 × 0.5 cm, length × width × thickness) was placed 12.5 cm away from the needle. The gelatin solution was dispensed from the syringe into the electrostatic field at a rate of 6.37 ml/h. The electrospun fibers were accumulated

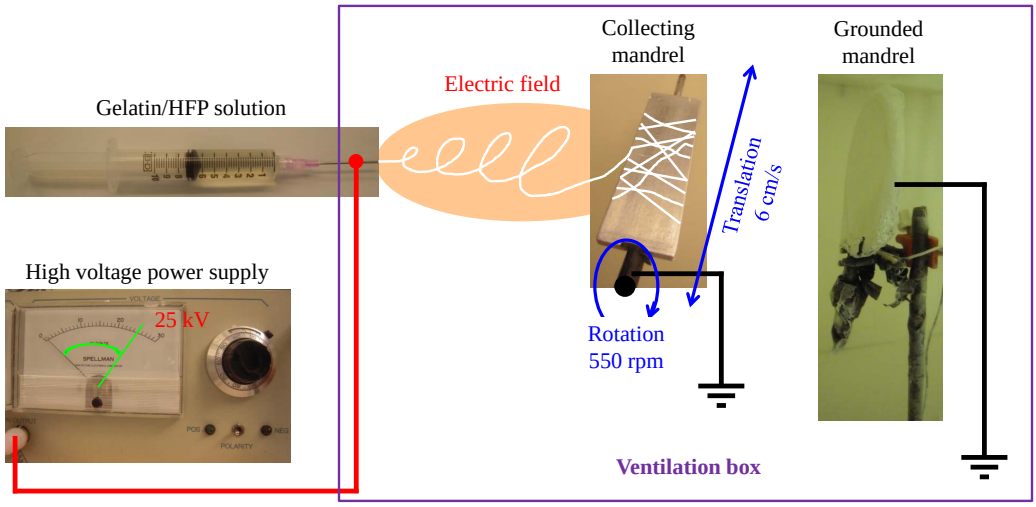


**Figure 3.1: Experimental setup to schematically illustrate the conventional electrospinning.**

on the collecting mandrel with a rotational speed of 550 rpm and a translational speeds of 6 cm/s over 12 cm.

### 3.2.5 Preparation of crosslinked GF and GIF

The eosin Y photoinitiator solution was prepared by 0.1% w/w eosin Y, 40% w/w TEOA and 4% w/w NVP in ethanol as previously reported [24, 29]. In order to obtain the optimal ratio of PEG-DA to eosin Y solution, (1) the electrospun GFs were cross-linked with various concentrations (0%, 1%, 2%, 5%, 10% and 20%; w/v) of PEG-DA in the presence of 5% (v/v) eosin Y solution; (2) the electrospun GFs were crosslinked with various concentrations (2%, 5% and 10%; v/v) of eosin Y solutions in the presence of 1% (w/v) PEG-DA. The crosslinking solutions were prepared by combining appropriate amounts of eosin Y solution and PEG-DA. Next, 600 $\mu$l of crosslinking solution was poured onto a 200 mg of eletrospun gelatin fiber, followed by a 10 min of incubation to allow equilibrium. The scaffold was held under ultraviolet (UV) light (UVP Blak-Ray Long Wave Lamp, 100 Watts) from a 14 cm distance for 5 min on each side of the scaffold and then air-dried as previously reported [2].

### 3.2.6 Cytocompatibility assay

To evaluate the cytocompatibility of PEG-DA and PEG, both polymers were dissolved in ethanol first. 3T3-L1 preadipocytes were treated with 0, 17.4, 87, 174, 696 and 1739 M of PEG-DA or PEG for 2 days. To evaluate the cytocompatibility of the fabricated scaffolds, 20 mg of the fabricated scaffold was weighed and then individually immersed into a 5 ml of cell culture medium at 37°C for 2 hours. The elution media were collected at the end of incubation. 3T3-L1 preadipocytes were treated with these elution media for 2 days. At the end of each treatment, the cell viability was determined by WST-1 proliferation assay following the manufacturers protocol.

### 3.2.7 In vitro degradation studies

In vitro degradation of the fabricated scaffolds was evaluated in cell culture medium and simulated saliva fluid (SSF; 12 mM $KH_2PO_4$, 40 mM NaCl, 1.5 mM $CaCl_2$, with NaOH adjusted to pH 6.2) at 37 °C as previously reported [2]. Briefly, 20 mg of the fabricated scaffolds (n = 5) were weighed and then individually immersed in a tube filled with 5 ml of one of the two media mentioned above. Samples were taken out at 15, 30, 60 and 120 min, and centrifuged at 5,000 rpm for 5 min. After centrifugation, the residue was collected, freeze-dried and weighed.

### 3.2.8 Uniaxial tensile testing

Uniaxial tensile testing of electrospun scaffolds was performed as previously described [2, 50]. Briefly, dog-bone-shaped samples (n = 12-18) were obtained using a punch die (ODC Testing & Molds) with a dimension of 19.05 mm in length, 3.175 mm at narrowest point and 6.1 mm at widest point. The thickness of the samples was determined using a digital caliper. Mechanical properties of the samples were tested using an MTS Bionix 200 testing system with 100 N load cell (MTS Systems, Eden Prairie, MN). Samples were tested to failure at a 1.33 $min^{-1}$ strain rate. TestWorks version 4 was used to calculate peak load, peak stress, modulus, strain at break and energy to break.

### 3.2.9 Scanning electron microscopy (SEM)

The fabricated and degraded scaffolds were placed on a 1 cm diameter stub, and the stub was placed on a specimen holder and prepared by gold sputtering. SEM images were taken on a JEOL LV-5610 scanning electron microscope in the Nanomaterials Core Characterization facility at Virginia Commonwealth University. The diameters of fibers were analyzed by NIH ImageJ (http://rsbweb.nih.gov/ij/) from 60 randomly chosen fibers in each SEM image as previously reported [2, 50].

### 3.2.10 Insulin release studies

Insulin released from the fabricated scaffolds was analyzed by ELISA and Western blot analysis as previously reported [84, 75, 70, 55]. Briefly, 20 mg of insulin-loaded scaffolds were immersed in 5 ml of DMEM at 37 °C. At the predetermined time points of 15, 30, 60, 120 and 240 min, a 25 $\mu$l aliquot was withdrawn from the release medium. The cumulative drug release was then determined by ELISA according to the manufactory standard protocol. In order to evaluate the bioactivity of released insulin in each sample, 3T3-L1 preadipocytes were used as our model. 3T3-L1 preadipocytes were seeded onto a 6-well plate at $5 \times 10^5$ cells/well and cultured for 3 days to allow their attachment. The 3T3-L1 preadipocytes were then treated with the insulin released media at these predetermined time points for 10 min. The intracellular AKT signaling transduction in response to insulin treatment was determined by Western blot analysis.

### 3.2.11 Western blot analysis

Western blot analysis of total cellular protein was carried out by standard procedures, as described previously [89, 88]. Briefly, total cell lysates (30 $\mu$g) were separated on a 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride (PVDF; Bio-Rad, Hercules, CA) membrane using Bio-Rad Mini-Blot transfer apparatus. The membrane was blocked for 2 hours in Tris-buffered solution (TBS) containing 5% of non-fat dried milk. The specific proteins on the membrane were determined by

incubating with the primary antibodies overnight at 4 °C with shaking. The membrane was washed three times and then incubated in a 1:3,000 dilution of a secondary antibody at room temperature for 1 hour in the washing buffer (Tris-buffered solution containing 0.5% Tween 20). The specific antigen-antibody interactions were detected using enhanced chemiluminescence (Pierce ECL Western Blotting Substrate; Thermo Scientific, Rockford, IL). The total cellular expression of $\beta$-actin was used as the loading control.

### 3.2.12 Oil red O staining

3T3-L1 cells were seeded onto a 6-well plate at $5 \times 10^5$ cells/well cultured for 3 days to allow their attachment. According to the standard protocol [65, 84], differentiation was initiated by incubating preseeded cells with DMEM containing 10% FBS, 10 g/mL of insulin, 10 g/mL of dexamethasone (DEX; Sigma-Aldrich, St. Louis, MO), and 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, St. Louis, MO) or with insulin released media containing with 10% FBS, 10 $\mu$g/mL of dexamethasone, and 0.5 mM of IBMX at Day 0. The induction media were replaced by DMEM containing 10% FBS and 10 $\mu$g/mL of insulin or insulin released media containing with 10% FBS at Day 2. The cells were further incubated with DMEM containing with 10% FBS at Day 4, Day 6 and Day 8. The full maturation into adipocytes was achieved at Day 10. Differentiated adipocytes were rinsed in PBS for three times and then fixed with 10% formaldehyde for 2 hours. The cells were washed three times and immersed with 60% isopropanol for 2 min and then stained with 0.2% Oil Red O in 60% of isopropanol for 10 min. The cells were washed three times and then imaged under the microscope (Olympus, Tokyo, Japan) equipped with image recorder under $\times$ 20 lenses [88, 45].

### 3.2.13 In vitro permeation studies

Porcine cheek tissues were obtained from freshly sacrificed pigs (Silver Ridge Slaughter House, Fredericksburg, VA) and transported to the lab within 2 hours. As previously

reported [91], the mucosa tissues were excised and cut into approximately 2 $cm^2$ and frozen on aluminum foil at -20 °C. Before permeation studies, the frozen specimens were equilibrated in PBS for 1 hour at room temperature to allow it completely thawed. Excesses of connective and adipose tissues were trimmed off to obtain a thickness of approximately 0.6 ± 0.1 mm. Buccal mucosa membrane was mounted onto a vertical Franz diffusion cell (PermeGear, Hellertown, PA) with the epithelium facing the donor chamber and the connective tissue facing the receiver chamber. The Franz diffusion cell had a diffusion area of 0.785 $cm^2$ with a donor chamber volume of 1 ml and a receiver chamber volume of 5 ml. The cell was placed in the water bath at 37 °C.

The permeation experiments were carried out using PBS in the receiver chamber and SSF in the donor chamber to mimic the in vivo physiological conditions. The sIPN GIF was placed on top of the mucosa membrane and immersed with 1 ml of SSF. At a given time point up to 4 hour, an aliquot of 1 ml from the receiver chamber was collected via syringe and analyzed with ELISA. Free insulin dissolved in SSF was used as a comparison. The permeability coefficient, $P$, was calculated as follows: $P = (dQ/dt)/AC$, where $dQ/dt$ is the steady-state slope of a cumulative flux curve, $C$ is the loading concentration of a permeant in the donor chamber, and $A$ is the effective cross-sectional area (0.785 $cm^2$) available for diffusion.

### 3.2.14 Statistical analysis

All the data were expressed as means ± standard deviation (SD) and subjected to one way analysis of variance (ANOVA) followed by Holm-Sidak method for significance using SigmaPlot 12. A value of $p < 0.05$ was considered as statistically significant. Statistical analysis data is provided in Appendix A (p120-141).

## 3.3 Results and discussion

### 3.3.1 Optimization of sINP GF scaffold

In order to optimize the sIPN GF scaffold, the fabricated scaffolds were evaluated in respect to two aspects: cytocompatibility and degradation rate. Polyethylene glycol (PEG) is widely used in many applications from industrial manufacturing to medicine due to its excellent biocompatible properties. PEG-DA, a derivative of PEG, has also been widely used in biomedical applications, including hydrogels for drug delivery, tissue engineering and wound healing due to its crosslinking property [11, 27, 34, 40, 67]. However, a recent report indicated that the molecular weight of PEG-DA could affect cell viability in other ways [49]. It has been shown that PEG-DA (MW = 400) retained about 30% cell viability; while PEG-DA (MW = 3400) retained about 60% cell viability. The reason of this phenomenon could be the different diffusion rate of PEG-DA into the cells. A higher diffusion rate can cause more cell damage [49]. To conform these observations, the cytotoxicity of PEG-DA and PEG was determined first on 3T3-L1 preadipocytes. As shown in Figure 3.2, PEG monomers exhibited no or slightly cytotoxic effect on 3T3-L1 preadipocytes up to 174 $\mu$M; in contrast, PEG-DA significantly reduced the 3T3-L1 preadipocyte viability at all the concentrations. The cytotoxic effect is most likely due to the functional terminals of PEG-DA chain. Thus, it is important to utilize these functional groups during the PEG-DA based photopolymerization process in order to make the material cytocompatible.

With the understanding of cytotoxicity of PEG-DA monomers, the cytocompatibility of the different formulation of GF scaffolds were determined. Eosin Y is a good photoinitiator candidate to crosslink polymers due to its excellent cytocompatibility as illustrated in Chapter 2. In this work, GF was crosslinked with a fixed 5% concentration of eosin Y and various concentrations from 0% to 20% of PEG-DA to obtain different formulations of GF scaffolds. An elution method was applied to test cytocompatibility of these fabricated GF scaffolds. As shown in Figure 3.3, GF

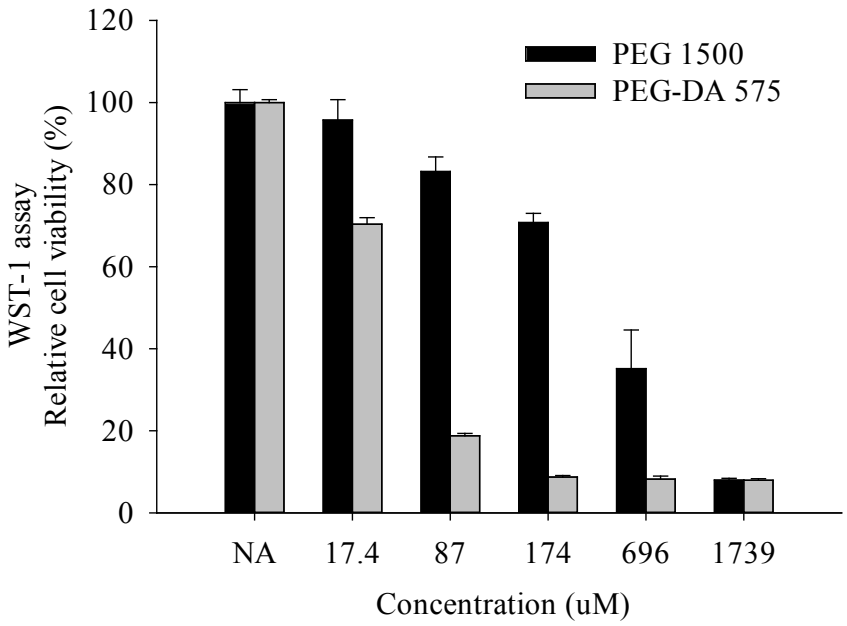


**Figure 3.2: Effects of PEG and PEG-DA on cell viability.**
3T3-L1 preadipocytes were treated with different concentrations of PEG or PEG-DA (0, 17.4, 87, 174, 696 and 1739 $\mu$M) for 2 days. The cell viabilities were measured by WST-1 assay at the end of the treatment (n = 4). The bars are mean $\pm$ standard deviation.

incubated with 5% eosin Y alone has no cytotoxic effect on 3T3-L1 preadipocytes. It is consistent with the previous studies that eosin Y and GF are both cytocompatible biomaterials [68, 2]. Interestingly, a significant cytotoxic effect was observed when the concentration of PEG-DA was above 2%. It suggests that a significant proportion of PEG-DA remains uncrosslinked after crosslinking process, which could be a severely cytotoxic reagent to the cells. In respect of cytocompatilibity, the optimal formulation of sIPN GF scaffold is GF crosslinked with 1% PEG-DA and 5% eosin Y photoinitiator solution. The ratio of eosin Y photoinitiator solution to PEG-DA is 5:1 (v/w), and it is sufficient to crosslink PEG-DA, subsequently to reduce the cytotoxic effect of PEG-DA monomers.

In order to determine the degradation rate, GF was crosslinked wtih a fixed 1% concentration of PEG-DA and various concentrations from 0% to 10% of eosin Y. The scaffold degradation rate was investigated in the cell culture media. As shown in Figure 3.4, the scaffold degradation rate was highly regulated by the concentration of eosin Y. The detail observation is explained as follows. GF was rapidly dissolved in the media within 30 min and totally lost its mass, and this is consistent with our previous results [2]. As expected, 1% PEG-DA could not crosslink with itself or GF in the absence of eosin Y in the crosslinking reaction, and GF/1% PEG-DA had same degradation rate as GF. The result has also shown crosslinking reaction was initiated in the presence of eosin Y and the crosslinking density was dependent on the concentration of eosin Y. GF/1% PEG-DA with 2% eosin Y scaffold was degraded within 60 min, but it could be potentially cytotoxic because the amount of eosin Y might be insufficient to utilize the functional group of PEG-DA. In turn, this scaffold could release the cytotoxic PEG-DA monomers into the cell culture media. In contrast, GF/1% PEG-DA with 10% eosin Y could be highly cytocompatible due to high crosslinking process and full utilization of functional groups of PEG-DA. However, GF/1% PEG-DA with 10% eosin Y exhibited a slow degradation rate, as the evidence showed that the mass of the scaffold was relatively stable during 120 min of incubation. This slow degradation rate could be insufficient to release

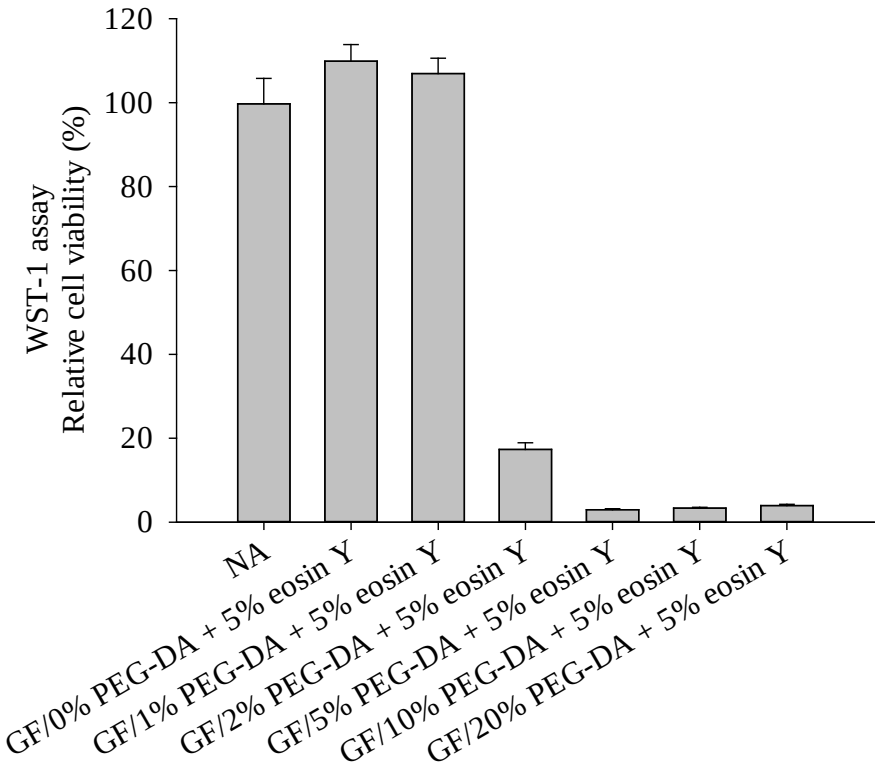


**Figure 3.3: Effects of different formulations of GF scaffolds on cell viability.** Different concentrations of PEG-DA were used to crosslink GF to achieve the corresponding GF scaffolds. The elution method was used to test the cytocompatibility of these scaffolds. These fabricated scaffolds were immersed into the cell culture media for 2 hours. 3T3-L1 preadipocytes were then treated with these individual elution media for 2 days. The cell viabilities were measured by WST-1 assay at the end of the treatment (n = 4). The bars are mean ± standard deviation.

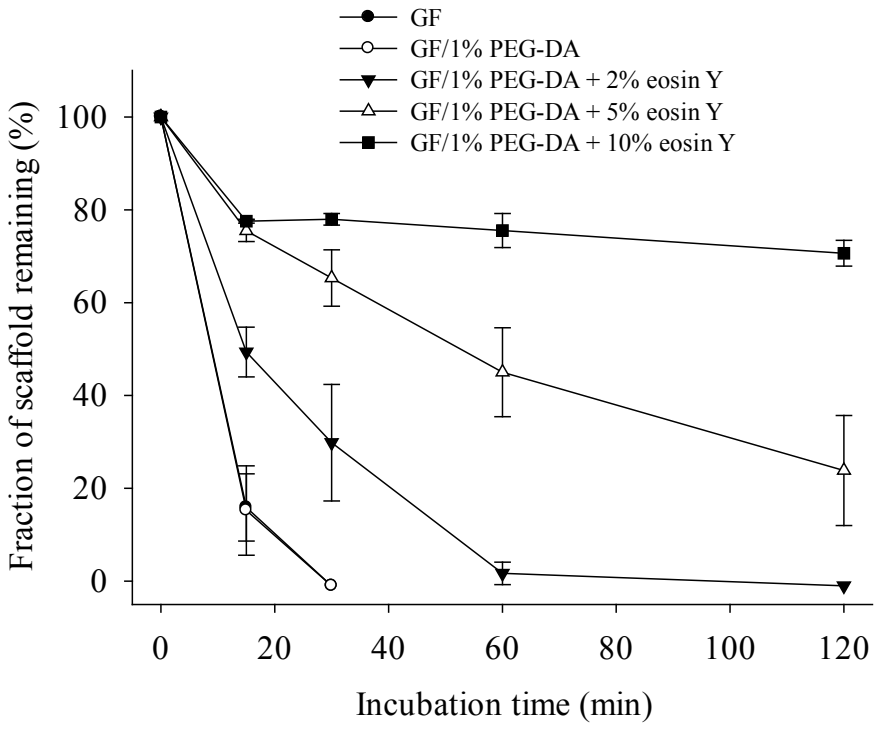


**Figure 3.4: In vitro degradation of different fabricated GF scaffolds in the cell culture media.**

20 mg of each fabricated GF scaffold was immersed into 5 ml of the cell culture media, and each sample was collected and weighed at the indicated time intervals (n = 5). The bars are mean ± standard deviation.

insulin that is required for transbuccal mucosal delivery of insulin within the oral cavity. The formulation of scaffold was optimized as GF crosslinked with 1% PEG-DA and 5% eosin Y in respect to both cytocompatibility and degradation rate, and this formulation of scaffold was referred to as sIPN GF in the rest of the work.

### 3.3.2 Mechanical properties of sINP GF scaffold

The mechanical properties were evaluated to confirm the crosslinking process of sIPN GF. As shown in Figure 3.5A, the thickness of sIPN GF was significantly smaller than the precursor GF. Most likely, the fibers fused together and crosslinked along or between the side chains. Similar observation was obtained using DMPA photoinitiator to crosslink GF with PEG-DA [2]. The mechanical properties, including the peak

load, peak stress, modulus, energy at break and strain at break, were significantly enhanced by 6.5, 7.9, 5.0, 17.1 and 2.4 fold in sIPN GF comparing with GF as shown in Figure 3.5B-F. The results confirmed 1% PEG-DA and 5% eosin Y were able to crosslink GF and increased its mechanical strength.

### 3.3.3 Morphology and fiber diameter of sIPN GF

The fiber morphology was evaluated by SEM to monitor the crosslinking process of sIPN GF. As shown in Figure 3.6, the morphology of GF displayed a well interconnective structure with fine continuous fibers and an undefined porosity, which was consistent with our previous reports [2, 7]. Interestingly, the morphology of sIPN GF showed that those continuous fibers of the scaffold were fused together and formed a much smaller porosity structure. At notice, some fibers were merged as side-by-side chains; while some fibers were crossed over the others. This observation supported the results from the tensile study that the thickness of sIPN GF was smaller than GF (Figure 3.5A). Such a small space between the crosslinked fibers was most likely resulted from the short chain of PEG-DA. Consequently, a significant increase of fiber diameter was observed in sIPN GF comparing to GF (Figure 3.7). It could also be observed clearly that two fibers were immersed together into one in sIPN GF (Figure 3.6). These results further confirmed the successful crosslinking process of sIPN GF.

### 3.3.4 Structure stability of sIPN GF

The structure stability of sIPN GF was investigated based on the degradation rate of sIPN GF in Figure 3.4, a set of SEM images of sIPN GF taken after 15, 30, 60 and 120 min of incubation in the cell culture media were compared to that of sIPN GF itself. As shown in Figure 3.8, the pore size significantly increased along with the incubation time. After 15 min of incubation, the fiber diameter was significantly reduced, and many small diameter fibers were observed, comparing to sIPN GF.

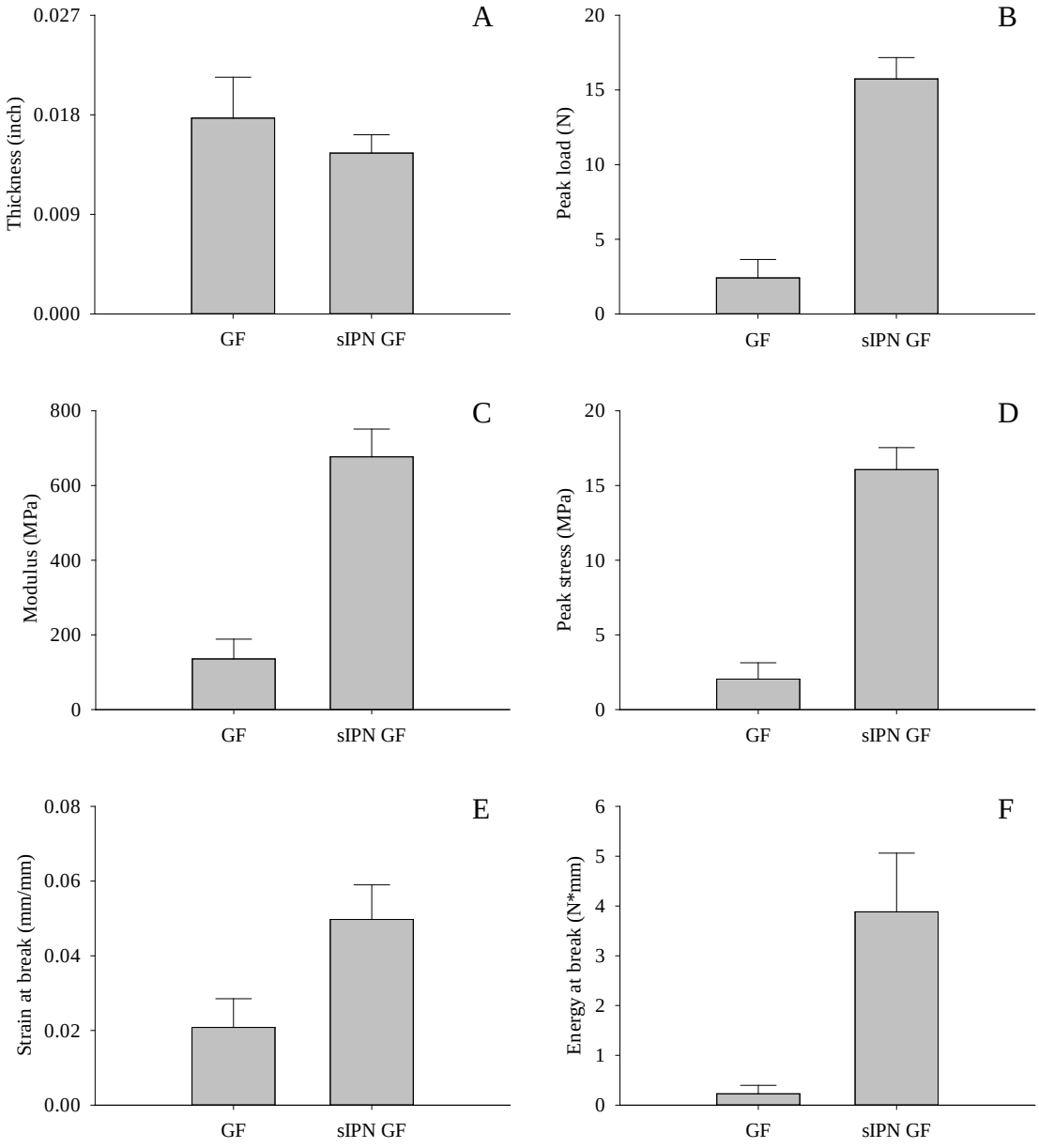


**Figure 3.5: Mechanical properties of GF and sIPN GF.**
The mechanical properties of GF (n = 18) and sIPN GF (n = 12) were evaluated by a uniaxial tensile test. (A) Thickness of the scaffolds. (B) Peak load of the scaffolds. (C) Peak stress of the scaffolds. (D) Modulus of the scaffolds. (E) Energy at break of the scaffolds. (F) Strain at break of the scaffolds. The bars are mean ± standard deviation.

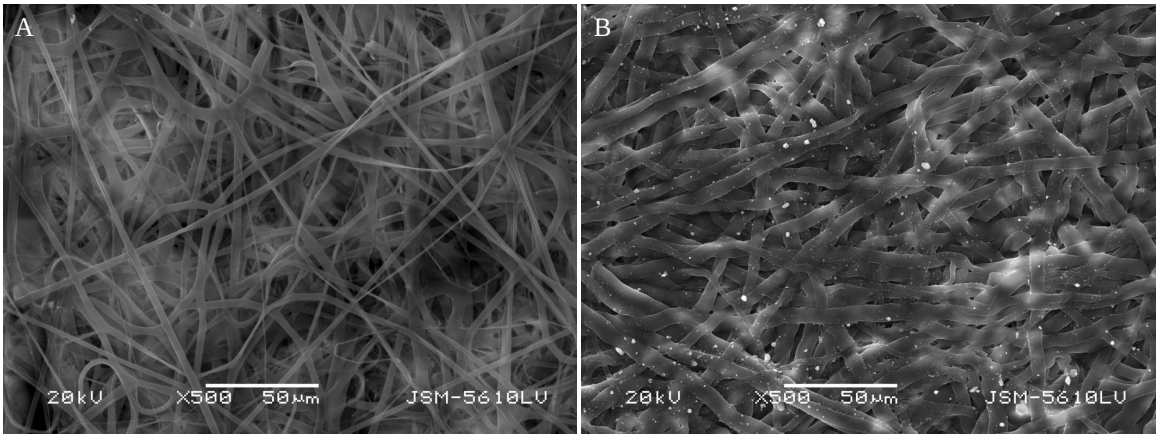


**Figure 3.6: Morphology of GF and sIPN GF.**
The scaffold morphology of (A) GF and (B) sIPN GF was observed by SEM (n = 3). Bars: 50 $\mu$m.

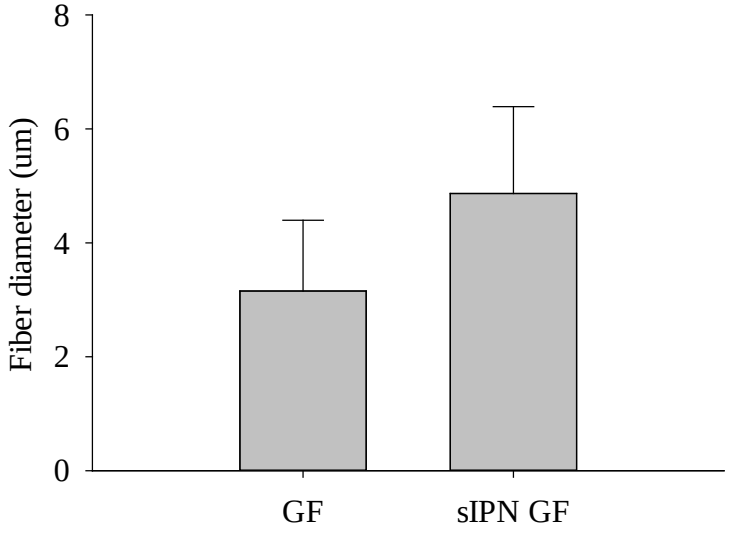


**Figure 3.7: Fiber diameter of GF and sIPN GF.**
Fiber diameters of GF and sIPN GF (n = 60) were measured by NIH ImageJ software from the SEM images. The bars are mean ± standard deviation.

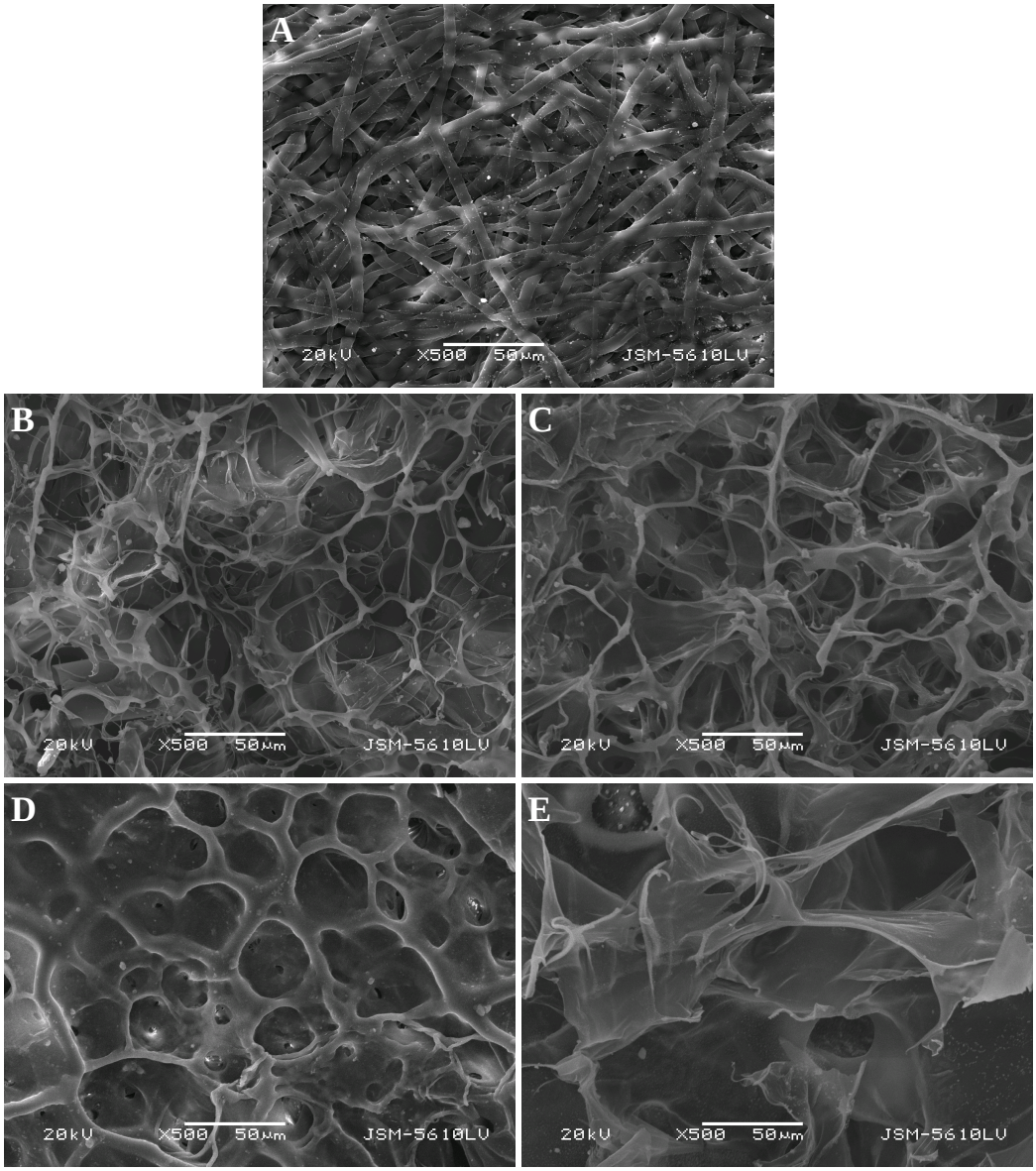


**Figure 3.8: Morphology of sIPN GF after degradation.**
20 mg of sIPN GF was immersed into 5 ml of cell culture media, and each sample was collected at the indicated time intervals of (A) 0 min, (B) 15 min, (C) 30 min, (D) 60 min and (E) 120 min. The remaining scaffold morphology was observed by SEM (n = 3). Bars: 50 $\mu$m.

After 30 min of incubation, most of the small diameter fibers were disappeared in the scaffold structure, while the structure remained three-dimensional. Most likely, these small diameter fibers were more easily degraded and dissolved into the media than the large diameter fibers. After 60 min of incubation, all the small diameter fibers were dissolved in the scaffold and the fiber diameter became larger. It was likely caused by the fact that some of the fibers were fused together to maintain the structure in the media. Lastly, after 120 min of incubation, the scaffold lost its defined three-dimensional structure and became separated pieces of polymers. These results were consistent with the degradation assay shown in Figure 3.4 that only 20% mass remained, and the scaffold lost its whole integrity after 120 min of incubation.

### 3.3.5 Insulin release kinetics of sIPN GIF

The fabricated sIPN GIF using optimized formulation described earlier was assessed to investigate the insulin release kinetics in response to the cell culture media. Cumulative insulin release studies were performed using ELISA. As shown in Figure 3.9, a fast insulin release was achieved from GIF that almost all insulin was released within 15 min. GIF, same as GF, can be instantaneously degraded and dissolved in the cell culture media within 30 min (Figure 3.4), which interprets all the insulin fabricated within GIF was released into the media within 30 min. The feeding ratio of insulin to gelatin was 5:1 (mg/g) according the formulation; and the resultant ratio of insulin to GIF was 3.25:1 (mg/g). Indeed, the loading efficiency of insulin after electrospinning was about 65%.

In contrast, a slower insulin release was observed from sIPN GIF within a 4-hour window of incubation in the cell culture media. These results were also consistent with the degradation rate (Figure 3.4) and the porosity of sIPN GF (Figure 3.8) described above. Nearly 20% of sIPN GIF mass has been degraded from the scaffold in the initial degradation at 15 min. The increase in porosity allows water molecules to come inside of scaffold, dissolve the gelatin fiber, and absorb insulin molecules. As shown in Figure 3.10, an initial burst release of insulin was observed about 70% of

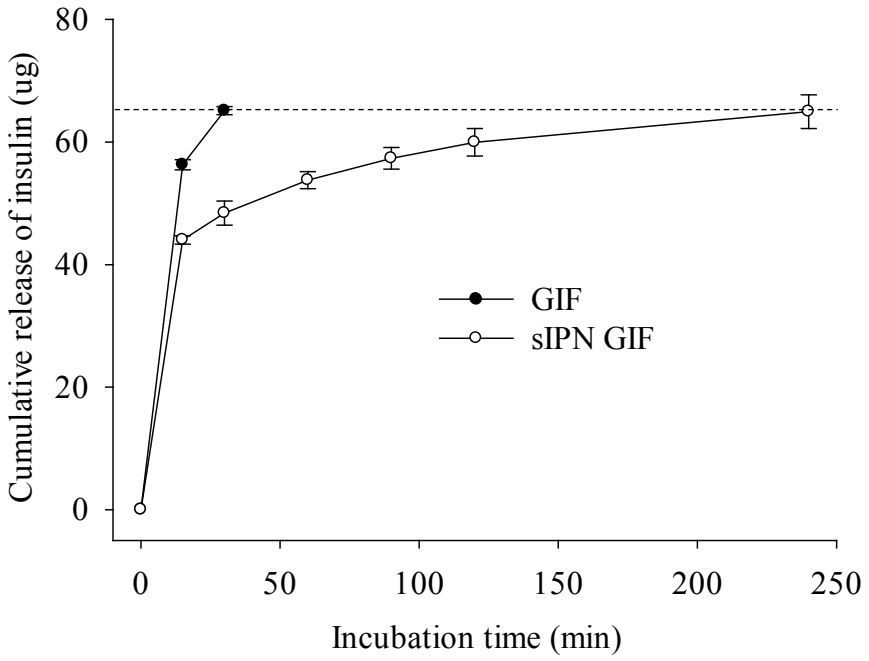


**Figure 3.9: In vitro insulin release kinetics.**
20 mg of GIF or sIPN GIF was immersed into 5 ml of cell culture media, and 25 $\mu$l of release media was collected at the indicated time intervals. ELISA was employed to evaluate the insulin concentration from the release media. The bars are mean $\pm$ standard deviation.

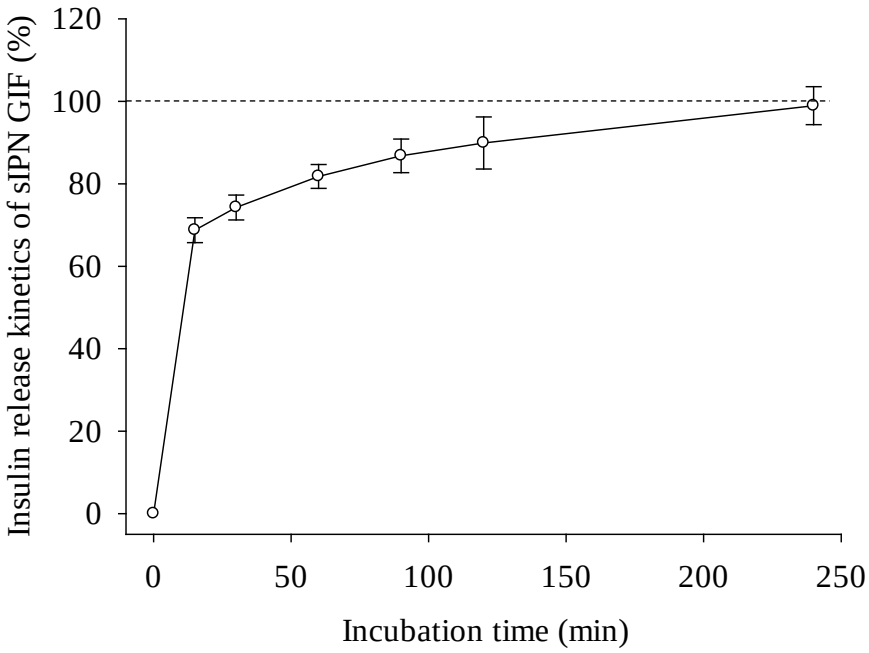


**Figure 3.10: In vitro insulin release kinetics of sIPN GIF.**
The insulin release kinetics of sIPN GIF was normalized by taking into account of 100% of insulin released from GIF within 30 min of incubation from Figure 3.10. The bars are mean ± standard deviation.

loading insulin from sIPN GIF within 15 min of incubation. The release rate of insulin was then reduced until 100% loading insulin was released from sIPN GIF, i.e., about 4 hours. This formulation of sIPN GF provides a fast release at the initial stage and a sustained slow release at the late stage, which can be benefit in oral mucosal delivery of insulin. Generally, an increase of both PEG-DA and eosin Y concentrations during the crosslinking process could help to enhance the scaffold stability and subsequently modulate the drug release kinetics. It is also important to keep the same ratio of PEG-DA to eosin Y as 1:5 (w/v) in order to maintain its biocompatibility.

### 3.3.6 Bioactivity of the insulin released from sIPN GIF

The bioactivity of insulin within sIPN GIF after electrospinning was examined in this work. It has been reported that proteins can be co-electrospun with polymers

without causing denature of the neutral protein. For instance, purified adipose tissue-derived extracellular matrix (At-ECM) can be electrospun alone or co-electrospun with PDO. The resultant scaffold forms a basement membrane-rich tissue engineering matrix that is capable of supporting stem cells [26]. Insulin is known as a peptide hormone that can bind to the extracellular portion of the alpha subunits of the insulin receptor and in turn trigger the signaling transduction within cells. Once the binding occurs, it causes a conformational change of the insulin receptor and then activates the kinase domain on the intracellular portion of the beta subunits. The activated kinase domain autophosphorylates IRS-1 protein, which subsequently activates phosphoinositol 3 kinase (PI3K). PI3K then catalyzes the reaction of PIP2 to PIP3, which can phosphorylate protein kinase B (PKB). PKB is also known as AKT that plays a key role in glucose metabolism, apoptosis, cell proliferation, transcription and cell migration [31, 80]. In this work, the phosphorylation of AKT was used as a marker to evaluate the bioactivity of released insulin. As shown in Figure 9, free insulin induced AKT phosphorylation as expected; while the release media from GIF also significantly induced AKT phosphorylation. It was also noticed that no significant change of p-AKT was observed in the release media from GF and sIPN GF. These results suggest that the insulin remains bioactive after electrospinning.

In the next step, sIPN GIF was examined for the ability to release bioactive insulin. As shown in Figure 3.12, phosphorylation of AKT displayed an incubation time-dependent manner of sIPN GIF. It was noticed that sIPN GIF in 15 min of incubation released a portion of insulin that was able to trigger AKT phosphorylation but not as high as that of 60 min of incubation. Together with insulin release kinetics (Figure 3.9), it supports that insulin bioactivity is close related to its release kinetics of sIPN GIF.

The differentiation of 3T3-L1 preadipocytes was also evaluated in regard to the insulin released from sIPN GIF. As expected, no adipocyte differentiation was observed in sIPN GF and negative control treatment groups; whereas a significant

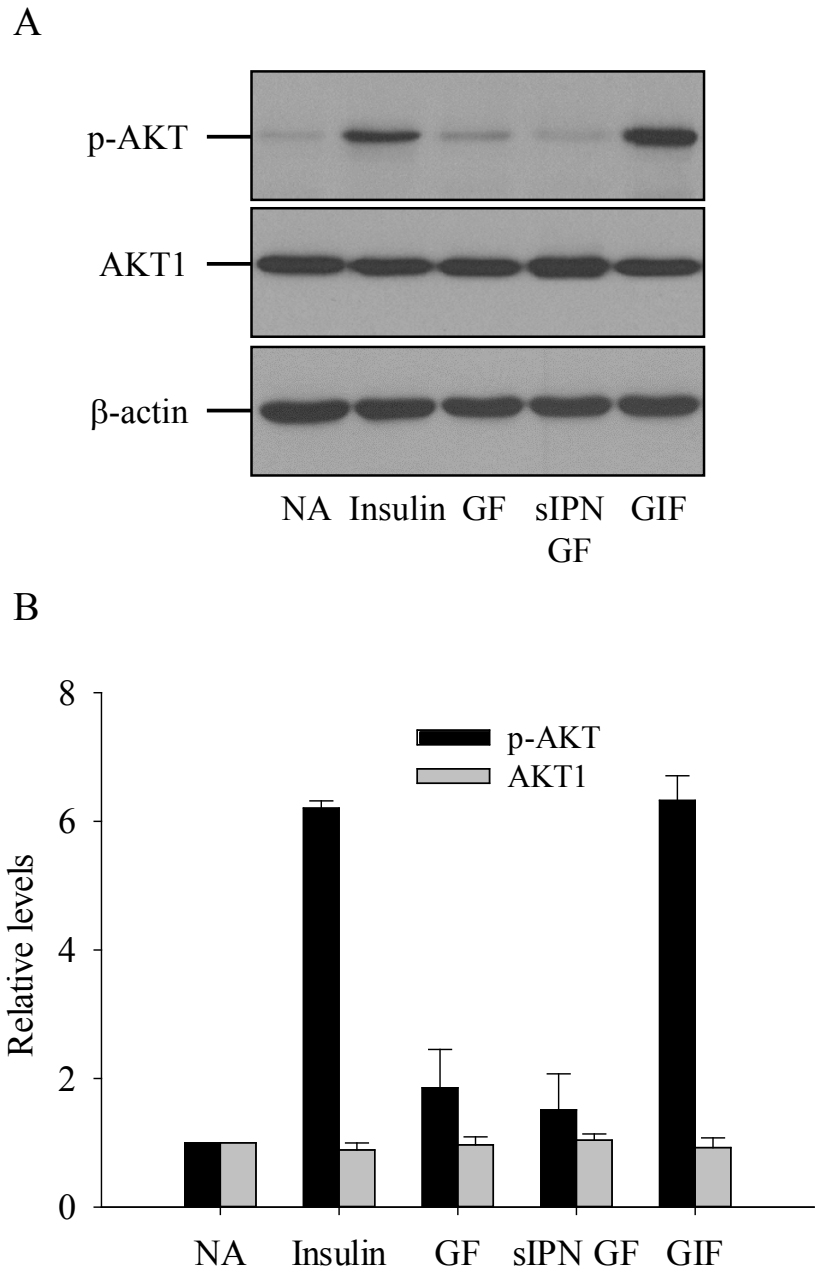


**Figure 3.11: Western blot analysis of intracellular AKT activity in responding to the fabricated GF scaffolds.**
20 mg of each fabricated GF scaffold (GF, sIPN GF and GIF) was immersed into 5 ml of cell culture media for 30 min. 3T3-L1 preadipocytes were then treated with these individual elution media or free insulin for 10 min. The intracellular phospho-AKT (p-AKT) and total AKT levels were analyzed by Western blot analysis (A). Each positive band was normalized to $\beta$-actin and was quantified by NIH ImageJ (B). The data represents typical one of three experiments. The bars are mean $\pm$ standard deviation.

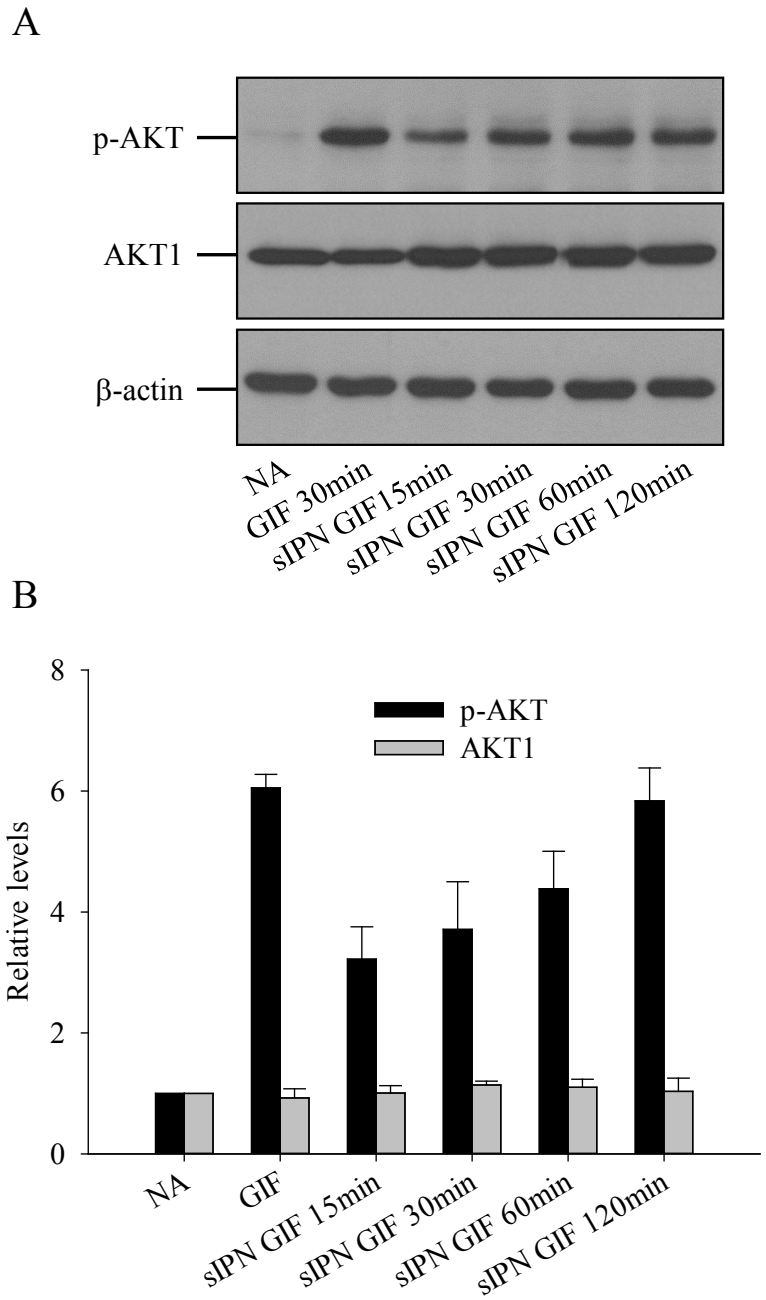


**Figure 3.12: Western blot analysis of intracellular AKT activity in responding to sIPN GIF.**

20 mg of sIPN GIF was immersed into 5 ml of cell culture media for 15 min, 30 min, 60 min and 120 min; while 20 mg of GIF was immersed into 5 ml of cell culture media for 30 min. 3T3-L1 preadipocytes were then treated with these individual elution media for 10 min. The intracellular phospho-AKT (p-AKT) and total AKT levels were analyzed by Western blot analysis (A). Each positive band was normalized to $\beta$-actin and was quantified by NIH ImageJ (B). The data represents typical one of three experiments. The bars are mean $\pm$ standard deviation.

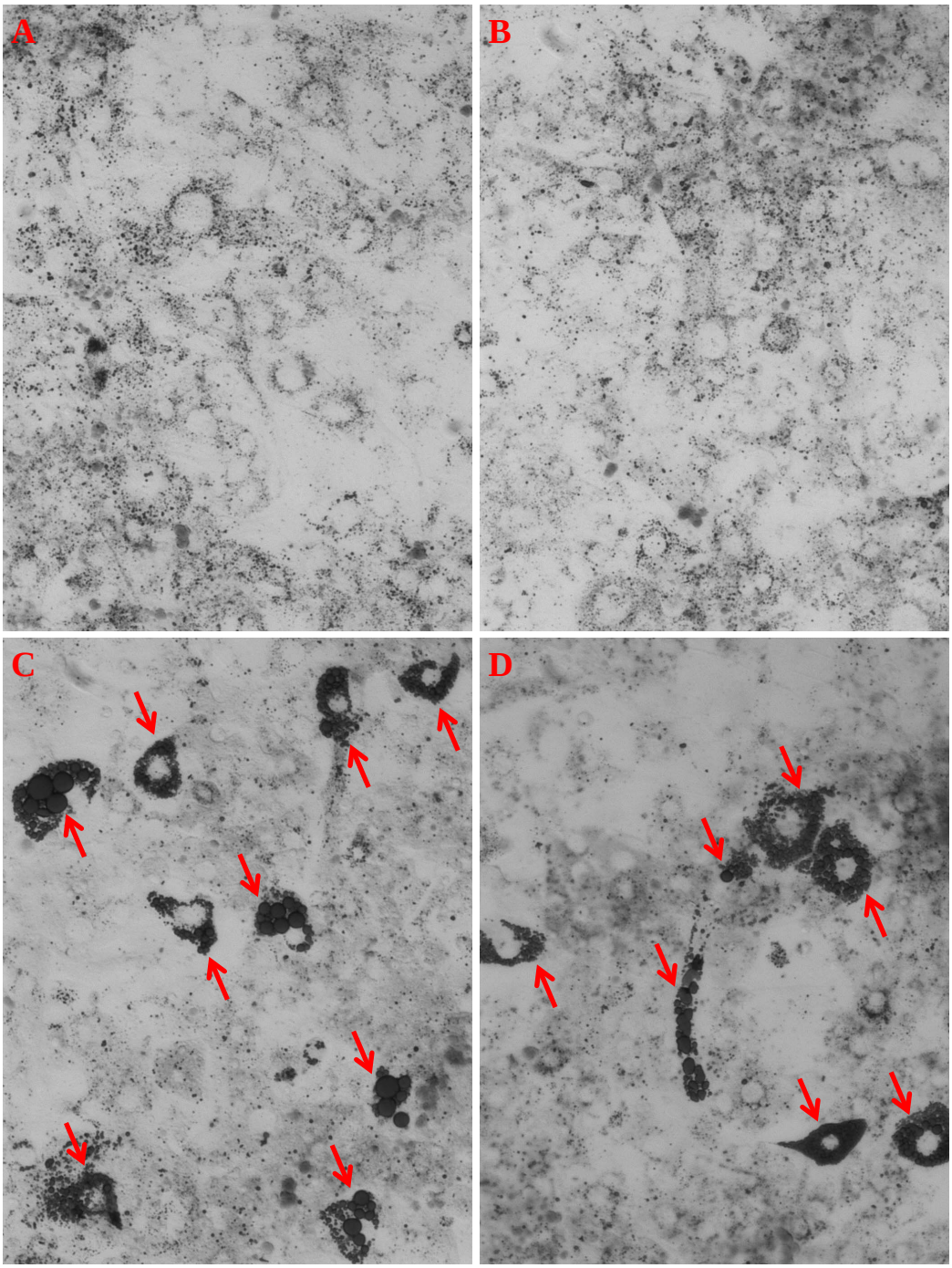


**Figure 3.13: Oil red O staining of 3T3-L1 preadipocytes in responding to sIPN GIF.**

20 mg of sIPN GIF or sIPN GF was immersed into 5 ml of cell culture media for 120 min. 3T3-L1 preadipocytes were then treated with these individual elution media or free insulin along with DEX and IBMX for 2 days. After additional 8 days incubation, the cells are fixed and total intracellular neutral lipids were stained with Oil Red O. (A) NA, (B) sIPN GF, (C) sIPN GIF and (D) free insulin. Arrows indicate stained lipid inclusions.

adipocyte differentiation was achieved in sIPN GIF and free insulin groups as shown in Figure 3.13. It confirmed from the biological aspect that the insulin can be co-electrospun with gelatin and remain its bioactivity. The insulin release kinetics can be modulated by applying crosslink reaction to GIF.

### 3.3.7 Diffusion rate of insulin from sIPN GIF across porcine buccal mucosa

Hydrophilic molecules across buccal membrane is generally via a paracellular route that is driven by passive diffusion. Due to its good biodegradability, moderate stiffness and satisfactory tissue adhesiveness properties [73, 92], gelatin/PEG sIPN are investigated to deliver biomacromolecules and release them in a controlled release manner [10]. Firstly, it has been reported that mucoadhesive hydrogels can open up the tight junctions of the epithelium by dehydrating the cells as they swell, which in turn increases the permeability of the loaded drug [60]. Secondly, a direct contact of gels and mucosa could give a high localization of drug onto the mucosal surface, which in turn facilitates drug buccal transport. Based on the understanding of these mechanisms, a gelatin/PEG sIPN was developed to enhance transbuccal delivery of dendritic nanoparticles through an in vitro model [91]. Although PEG-only hydrogel has shown a slightly higher degree of enhanced transbuccal delivery of dendritic nanoparticles, it is a lack of mucosal adhesiveness property that makes PEG-only hydrogel unsuitable platform for buccal administration formulations [91].

In this work, the peameability of insulin from sIPN GIF was evaluated via a vertical Franz diffusion cell with a porcine epithelium membrane. First, the degradation rate of sIPN GF was determined in SSF, which simulates oral saliva condition. As shown in Figure 3.14, the degradation rate of sIPN GF in SSF is similar to that in the cell culture media (Figure 3.4). However, the composition of in vivo saliva remains different as that of in vitro SSF. The saliva contains the enzymes secreted from the salivary glands, such as amylase and lipase. These enzymes may have the potential to accelerate the degradation of sIPN GF. Second, the diffusion rates of in-

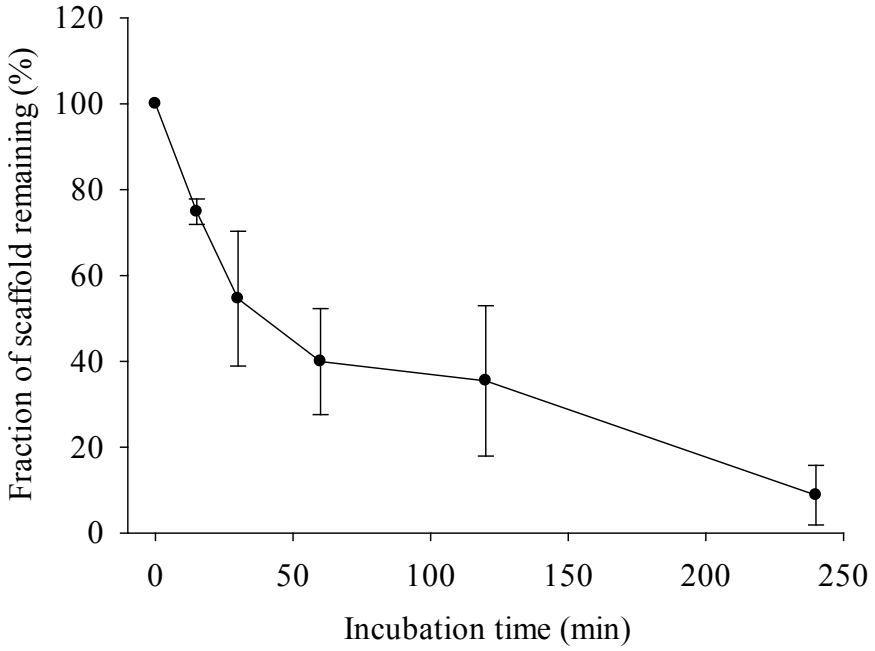


**Figure 3.14: In vitro degradation of sIPN GF in SSF.**
20 mg of sIPN GF was immersed into 5 ml of SSF, and each sample was collected and weighed at the indicated time intervals (n = 8). The bars are mean ± standard deviation.

sulin from sIPN GF and free insulin were determined through porcine buccal mucosa. As shown in Figure 3.15, the positive control benzylamine displayed a high transport rate, consistent with the previous report [2]. As shown in Figure 3.16, the cumulative flux curves of both sIPN GIF and free insulin exhibited in a linear range during the first 2 hours, indicating a steady state transport of the insulin via the paracellular route. Permeability was then calculated from the linear range of the cumulative flux curves. The permeability of insulin from sIPN GIF was determined to be $1.00 \times 10^{-7}$ cm/s; whereas the permeability of free insulin was $7.67 \times 10^{-8}$ cm/s. A 30% enhancement of permeability was achieved by sIPN GIF.

Although the enhanced permeability of insulin from sIPN GIF has been shown, some challenges and future directions remain as follow. (1) It would be critical if the mucosal endothelial cells remain as intact and tight conjunct after freeze-thaw cycle.

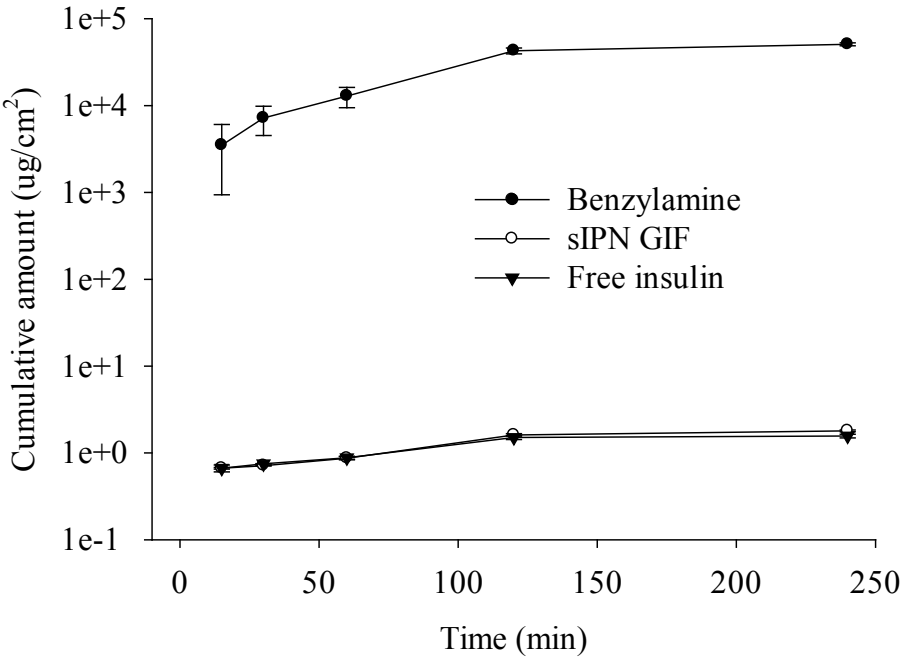


**Figure 3.15: Transport of insulin or benzylamine across porcine buccal mucosa.**

At the indicated time intervals, the transbuccal mucosal insulin from sIPN GIF or free insulin was determined by ELISA, and benzylamine was determined by UV-Vis (n = 3). The bars are mean  standard deviation.

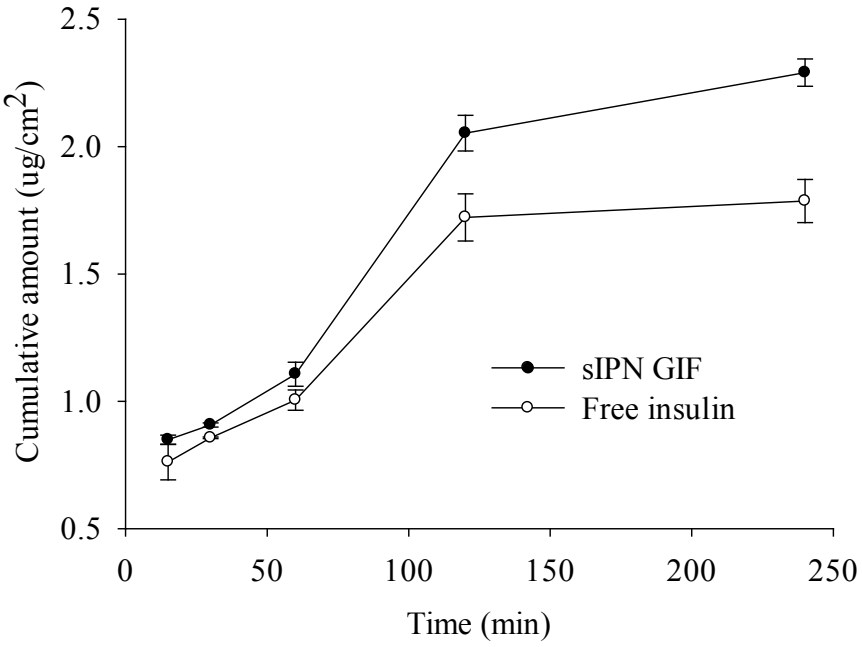


**Figure 3.16: Transport of insulin across porcine buccal mucosa.**
At the indicated time intervals, the transbuccal mucosal insulin from sIPN GIF or free insulin was determined by ELISA (n = 3). The bars are mean  standard deviation.

(2) It is of interest to investigate whether the mucosal endothelial cells can uptake insulin during the diffusion process. (3) The continued work could be determination of the mucosal adhesion properties of sIPN GF. (4) Future studies include optimization of sIPN GIF loaded with permeation enhancer, such as sodium glycodeoxycholate (NaGDC), since it has been reported that bile salts hold a great potential to enhance transbuccal permeability of drug [91]. (5) The studies may also include the testing of pharmacokinetics and pharmacodynamics (PK/PD) of sIPN GIF following buccal administration in pig.

## 3.4 Conclusions

In this work, sIPN GF was fabricated by crosslinking GF with PEG-DA via photopolymerization process. The optimal ratio of PEG-DA to eosin Y is 1:5 (w/v) with respect to biocompatibility, degradation rate and structure stability. This formulation of sIPN GF exhibits high biocompatibility and possesses mucoadhesion property. It could be a good candidate for buccal mucosal delivery of insulin. The insulin release kinetics was modulated to achieve a desired bioactivity. Based on our preliminary in vitro investigation, sIPN GIF significantly enhanced transbuccal diffusion rate of insulin through the porcine mucosal membrane. The in vivo mucosal administration of insulin on sIPN GIF will be evaluated in our future studies.

# Chapter 4

# General conclusions and future directions

## 4.1 General conclusions

To explore the potential application of sIPN GIF in transbuccal mucosal delivery of insulin, a general review was provided on adhesion property and release kinetics from polymeric insulin delivery systems (Chapter 3). Several factors may impact insulin transbuccal mucosal behaviors from polymeric matrices, including matrix factors, solute factors and experimental conditions. Among these factors, material degradation rate and insulin release kinetics and permeability are the key driving forces. With a better understanding of buccal mucosal transport mechanism, our objective was to optimize sIPN GF with the most appropriate biological and physical properties for transbuccal mucosal delivery of insulin.

sIPN GF is commonly formed by crosslinking GF with PEG-DA. This crosslinking process is driven by photopolymerization. Photoinitiators play an important role within photopolymerization. Photoinitiators can be dissociated into high-energy free radicals by exposure to specific wavelengths of light. Subsequently, these free radicals induce the polymerization of a macromer solution. However, these high-energy free radicals are toxic molecules that can induce oxidative stress to the cells. Chapter 2 illustrated that eosin Y was less cytotoxic than Irgacure 2959 and DMPA, and eosin Y

possessed minimal effect on intracellular AKT signaling transduction up to 25 $\mu$l/ml. It has been shown that the cytocompatibility of photoinitiators was closely correlated to the intracellular AKT signaling transduction within the cells. Free radicals can directly or indirectly interact with cells, inhibit the intracellular AKT signaling transduction and decrease cell viability. In summary, eosin Y can serve as a photoinitiator for the photopolymerization of sIPN GF, by owing its high cytocompatible property. The amount ratio of eosin Y to GF and PEG-DA needs to be optimized in order to minimize the cytotoxic effect induced by free radicals.

PEG-DA and PEG are widely used in biomedical applications, including drug delivery, tissue engineering and wound healing. A smaller molecular weight of PEG-DA has a higher diffusion rate into the cells, which can cause more damage of the cells. It has been shown the PEG-DA enhanced cytotoxic effect to the cells comparing to its precursor PEG (Chapter 3). This increase of toxicity is most likely caused by the acrylate modification at the terminals of PEG monomer chains. It becomes important to optimize the amount of both PEG-DA and eosin Y in the formulation of sIPN GF in respect to cytocompatibility.

Another important factor for transbuccal mucosal delivery of insulin is the degradation rate of polymeric matrices. Photopolymerization can physically enhance the entanglement of polymer chains in a three-dimensional structure. By controlling the crosslinking density, polymeric matrices can achieve a desired degradation rate.

In respect to both cytocompatibility and degradation rate of the scaffold, the formulation of sIPN GF was optimized by crosslinking GF with 1% PEG-DA and 5% eosin Y (Chapter 3). The optimized sIPN GF possessed no cytotoxic effect and exhibited a moderate degradation rate within 4-hour window (Chapter 3).

This optimized formulation was used to fabricate sIPN GIF for transbuccal mucosal delivery of insulin. Several important factors of sIPN GIF have been determined. 1) sIPN GIF achieved a 65% loading efficiency of insulin after electrospinning and crosslinking. 2) The insulin release kinetics was modulated after crosslinking in sIPN GIF. 3) The insulin released from sIPN GIF remained bioactive to activate intracellular AKT signaling transduction and differentiate 3T3-L1 preadipocytes. 4)

The permeability of the insulin released from sIPN GIF was on the order of $10^{-7}$ cm/s using a vertical Franz diffusion cell system mounted with porcine buccal mucosa (Chapter 3).

Insulin is commercially available in a concentration of 100 or 500 units/ml, which is designated U-100 and U-500, respectively. U-500 is only used in rare cases of insulin resistance when the patient requires extremely large doses of insulin [5]. The bolus dose for food coverage is prescribed as an insulin to carbohydrate ratio. The insulin to carbohydrate ratio represents the amounts of carbohydrate are covered by 1 unit of insulin. Here, 1 unit of insulin is equivalent to about 36 $\mu$g of insulin. Generally, 1 unit of insulin will dispose of 12-15 g of carbohydrate. This range can vary from 6-30 g or more of carbohydrate depending on the insulin sensitivity, which can vary according to the time of day, from person to person, and is affected by physical activity and stress. According to the formulation of sIPN GIF, 65 $\mu$g of insulin was incorporated within 20 mg of sIPN GIF, which is equal to 1.8 units of insulin. In order to achieve a common unit of insulin (10 units) taken each time, about 110 mg of sIPN GIF is required. The size of 110 of sIPN GIF is within 5 $cm^2$ range, which is a reasonable size of pill for oral mucosal administration. These results suggest that sIPN GIF holds a great potential for transbuccal mucosal delivery of insulin.

## 4.2 Future directions

The accomplishments from this work can inspire the further suggestions for continuing this work.

### 4.2.1 Evaluation of sIPN GIF in in vivo model

Porcine buccal tissues were utilized for studing the permeability of insulin from sIPN GIF in this work. Porcine buccal mucosa has been well established to be the closest to human mucosa. In vivo evaluation of sIPN FIG in transbuccal mucosal delivery of insulin will be conducted in pig in our future investigations. The bioavailability of

insulin will be studied by pharmacokinetics and pharmacodynamics. This in vivo pig study can provide direct evidence to evaluate the buccal mucosal permeability and biocompatibility from sIPN GIF formulation.

### 4.2.2 Potential application of sIPN GF for wound healing

The outcome of these studies demonstrated the potential of developing such natural/synthetic hybrid materials into dermal equivalence. As a natural protein, insulin was successfully co-electrospun with gelatin in this work. The loading efficacy of insulin was about 65%, and the insulin remained bioactive after electrospinning and crosslinking process. These results inspire our great interests to further explore sIPN GF in the biomedical applications, such as wound healing.

Wounds can extend partially through the dermis and have capabilities to regenerate. However, the body cannot heal deep dermal injuries adequately. In some cases, such as full thickness burns or deep ulcers, there are no remaining sources of cells for regeneration except peripheral cells from the wound. Therefore, it takes a long time to complete re-epithelialization, and it is complicated by scarring of the base [18].

Matrix metalloproteinases, referred to as MMPs, play a major role in wound healing. MMPs can degrade all components of the extracellular matrix as well as regulate vascularization and inflammatory response [61, 53, 30]. It has been reported that MMP-2 regulates the formation of a glial scar and white matter sparing and/or axonal plasticity [30]. MMP-2 in turn can facilitate wound healing and promote functional recovery after spinal cord injury [61]. Thus, MMP-2 is exploited as a therapeutic target in the treatment of wound healing.

Electrospun nano- or macro-fiber scaffolds hold a great potential in the treatment of dermal wounds through layered application [33, 36, 18, 16]. It is of interest to explore sIPN GF incorporating with MMP-2 (sIPN GF/MMP-2) in the treatment of wound healing. The formulation of sIPN GF is required to be optimized in respect to the degradation rate. In the treatment of wound healing, the ratio of PEG-DA to eosin Y will be further modulated in order to achieve a slow degradation rate of the

sIPN GF and a slow release kinetics of MMP-2. It is important to investigate the efficacy of sIPN GF/MMP-2 in modulating host inflammatory response and healing outcome in a disease-relevant animal model, such as db/db mice with full thickness wounds.

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# Appendix A

# Statistical analysis of the experimental data

Figure 2.1 eosin Y

One Way Analysis of Variance

Data source: Data 1 in Fig.1 eosinY

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.094)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 0d | 3 | 0 | 100 | 6.811 | 3.932 |
| NA 1d | 3 | 0 | 144.147 | 13.87 | 8.008 |
| 2.5ul/ml 1d | 3 | 0 | 148.997 | 4.013 | 2.317 |
| 5ul/ml 1d | 3 | 0 | 128.93 | 3.29 | 1.899 |
| 10ul/ml 1d | 3 | 0 | 141.472 | 8.704 | 5.025 |
| 25ul/ml 1d | 3 | 0 | 72.408 | 10.81 | 6.241 |
| NA 2d | 3 | 0 | 206.02 | 53.979 | 31.165 |
| 2.5ul/ml 2d | 3 | 0 | 202.174 | 39.727 | 22.937 |
| 5ul/ml 2d | 3 | 0 | 139.13 | 18.966 | 10.95 |
| 10ul/ml 2d | 3 | 0 | 121.906 | 24.643 | 14.228 |
| 25ul/ml 2d | 3 | 0 | 18.896 | 2.027 | 1.171 |
| NA 3d | 3 | 0 | 259.532 | 18.813 | 10.862 |
| 2.5ul/ml 3d | 3 | 0 | 239.632 | 32.436 | 18.727 |
| 5ul/ml 3d | 3 | 0 | 187.291 | 20.331 | 11.738 |
| 10ul/ml 3d | 3 | 0 | 91.806 | 18.724 | 10.81 |
| 25ul/ml 3d | 3 | 0 | 4.849 | 1.613 | 0.931 |
| NA 4d | 3 | 0 | 323.077 | 31.883 | 18.408 |
| 2.5ul/ml 4d | 3 | 0 | 290.468 | 28.254 | 16.313 |
| 5ul/ml 4d | 3 | 0 | 143.144 | 18.208 | 10.513 |
| 10ul/ml 4d | 3 | 0 | 38.127 | 7.287 | 4.207 |
| 25ul/ml 4d | 3 | 0 | 5.017 | 0.869 | 0.502 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 20 | 483604.7 | 24180.23 | 49.327 | <0.001 |
| Residual | 42 | 20588.36 | 490.199 | | |
| Total | 62 | 504193 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| NA 4d vs. 25ul/ml 3d | 318.227 | 17.603 | <0.001 | Yes |
| NA 4d vs. 25ul/ml 4d | 318.06 | 17.594 | <0.001 | Yes |
| NA 4d vs. 25ul/ml 2d | 304.181 | 16.826 | <0.001 | Yes |
| 2.5ul/ml 4d vs. 25ul/ml 3d | 285.619 | 15.8 | <0.001 | Yes |
| 2.5ul/ml 4d vs. 25ul/ml 4d | 285.452 | 15.79 | <0.001 | Yes |
| NA 4d vs. 10ul/ml 4d | 284.95 | 15.763 | <0.001 | Yes |
| 2.5ul/ml 4d vs. 25ul/ml 2d | 271.572 | 15.023 | <0.001 | Yes |
| NA 3d vs. 25ul/ml 3d | 254.682 | 14.088 | <0.001 | Yes |
| NA 3d vs. 25ul/ml 4d | 254.515 | 14.079 | <0.001 | Yes |
| 2.5ul/ml 4d vs. 10ul/ml 4d | 252.341 | 13.959 | <0.001 | Yes |
| NA 4d vs. 25ul/ml 1d | 250.669 | 13.866 | <0.001 | Yes |
| NA 3d vs. 25ul/ml 2d | 240.635 | 13.311 | <0.001 | Yes |
| 2.5ul/ml 3d vs. 25ul/ml 3d | 234.783 | 12.987 | <0.001 | Yes |
| 2.5ul/ml 3d vs. 25ul/ml 4d | 234.615 | 12.978 | <0.001 | Yes |
| NA 4d vs. 10ul/ml 3d | 231.271 | 12.793 | <0.001 | Yes |
| NA 4d vs. 0d | 223.077 | 12.34 | <0.001 | Yes |
| NA 3d vs. 10ul/ml 4d | 221.405 | 12.247 | <0.001 | Yes |
| 2.5ul/ml 3d vs. 25ul/ml 2d | 220.736 | 12.21 | <0.001 | Yes |
| 2.5ul/ml 4d vs. 25ul/ml 1d | 218.06 | 12.062 | <0.001 | Yes |
| 2.5ul/ml 3d vs. 10ul/ml 4d | 201.505 | 11.147 | <0.001 | Yes |
| NA 4d vs. 10ul/ml 2d | 201.171 | 11.128 | <0.001 | Yes |
| NA 2d vs. 25ul/ml 3d | 201.171 | 11.128 | <0.001 | Yes |
| NA 2d vs. 25ul/ml 4d | 201.003 | 11.119 | <0.001 | Yes |
| 2.5ul/ml 4d vs. 10ul/ml 3d | 198.662 | 10.989 | <0.001 | Yes |
| 2.5ul/ml 2d vs. 25ul/ml 3d | 197.324 | 10.915 | <0.001 | Yes |
| 2.5ul/ml 2d vs. 25ul/ml 4d | 197.157 | 10.906 | <0.001 | Yes |
| NA 4d vs. 5ul/ml 1d | 194.147 | 10.74 | <0.001 | Yes |
| 2.5ul/ml 4d vs. 0d | 190.468 | 10.536 | <0.001 | Yes |
| NA 3d vs. 25ul/ml 1d | 187.124 | 10.351 | <0.001 | Yes |
| NA 2d vs. 25ul/ml 2d | 187.124 | 10.351 | <0.001 | Yes |
| NA 4d vs. 5ul/ml 2d | 183.946 | 10.175 | <0.001 | Yes |
| 2.5ul/ml 2d vs. 25ul/ml 2d | 183.278 | 10.138 | <0.001 | Yes |
| 5ul/ml 3d vs. 25ul/ml 3d | 182.441 | 10.092 | <0.001 | Yes |
| 5ul/ml 3d vs. 25ul/ml 4d | 182.274 | 10.083 | <0.001 | Yes |
| NA 4d vs. 10ul/ml 1d | 181.605 | 10.046 | <0.001 | Yes |
| NA 4d vs. 5ul/ml 4d | 179.933 | 9.953 | <0.001 | Yes |
| NA 4d vs. NA 1d | 178.93 | 9.898 | <0.001 | Yes |
| NA 4d vs. 2.5ul/ml 1d | 174.08 | 9.63 | <0.001 | Yes |
| 2.5ul/ml 4d vs. 10ul/ml 2d | 168.562 | 9.324 | <0.001 | Yes |
| 5ul/ml 3d vs. 25ul/ml 2d | 168.395 | 9.315 | <0.001 | Yes |
| NA 2d vs. 10ul/ml 4d | 167.893 | 9.287 | <0.001 | Yes |
| NA 3d vs. 10ul/ml 3d | 167.726 | 9.278 | <0.001 | Yes |
| 2.5ul/ml 3d vs. 25ul/ml 1d | 167.224 | 9.25 | <0.001 | Yes |
| 2.5ul/ml 2d vs. 10ul/ml 4d | 164.047 | 9.075 | <0.001 | Yes |
| 2.5ul/ml 4d vs. 5ul/ml 1d | 161.538 | 8.936 | <0.001 | Yes |
| NA 3d vs. 0d | 159.532 | 8.825 | <0.001 | Yes |
| 2.5ul/ml 4d vs. 5ul/ml 2d | 151.338 | 8.372 | <0.001 | Yes |
| 5ul/ml 3d vs. 10ul/ml 4d | 149.164 | 8.251 | <0.001 | Yes |
| 2.5ul/ml 4d vs. 10ul/ml 1d | 148.997 | 8.242 | <0.001 | Yes |

| | | | | |
|---|---|---|---|---|
| 2.5ul/ml 3d vs. 10ul/ml 3d | 147.826 | 8.177 | <0.001 | Yes |
| 2.5ul/ml 4d vs. 5ul/ml 4d | 147.324 | 8.15 | <0.001 | Yes |
| 2.5ul/ml 4d vs. NA 1d | 146.321 | 8.094 | <0.001 | Yes |
| 2.5ul/ml 1d vs. 25ul/ml 3d | 144.147 | 7.974 | <0.001 | Yes |
| 2.5ul/ml 1d vs. 25ul/ml 4d | 143.98 | 7.965 | <0.001 | Yes |
| 2.5ul/ml 4d vs. 2.5ul/ml 1d | 141.472 | 7.826 | <0.001 | Yes |
| 2.5ul/ml 3d vs. 0d | 139.632 | 7.724 | <0.001 | Yes |
| NA 1d vs. 25ul/ml 3d | 139.298 | 7.706 | <0.001 | Yes |
| NA 1d vs. 25ul/ml 4d | 139.13 | 7.696 | <0.001 | Yes |
| 5ul/ml 4d vs. 25ul/ml 3d | 138.294 | 7.65 | <0.001 | Yes |
| 5ul/ml 4d vs. 25ul/ml 4d | 138.127 | 7.641 | <0.001 | Yes |
| NA 3d vs. 10ul/ml 2d | 137.625 | 7.613 | <0.001 | Yes |
| 10ul/ml 1d vs. 25ul/ml 3d | 136.622 | 7.558 | <0.001 | Yes |
| 10ul/ml 1d vs. 25ul/ml 4d | 136.455 | 7.548 | <0.001 | Yes |
| NA 4d vs. 5ul/ml 3d | 135.786 | 7.511 | <0.001 | Yes |
| 5ul/ml 2d vs. 25ul/ml 3d | 134.281 | 7.428 | <0.001 | Yes |
| 5ul/ml 2d vs. 25ul/ml 4d | 134.114 | 7.419 | <0.001 | Yes |
| NA 2d vs. 25ul/ml 1d | 133.612 | 7.391 | <0.001 | Yes |
| NA 3d vs. 5ul/ml 1d | 130.602 | 7.225 | <0.001 | Yes |
| 2.5ul/ml 1d vs. 25ul/ml 2d | 130.1 | 7.197 | <0.001 | Yes |
| 2.5ul/ml 2d vs. 25ul/ml 1d | 129.766 | 7.178 | <0.001 | Yes |
| NA 1d vs. 25ul/ml 2d | 125.251 | 6.929 | <0.001 | Yes |
| 5ul/ml 4d vs. 25ul/ml 2d | 124.247 | 6.873 | <0.001 | Yes |
| 5ul/ml 1d vs. 25ul/ml 3d | 124.08 | 6.864 | <0.001 | Yes |
| 5ul/ml 1d vs. 25ul/ml 4d | 123.913 | 6.855 | <0.001 | Yes |
| 10ul/ml 1d vs. 25ul/ml 2d | 122.575 | 6.781 | <0.001 | Yes |
| NA 4d vs. 2.5ul/ml 2d | 120.903 | 6.688 | <0.001 | Yes |
| NA 3d vs. 5ul/ml 2d | 120.401 | 6.66 | <0.001 | Yes |
| 5ul/ml 2d vs. 25ul/ml 2d | 120.234 | 6.651 | <0.001 | Yes |
| NA 3d vs. 10ul/ml 1d | 118.06 | 6.531 | <0.001 | Yes |
| 2.5ul/ml 3d vs. 10ul/ml 2d | 117.726 | 6.512 | <0.001 | Yes |
| NA 4d vs. NA 2d | 117.057 | 6.475 | <0.001 | Yes |
| 10ul/ml 2d vs. 25ul/ml 3d | 117.057 | 6.475 | <0.001 | Yes |
| 10ul/ml 2d vs. 25ul/ml 4d | 116.89 | 6.466 | <0.001 | Yes |
| NA 3d vs. 5ul/ml 4d | 116.388 | 6.438 | <0.001 | Yes |
| NA 3d vs. NA 1d | 115.385 | 6.383 | <0.001 | Yes |
| 5ul/ml 3d vs. 25ul/ml 1d | 114.883 | 6.355 | <0.001 | Yes |
| NA 2d vs. 10ul/ml 3d | 114.214 | 6.318 | <0.001 | Yes |
| 2.5ul/ml 1d vs. 10ul/ml 4d | 110.87 | 6.133 | <0.001 | Yes |
| 2.5ul/ml 3d vs. 5ul/ml 1d | 110.702 | 6.124 | <0.001 | Yes |
| NA 3d vs. 2.5ul/ml 1d | 110.535 | 6.114 | <0.001 | Yes |
| 2.5ul/ml 2d vs. 10ul/ml 3d | 110.368 | 6.105 | <0.001 | Yes |
| 5ul/ml 1d vs. 25ul/ml 2d | 110.033 | 6.087 | <0.001 | Yes |
| NA 1d vs. 10ul/ml 4d | 106.02 | 5.865 | <0.001 | Yes |
| NA 2d vs. 0d | 106.02 | 5.865 | <0.001 | Yes |
| 5ul/ml 4d vs. 10ul/ml 4d | 105.017 | 5.809 | <0.001 | Yes |
| 10ul/ml 1d vs. 10ul/ml 4d | 103.344 | 5.717 | <0.001 | Yes |
| 2.5ul/ml 4d vs. 5ul/ml 3d | 103.177 | 5.707 | <0.001 | Yes |
| 10ul/ml 2d vs. 25ul/ml 2d | 103.01 | 5.698 | <0.001 | Yes |

| | | | | |
|---|---|---|---|---|
| 2.5ul/ml 2d vs. 0d | 102.174 | 5.652 | <0.001 | Yes |
| 5ul/ml 2d vs. 10ul/ml 4d | 101.003 | 5.587 | <0.001 | Yes |
| 2.5ul/ml 3d vs. 5ul/ml 2d | 100.502 | 5.559 | <0.001 | Yes |
| 2.5ul/ml 3d vs. 10ul/ml 1d | 98.161 | 5.43 | <0.001 | Yes |
| 2.5ul/ml 3d vs. 5ul/ml 4d | 96.488 | 5.337 | <0.001 | Yes |
| 5ul/ml 3d vs. 10ul/ml 3d | 95.485 | 5.282 | <0.001 | Yes |
| 2.5ul/ml 3d vs. NA 1d | 95.485 | 5.282 | <0.001 | Yes |
| 0d vs. 25ul/ml 3d | 95.151 | 5.263 | <0.001 | Yes |
| 0d vs. 25ul/ml 4d | 94.983 | 5.254 | <0.001 | Yes |
| 5ul/ml 1d vs. 10ul/ml 4d | 90.803 | 5.023 | 0.001 | Yes |
| 2.5ul/ml 3d vs. 2.5ul/ml 1d | 90.635 | 5.014 | 0.001 | Yes |
| 2.5ul/ml 4d vs. 2.5ul/ml 2d | 88.294 | 4.884 | 0.002 | Yes |
| 5ul/ml 3d vs. 0d | 87.291 | 4.829 | 0.002 | Yes |
| 10ul/ml 3d vs. 25ul/ml 3d | 86.957 | 4.81 | 0.002 | Yes |
| 10ul/ml 3d vs. 25ul/ml 4d | 86.789 | 4.801 | 0.002 | Yes |
| 2.5ul/ml 4d vs. NA 2d | 84.448 | 4.671 | 0.003 | Yes |
| NA 2d vs. 10ul/ml 2d | 84.114 | 4.653 | 0.003 | Yes |
| 10ul/ml 2d vs. 10ul/ml 4d | 83.779 | 4.634 | 0.003 | Yes |
| NA 4d vs. 2.5ul/ml 3d | 83.445 | 4.616 | 0.003 | Yes |
| 0d vs. 25ul/ml 2d | 81.104 | 4.486 | 0.005 | Yes |
| 2.5ul/ml 2d vs. 10ul/ml 2d | 80.268 | 4.44 | 0.006 | Yes |
| NA 2d vs. 5ul/ml 1d | 77.09 | 4.264 | 0.01 | Yes |
| 2.5ul/ml 1d vs. 25ul/ml 1d | 76.589 | 4.237 | 0.011 | Yes |
| 2.5ul/ml 2d vs. 5ul/ml 1d | 73.244 | 4.052 | 0.019 | Yes |
| 10ul/ml 3d vs. 25ul/ml 2d | 72.91 | 4.033 | 0.02 | Yes |
| NA 3d vs. 5ul/ml 3d | 72.241 | 3.996 | 0.022 | Yes |
| NA 1d vs. 25ul/ml 1d | 71.739 | 3.968 | 0.024 | Yes |
| 5ul/ml 4d vs. 25ul/ml 1d | 70.736 | 3.913 | 0.027 | Yes |
| 10ul/ml 1d vs. 25ul/ml 1d | 69.064 | 3.82 | 0.036 | Yes |
| 25ul/ml 1d vs. 25ul/ml 3d | 67.559 | 3.737 | 0.045 | Yes |
| 25ul/ml 1d vs. 25ul/ml 4d | 67.391 | 3.728 | 0.046 | Yes |
| NA 2d vs. 5ul/ml 2d | 66.89 | 3.7 | 0.049 | Yes |
| 5ul/ml 2d vs. 25ul/ml 1d | 66.722 | 3.691 | 0.05 | Yes |
| 5ul/ml 3d vs. 10ul/ml 2d | 65.385 | 3.617 | 0.061 | No |
| NA 2d vs. 10ul/ml 1d | 64.548 | 3.571 | 0.068 | No |
| NA 4d vs. NA 3d | 63.545 | 3.515 | 0.079 | No |
| 2.5ul/ml 2d vs. 5ul/ml 2d | 63.043 | 3.487 | 0.084 | No |
| NA 2d vs. 5ul/ml 4d | 62.876 | 3.478 | 0.085 | No |
| NA 2d vs. NA 1d | 61.873 | 3.423 | 0.098 | No |
| 0d vs. 10ul/ml 4d | 61.873 | 3.423 | 0.097 | No |
| 2.5ul/ml 2d vs. 10ul/ml 1d | 60.702 | 3.358 | 0.114 | No |
| 2.5ul/ml 2d vs. 5ul/ml 4d | 59.03 | 3.265 | 0.144 | No |
| 5ul/ml 3d vs. 5ul/ml 1d | 58.361 | 3.228 | 0.156 | No |
| 2.5ul/ml 2d vs. NA 1d | 58.027 | 3.21 | 0.161 | No |
| NA 3d vs. 2.5ul/ml 2d | 57.358 | 3.173 | 0.175 | No |
| 2.5ul/ml 1d vs. 10ul/ml 3d | 57.191 | 3.164 | 0.177 | No |
| NA 2d vs. 2.5ul/ml 1d | 57.023 | 3.154 | 0.178 | No |
| 5ul/ml 1d vs. 25ul/ml 1d | 56.522 | 3.127 | 0.188 | No |
| 10ul/ml 3d vs. 10ul/ml 4d | 53.679 | 2.969 | 0.27 | No |

| | | | | |
|---|---|---|---|---|
| NA 3d vs. NA 2d | 53.512 | 2.96 | 0.273 | No |
| 25ul/ml 1d vs. 25ul/ml 2d | 53.512 | 2.96 | 0.269 | No |
| 2.5ul/ml 2d vs. 2.5ul/ml 1d | 53.177 | 2.942 | 0.277 | No |
| NA 1d vs. 10ul/ml 3d | 52.341 | 2.895 | 0.303 | No |
| 2.5ul/ml 3d vs. 5ul/ml 3d | 52.341 | 2.895 | 0.298 | No |
| 5ul/ml 4d vs. 10ul/ml 3d | 51.338 | 2.84 | 0.332 | No |
| 2.5ul/ml 4d vs. 2.5ul/ml 3d | 50.836 | 2.812 | 0.347 | No |
| 10ul/ml 1d vs. 10ul/ml 3d | 49.666 | 2.747 | 0.391 | No |
| 10ul/ml 2d vs. 25ul/ml 1d | 49.498 | 2.738 | 0.393 | No |
| 2.5ul/ml 1d vs. 0d | 48.997 | 2.71 | 0.409 | No |
| 5ul/ml 3d vs. 5ul/ml 2d | 48.161 | 2.664 | 0.441 | No |
| 5ul/ml 2d vs. 10ul/ml 3d | 47.324 | 2.618 | 0.473 | No |
| 5ul/ml 3d vs. 10ul/ml 1d | 45.819 | 2.535 | 0.539 | No |
| 5ul/ml 3d vs. 5ul/ml 4d | 44.147 | 2.442 | 0.615 | No |
| NA 1d vs. 0d | 44.147 | 2.442 | 0.607 | No |
| 5ul/ml 3d vs. NA 1d | 43.144 | 2.387 | 0.649 | No |
| 5ul/ml 4d vs. 0d | 43.144 | 2.387 | 0.641 | No |
| 10ul/ml 1d vs. 0d | 41.472 | 2.294 | 0.714 | No |
| 5ul/ml 2d vs. 0d | 39.13 | 2.165 | 0.809 | No |
| 5ul/ml 3d vs. 2.5ul/ml 1d | 38.294 | 2.118 | 0.835 | No |
| 2.5ul/ml 3d vs. 2.5ul/ml 2d | 37.458 | 2.072 | 0.858 | No |
| 5ul/ml 1d vs. 10ul/ml 3d | 37.124 | 2.054 | 0.863 | No |
| 25ul/ml 1d vs. 10ul/ml 4d | 34.281 | 1.896 | 0.936 | No |
| 2.5ul/ml 3d vs. NA 2d | 33.612 | 1.859 | 0.945 | No |
| 10ul/ml 4d vs. 25ul/ml 3d | 33.278 | 1.841 | 0.947 | No |
| 10ul/ml 4d vs. 25ul/ml 4d | 33.11 | 1.832 | 0.946 | No |
| NA 4d vs. 2.5ul/ml 4d | 32.609 | 1.804 | 0.951 | No |
| 2.5ul/ml 4d vs. NA 3d | 30.936 | 1.711 | 0.972 | No |
| 10ul/ml 2d vs. 10ul/ml 3d | 30.1 | 1.665 | 0.978 | No |
| 5ul/ml 1d vs. 0d | 28.93 | 1.6 | 0.985 | No |
| 0d vs. 25ul/ml 1d | 27.592 | 1.526 | 0.991 | No |
| 2.5ul/ml 1d vs. 10ul/ml 2d | 27.09 | 1.499 | 0.992 | No |
| NA 1d vs. 10ul/ml 2d | 22.241 | 1.23 | 1 | No |
| 10ul/ml 2d vs. 0d | 21.906 | 1.212 | 1 | No |
| 5ul/ml 4d vs. 10ul/ml 2d | 21.237 | 1.175 | 1 | No |
| 2.5ul/ml 1d vs. 5ul/ml 1d | 20.067 | 1.11 | 1 | No |
| NA 3d vs. 2.5ul/ml 3d | 19.9 | 1.101 | 1 | No |
| 10ul/ml 1d vs. 10ul/ml 2d | 19.565 | 1.082 | 1 | No |
| 10ul/ml 3d vs. 25ul/ml 1d | 19.398 | 1.073 | 1 | No |
| 10ul/ml 4d vs. 25ul/ml 2d | 19.231 | 1.064 | 1 | No |
| NA 2d vs. 5ul/ml 3d | 18.729 | 1.036 | 1 | No |
| 5ul/ml 2d vs. 10ul/ml 2d | 17.224 | 0.953 | 1 | No |
| NA 1d vs. 5ul/ml 1d | 15.217 | 0.842 | 1 | No |
| 2.5ul/ml 2d vs. 5ul/ml 3d | 14.883 | 0.823 | 1 | No |
| 5ul/ml 4d vs. 5ul/ml 1d | 14.214 | 0.786 | 1 | No |
| 25ul/ml 2d vs. 25ul/ml 3d | 14.047 | 0.777 | 1 | No |
| 25ul/ml 2d vs. 25ul/ml 4d | 13.88 | 0.768 | 1 | No |
| 10ul/ml 1d vs. 5ul/ml 1d | 12.542 | 0.694 | 1 | No |
| 5ul/ml 2d vs. 5ul/ml 1d | 10.201 | 0.564 | 1 | No |

| | | | | |
|---|---|---|---|---|
| 2.5ul/ml 1d vs. 5ul/ml 2d | 9.866 | 0.546 | 1 | No |
| 0d vs. 10ul/ml 3d | 8.194 | 0.453 | 1 | No |
| 2.5ul/ml 1d vs. 10ul/ml 1d | 7.525 | 0.416 | 1 | No |
| 5ul/ml 1d vs. 10ul/ml 2d | 7.023 | 0.389 | 1 | No |
| 2.5ul/ml 1d vs. 5ul/ml 4d | 5.853 | 0.324 | 1 | No |
| NA 1d vs. 5ul/ml 2d | 5.017 | 0.278 | 1 | No |
| 2.5ul/ml 1d vs. NA 1d | 4.849 | 0.268 | 1 | No |
| 5ul/ml 4d vs. 5ul/ml 2d | 4.013 | 0.222 | 1 | No |
| NA 2d vs. 2.5ul/ml 2d | 3.846 | 0.213 | 1 | No |
| NA 1d vs. 10ul/ml 1d | 2.676 | 0.148 | 1 | No |
| 10ul/ml 1d vs. 5ul/ml 2d | 2.341 | 0.13 | 1 | No |
| 5ul/ml 4d vs. 10ul/ml 1d | 1.672 | 0.0925 | 1 | No |
| NA 1d vs. 5ul/ml 4d | 1.003 | 0.0555 | 0.998 | No |
| 25ul/ml 4d vs. 25ul/ml 3d | 1.67E-01 | 9.25E-03 | 0.993 | No |

Figure 2.2 Irgacure2959

One Way Analysis of Variance

Data source: Data 1 in Irgacure 2959 viability

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.147)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 0d | 3 | 0 | 100 | 6.811 | 3.932 |
| NA 1d | 3 | 0 | 141.472 | 6.56 | 3.788 |
| 1mg/ml 1d | 3 | 0 | 144.649 | 13.76 | 7.944 |
| 2mg/ml 1d | 3 | 0 | 96.488 | 7.396 | 4.27 |
| 5mg/ml 1d | 3 | 0 | 8.528 | 1.003 | 0.579 |
| 10mg/ml 1d | 3 | 0 | 5.518 | 2.299 | 1.327 |
| NA 2d | 3 | 0 | 207.191 | 16.147 | 9.323 |
| 1mg/ml 2d | 3 | 0 | 208.696 | 9.291 | 5.364 |
| 2mg/ml 2d | 3 | 0 | 148.662 | 21.132 | 12.2 |
| 5mg/ml 2d | 3 | 0 | 2.843 | 0.766 | 0.442 |
| 10mg/ml 2d | 3 | 0 | 5.351 | 2.262 | 1.306 |
| NA 3d | 3 | 0 | 265.552 | 14.491 | 8.366 |
| 1mg/ml 3d | 3 | 0 | 236.789 | 22.17 | 12.8 |
| 2mg/ml 3d | 3 | 0 | 193.478 | 7.206 | 4.16 |
| 5mg/ml 3d | 3 | 0 | 7.358 | 1.263 | 0.729 |
| 10mg/ml 3d | 3 | 0 | 9.699 | 1.159 | 0.669 |
| NA 4d | 3 | 0 | 350.334 | 17.668 | 10.201 |
| 1mg/ml 4d | 3 | 0 | 348.328 | 53.584 | 30.936 |
| 2mg/ml 4d | 3 | 0 | 220.736 | 14.618 | 8.439 |
| 5mg/ml 4d | 3 | 0 | 5.184 | 2.262 | 1.306 |
| 10mg/ml 4d | 3 | 0 | 2.508 | 2.508 | 1.448 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 20 | 829222.5 | 41461.13 | 164.16 | <0.001 |
| Residual | 42 | 10607.77 | 252.566 | | |
| Total | 62 | 839830.3 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| NA 4d vs. 10mg/ml 4d | 347.826 | 26.805 | <0.001 | Yes |
| NA 4d vs. 5mg/ml 2d | 347.492 | 26.78 | <0.001 | Yes |
| 1mg/ml 4d vs. 10mg/ml 4d | 345.819 | 26.651 | <0.001 | Yes |
| 1mg/ml 4d vs. 5mg/ml 2d | 345.485 | 26.625 | <0.001 | Yes |
| NA 4d vs. 5mg/ml 4d | 345.151 | 26.599 | <0.001 | Yes |
| NA 4d vs. 10mg/ml 2d | 344.983 | 26.586 | <0.001 | Yes |
| NA 4d vs. 10mg/ml 1d | 344.816 | 26.573 | <0.001 | Yes |
| 1mg/ml 4d vs. 5mg/ml 4d | 343.144 | 26.444 | <0.001 | Yes |
| NA 4d vs. 5mg/ml 3d | 342.977 | 26.432 | <0.001 | Yes |
| 1mg/ml 4d vs. 10mg/ml 2d | 342.977 | 26.432 | <0.001 | Yes |
| 1mg/ml 4d vs. 10mg/ml 1d | 342.809 | 26.419 | <0.001 | Yes |
| NA 4d vs. 5mg/ml 1d | 341.806 | 26.341 | <0.001 | Yes |
| 1mg/ml 4d vs. 5mg/ml 3d | 340.97 | 26.277 | <0.001 | Yes |
| NA 4d vs. 10mg/ml 3d | 340.635 | 26.251 | <0.001 | Yes |
| 1mg/ml 4d vs. 5mg/ml 1d | 339.799 | 26.187 | <0.001 | Yes |
| 1mg/ml 4d vs. 10mg/ml 3d | 338.629 | 26.096 | <0.001 | Yes |
| NA 3d vs. 10mg/ml 4d | 263.043 | 20.271 | <0.001 | Yes |
| NA 3d vs. 5mg/ml 2d | 262.709 | 20.246 | <0.001 | Yes |
| NA 3d vs. 5mg/ml 4d | 260.368 | 20.065 | <0.001 | Yes |
| NA 3d vs. 10mg/ml 2d | 260.201 | 20.052 | <0.001 | Yes |
| NA 3d vs. 10mg/ml 1d | 260.033 | 20.04 | <0.001 | Yes |
| NA 3d vs. 5mg/ml 3d | 258.194 | 19.898 | <0.001 | Yes |
| NA 3d vs. 5mg/ml 1d | 257.023 | 19.808 | <0.001 | Yes |
| NA 3d vs. 10mg/ml 3d | 255.853 | 19.717 | <0.001 | Yes |
| NA 4d vs. 2mg/ml 1d | 253.846 | 19.563 | <0.001 | Yes |
| 1mg/ml 4d vs. 2mg/ml 1d | 251.839 | 19.408 | <0.001 | Yes |
| NA 4d vs. 0d | 250.334 | 19.292 | <0.001 | Yes |
| 1mg/ml 4d vs. 0d | 248.328 | 19.137 | <0.001 | Yes |
| 1mg/ml 3d vs. 10mg/ml 4d | 234.281 | 18.055 | <0.001 | Yes |
| 1mg/ml 3d vs. 5mg/ml 2d | 233.946 | 18.029 | <0.001 | Yes |
| 1mg/ml 3d vs. 5mg/ml 4d | 231.605 | 17.849 | <0.001 | Yes |
| 1mg/ml 3d vs. 10mg/ml 2d | 231.438 | 17.836 | <0.001 | Yes |
| 1mg/ml 3d vs. 10mg/ml 1d | 231.271 | 17.823 | <0.001 | Yes |
| 1mg/ml 3d vs. 5mg/ml 3d | 229.431 | 17.681 | <0.001 | Yes |
| 1mg/ml 3d vs. 5mg/ml 1d | 228.261 | 17.591 | <0.001 | Yes |
| 1mg/ml 3d vs. 10mg/ml 3d | 227.09 | 17.501 | <0.001 | Yes |
| 2mg/ml 4d vs. 10mg/ml 4d | 218.227 | 16.818 | <0.001 | Yes |
| 2mg/ml 4d vs. 5mg/ml 2d | 217.893 | 16.792 | <0.001 | Yes |
| 2mg/ml 4d vs. 5mg/ml 4d | 215.552 | 16.612 | <0.001 | Yes |
| 2mg/ml 4d vs. 10mg/ml 2d | 215.385 | 16.599 | <0.001 | Yes |
| 2mg/ml 4d vs. 10mg/ml 1d | 215.217 | 16.586 | <0.001 | Yes |
| 2mg/ml 4d vs. 5mg/ml 3d | 213.378 | 16.444 | <0.001 | Yes |
| 2mg/ml 4d vs. 5mg/ml 1d | 212.207 | 16.354 | <0.001 | Yes |
| 2mg/ml 4d vs. 10mg/ml 3d | 211.037 | 16.264 | <0.001 | Yes |
| NA 4d vs. NA 1d | 208.863 | 16.096 | <0.001 | Yes |
| 1mg/ml 4d vs. NA 1d | 206.856 | 15.941 | <0.001 | Yes |
| 1mg/ml 2d vs. 10mg/ml 4d | 206.187 | 15.89 | <0.001 | Yes |
| 1mg/ml 2d vs. 5mg/ml 2d | 205.853 | 15.864 | <0.001 | Yes |
| NA 4d vs. 1mg/ml 1d | 205.686 | 15.851 | <0.001 | Yes |

| | | | | |
|---|---|---|---|---|
| NA 2d vs. 10mg/ml 4d | 204.682 | 15.774 | <0.001 | Yes |
| NA 2d vs. 5mg/ml 2d | 204.348 | 15.748 | <0.001 | Yes |
| 1mg/ml 4d vs. 1mg/ml 1d | 203.679 | 15.697 | <0.001 | Yes |
| 1mg/ml 2d vs. 5mg/ml 4d | 203.512 | 15.684 | <0.001 | Yes |
| 1mg/ml 2d vs. 10mg/ml 2d | 203.344 | 15.671 | <0.001 | Yes |
| 1mg/ml 2d vs. 10mg/ml 1d | 203.177 | 15.658 | <0.001 | Yes |
| NA 2d vs. 5mg/ml 4d | 202.007 | 15.568 | <0.001 | Yes |
| NA 2d vs. 10mg/ml 2d | 201.839 | 15.555 | <0.001 | Yes |
| NA 4d vs. 2mg/ml 2d | 201.672 | 15.542 | <0.001 | Yes |
| NA 2d vs. 10mg/ml 1d | 201.672 | 15.542 | <0.001 | Yes |
| 1mg/ml 2d vs. 5mg/ml 3d | 201.338 | 15.516 | <0.001 | Yes |
| 1mg/ml 2d vs. 5mg/ml 1d | 200.167 | 15.426 | <0.001 | Yes |
| NA 2d vs. 5mg/ml 3d | 199.833 | 15.4 | <0.001 | Yes |
| 1mg/ml 4d vs. 2mg/ml 2d | 199.666 | 15.387 | <0.001 | Yes |
| 1mg/ml 2d vs. 10mg/ml 3d | 198.997 | 15.336 | <0.001 | Yes |
| NA 2d vs. 5mg/ml 1d | 198.662 | 15.31 | <0.001 | Yes |
| NA 2d vs. 10mg/ml 3d | 197.492 | 15.22 | <0.001 | Yes |
| 2mg/ml 3d vs. 10mg/ml 4d | 190.97 | 14.717 | <0.001 | Yes |
| 2mg/ml 3d vs. 5mg/ml 2d | 190.635 | 14.691 | <0.001 | Yes |
| 2mg/ml 3d vs. 5mg/ml 4d | 188.294 | 14.511 | <0.001 | Yes |
| 2mg/ml 3d vs. 10mg/ml 2d | 188.127 | 14.498 | <0.001 | Yes |
| 2mg/ml 3d vs. 10mg/ml 1d | 187.96 | 14.485 | <0.001 | Yes |
| 2mg/ml 3d vs. 5mg/ml 3d | 186.12 | 14.343 | <0.001 | Yes |
| 2mg/ml 3d vs. 5mg/ml 1d | 184.95 | 14.253 | <0.001 | Yes |
| 2mg/ml 3d vs. 10mg/ml 3d | 183.779 | 14.163 | <0.001 | Yes |
| NA 3d vs. 2mg/ml 1d | 169.064 | 13.029 | <0.001 | Yes |
| NA 3d vs. 0d | 165.552 | 12.758 | <0.001 | Yes |
| NA 4d vs. 2mg/ml 3d | 156.856 | 12.088 | <0.001 | Yes |
| 1mg/ml 4d vs. 2mg/ml 3d | 154.849 | 11.934 | <0.001 | Yes |
| 2mg/ml 2d vs. 10mg/ml 4d | 146.154 | 11.263 | <0.001 | Yes |
| 2mg/ml 2d vs. 5mg/ml 2d | 145.819 | 11.238 | <0.001 | Yes |
| 2mg/ml 2d vs. 5mg/ml 4d | 143.478 | 11.057 | <0.001 | Yes |
| 2mg/ml 2d vs. 10mg/ml 2d | 143.311 | 11.044 | <0.001 | Yes |
| NA 4d vs. NA 2d | 143.144 | 11.031 | <0.001 | Yes |
| 2mg/ml 2d vs. 10mg/ml 1d | 143.144 | 11.031 | <0.001 | Yes |
| 1mg/ml 1d vs. 10mg/ml 4d | 142.14 | 10.954 | <0.001 | Yes |
| 1mg/ml 1d vs. 5mg/ml 2d | 141.806 | 10.928 | <0.001 | Yes |
| NA 4d vs. 1mg/ml 2d | 141.639 | 10.915 | <0.001 | Yes |
| 2mg/ml 2d vs. 5mg/ml 3d | 141.304 | 10.89 | <0.001 | Yes |
| 1mg/ml 4d vs. NA 2d | 141.137 | 10.877 | <0.001 | Yes |
| 1mg/ml 3d vs. 2mg/ml 1d | 140.301 | 10.812 | <0.001 | Yes |
| 2mg/ml 2d vs. 5mg/ml 1d | 140.134 | 10.799 | <0.001 | Yes |
| 1mg/ml 4d vs. 1mg/ml 2d | 139.632 | 10.761 | <0.001 | Yes |
| 1mg/ml 1d vs. 5mg/ml 4d | 139.465 | 10.748 | <0.001 | Yes |
| 1mg/ml 1d vs. 10mg/ml 2d | 139.298 | 10.735 | <0.001 | Yes |
| 1mg/ml 1d vs. 10mg/ml 1d | 139.13 | 10.722 | <0.001 | Yes |
| NA 1d vs. 10mg/ml 4d | 138.963 | 10.709 | <0.001 | Yes |
| 2mg/ml 2d vs. 10mg/ml 3d | 138.963 | 10.709 | <0.001 | Yes |
| NA 1d vs. 5mg/ml 2d | 138.629 | 10.683 | <0.001 | Yes |

| | | | | |
|---|---|---|---|---|
| 1mg/ml 1d vs. 5mg/ml 3d | 137.291 | 10.58 | <0.001 | Yes |
| 1mg/ml 3d vs. 0d | 136.789 | 10.542 | <0.001 | Yes |
| NA 1d vs. 5mg/ml 4d | 136.288 | 10.503 | <0.001 | Yes |
| NA 1d vs. 10mg/ml 2d | 136.12 | 10.49 | <0.001 | Yes |
| 1mg/ml 1d vs. 5mg/ml 1d | 136.12 | 10.49 | <0.001 | Yes |
| NA 1d vs. 10mg/ml 1d | 135.953 | 10.477 | <0.001 | Yes |
| 1mg/ml 1d vs. 10mg/ml 3d | 134.95 | 10.4 | <0.001 | Yes |
| NA 1d vs. 5mg/ml 3d | 134.114 | 10.335 | <0.001 | Yes |
| NA 1d vs. 5mg/ml 1d | 132.943 | 10.245 | <0.001 | Yes |
| NA 1d vs. 10mg/ml 3d | 131.773 | 10.155 | <0.001 | Yes |
| NA 4d vs. 2mg/ml 4d | 129.599 | 9.988 | <0.001 | Yes |
| 1mg/ml 4d vs. 2mg/ml 4d | 127.592 | 9.833 | <0.001 | Yes |
| 2mg/ml 4d vs. 2mg/ml 1d | 124.247 | 9.575 | <0.001 | Yes |
| NA 3d vs. NA 1d | 124.08 | 9.562 | <0.001 | Yes |
| NA 3d vs. 1mg/ml 1d | 120.903 | 9.317 | <0.001 | Yes |
| 2mg/ml 4d vs. 0d | 120.736 | 9.305 | <0.001 | Yes |
| NA 3d vs. 2mg/ml 2d | 116.89 | 9.008 | <0.001 | Yes |
| NA 4d vs. 1mg/ml 3d | 113.545 | 8.75 | <0.001 | Yes |
| 1mg/ml 2d vs. 2mg/ml 1d | 112.207 | 8.647 | <0.001 | Yes |
| 1mg/ml 4d vs. 1mg/ml 3d | 111.538 | 8.596 | <0.001 | Yes |
| NA 2d vs. 2mg/ml 1d | 110.702 | 8.531 | <0.001 | Yes |
| 1mg/ml 2d vs. 0d | 108.696 | 8.377 | <0.001 | Yes |
| NA 2d vs. 0d | 107.191 | 8.261 | <0.001 | Yes |
| 0d vs. 10mg/ml 4d | 97.492 | 7.513 | <0.001 | Yes |
| 0d vs. 5mg/ml 2d | 97.157 | 7.487 | <0.001 | Yes |
| 2mg/ml 3d vs. 2mg/ml 1d | 96.99 | 7.475 | <0.001 | Yes |
| 1mg/ml 3d vs. NA 1d | 95.318 | 7.346 | <0.001 | Yes |
| 0d vs. 5mg/ml 4d | 94.816 | 7.307 | <0.001 | Yes |
| 0d vs. 10mg/ml 2d | 94.649 | 7.294 | <0.001 | Yes |
| 0d vs. 10mg/ml 1d | 94.482 | 7.281 | <0.001 | Yes |
| 2mg/ml 1d vs. 10mg/ml 4d | 93.98 | 7.243 | <0.001 | Yes |
| 2mg/ml 1d vs. 5mg/ml 2d | 93.645 | 7.217 | <0.001 | Yes |
| 2mg/ml 3d vs. 0d | 93.478 | 7.204 | <0.001 | Yes |
| 0d vs. 5mg/ml 3d | 92.642 | 7.139 | <0.001 | Yes |
| 1mg/ml 3d vs. 1mg/ml 1d | 92.14 | 7.101 | <0.001 | Yes |
| 0d vs. 5mg/ml 1d | 91.472 | 7.049 | <0.001 | Yes |
| 2mg/ml 1d vs. 5mg/ml 4d | 91.304 | 7.036 | <0.001 | Yes |
| 2mg/ml 1d vs. 10mg/ml 2d | 91.137 | 7.024 | <0.001 | Yes |
| 2mg/ml 1d vs. 10mg/ml 1d | 90.97 | 7.011 | <0.001 | Yes |
| 0d vs. 10mg/ml 3d | 90.301 | 6.959 | <0.001 | Yes |
| 2mg/ml 1d vs. 5mg/ml 3d | 89.13 | 6.869 | <0.001 | Yes |
| 1mg/ml 3d vs. 2mg/ml 2d | 88.127 | 6.792 | <0.001 | Yes |
| 2mg/ml 1d vs. 5mg/ml 1d | 87.96 | 6.779 | <0.001 | Yes |
| 2mg/ml 1d vs. 10mg/ml 3d | 86.789 | 6.688 | <0.001 | Yes |
| NA 4d vs. NA 3d | 84.783 | 6.534 | <0.001 | Yes |
| 1mg/ml 4d vs. NA 3d | 82.776 | 6.379 | <0.001 | Yes |
| 2mg/ml 4d vs. NA 1d | 79.264 | 6.109 | <0.001 | Yes |
| 2mg/ml 4d vs. 1mg/ml 1d | 76.087 | 5.864 | <0.001 | Yes |
| NA 3d vs. 2mg/ml 3d | 72.074 | 5.554 | <0.001 | Yes |

| | | | | |
|---|---|---|---|---|
| 2mg/ml 4d vs. 2mg/ml 2d | 72.074 | 5.554 | <0.001 | Yes |
| 1mg/ml 2d vs. NA 1d | 67.224 | 5.181 | <0.001 | Yes |
| NA 2d vs. NA 1d | 65.719 | 5.065 | <0.001 | Yes |
| 1mg/ml 2d vs. 1mg/ml 1d | 64.047 | 4.936 | <0.001 | Yes |
| NA 2d vs. 1mg/ml 1d | 62.542 | 4.82 | 0.001 | Yes |
| 1mg/ml 2d vs. 2mg/ml 2d | 60.033 | 4.626 | 0.002 | Yes |
| NA 2d vs. 2mg/ml 2d | 58.528 | 4.511 | 0.003 | Yes |
| NA 3d vs. NA 2d | 58.361 | 4.498 | 0.003 | Yes |
| NA 3d vs. 1mg/ml 2d | 56.856 | 4.382 | 0.004 | Yes |
| 2mg/ml 2d vs. 2mg/ml 1d | 52.174 | 4.021 | 0.013 | Yes |
| 2mg/ml 3d vs. NA 1d | 52.007 | 4.008 | 0.013 | Yes |
| 2mg/ml 3d vs. 1mg/ml 1d | 48.829 | 3.763 | 0.026 | Yes |
| 2mg/ml 2d vs. 0d | 48.662 | 3.75 | 0.027 | Yes |
| 1mg/ml 1d vs. 2mg/ml 1d | 48.161 | 3.712 | 0.03 | Yes |
| NA 1d vs. 2mg/ml 1d | 44.983 | 3.467 | 0.058 | No |
| NA 3d vs. 2mg/ml 4d | 44.816 | 3.454 | 0.059 | No |
| 2mg/ml 3d vs. 2mg/ml 2d | 44.816 | 3.454 | 0.058 | No |
| 1mg/ml 1d vs. 0d | 44.649 | 3.441 | 0.059 | No |
| 1mg/ml 3d vs. 2mg/ml 3d | 43.311 | 3.338 | 0.077 | No |
| NA 1d vs. 0d | 41.472 | 3.196 | 0.11 | No |
| 1mg/ml 3d vs. NA 2d | 29.599 | 2.281 | 0.701 | No |
| NA 3d vs. 1mg/ml 3d | 28.763 | 2.217 | 0.746 | No |
| 1mg/ml 3d vs. 1mg/ml 2d | 28.094 | 2.165 | 0.779 | No |
| 2mg/ml 4d vs. 2mg/ml 3d | 27.258 | 2.101 | 0.818 | No |
| 1mg/ml 3d vs. 2mg/ml 4d | 16.054 | 1.237 | 1 | No |
| 1mg/ml 2d vs. 2mg/ml 3d | 15.217 | 1.173 | 1 | No |
| NA 2d vs. 2mg/ml 3d | 13.712 | 1.057 | 1 | No |
| 2mg/ml 4d vs. NA 2d | 13.545 | 1.044 | 1 | No |
| 2mg/ml 4d vs. 1mg/ml 2d | 12.04 | 0.928 | 1 | No |
| 10mg/ml 3d vs. 10mg/ml 4d | 7.191 | 0.554 | 1 | No |
| 2mg/ml 2d vs. NA 1d | 7.191 | 0.554 | 1 | No |
| 10mg/ml 3d vs. 5mg/ml 2d | 6.856 | 0.528 | 1 | No |
| 5mg/ml 1d vs. 10mg/ml 4d | 6.02 | 0.464 | 1 | No |
| 5mg/ml 1d vs. 5mg/ml 2d | 5.686 | 0.438 | 1 | No |
| 5mg/ml 3d vs. 10mg/ml 4d | 4.849 | 0.374 | 1 | No |
| 5mg/ml 3d vs. 5mg/ml 2d | 4.515 | 0.348 | 1 | No |
| 10mg/ml 3d vs. 5mg/ml 4d | 4.515 | 0.348 | 1 | No |
| 10mg/ml 3d vs. 10mg/ml 2d | 4.348 | 0.335 | 1 | No |
| 10mg/ml 3d vs. 10mg/ml 1d | 4.181 | 0.322 | 1 | No |
| 2mg/ml 2d vs. 1mg/ml 1d | 4.013 | 0.309 | 1 | No |
| 0d vs. 2mg/ml 1d | 3.512 | 0.271 | 1 | No |
| 5mg/ml 1d vs. 5mg/ml 4d | 3.344 | 0.258 | 1 | No |
| 5mg/ml 1d vs. 10mg/ml 2d | 3.177 | 0.245 | 1 | No |
| 1mg/ml 1d vs. NA 1d | 3.177 | 0.245 | 1 | No |
| 10mg/ml 1d vs. 10mg/ml 4d | 3.01 | 0.232 | 1 | No |
| 5mg/ml 1d vs. 10mg/ml 1d | 3.01 | 0.232 | 1 | No |
| 10mg/ml 2d vs. 10mg/ml 4d | 2.843 | 0.219 | 1 | No |
| 10mg/ml 1d vs. 5mg/ml 2d | 2.676 | 0.206 | 1 | No |
| 5mg/ml 4d vs. 10mg/ml 4d | 2.676 | 0.206 | 1 | No |

| | | | | |
|---|---|---|---|---|
| 10mg/ml 2d vs. 5mg/ml 2d | 2.508 | 0.193 | 1 | No |
| 5mg/ml 4d vs. 5mg/ml 2d | 2.341 | 0.18 | 1 | No |
| 10mg/ml 3d vs. 5mg/ml 3d | 2.341 | 0.18 | 1 | No |
| 5mg/ml 3d vs. 5mg/ml 4d | 2.174 | 0.168 | 1 | No |
| NA 4d vs. 1mg/ml 4d | 2.007 | 0.155 | 1 | No |
| 5mg/ml 3d vs. 10mg/ml 2d | 2.007 | 0.155 | 1 | No |
| 5mg/ml 3d vs. 10mg/ml 1d | 1.839 | 0.142 | 1 | No |
| 1mg/ml 2d vs. NA 2d | 1.505 | 0.116 | 1 | No |
| 5mg/ml 1d vs. 5mg/ml 3d | 1.171 | 0.0902 | 1 | No |
| 10mg/ml 3d vs. 5mg/ml 1d | 1.171 | 0.0902 | 1 | No |
| 10mg/ml 1d vs. 5mg/ml 4d | 0.334 | 0.0258 | 1 | No |
| 5mg/ml 2d vs. 10mg/ml 4d | 0.334 | 0.0258 | 1 | No |
| 10mg/ml 1d vs. 10mg/ml 2d | 0.167 | 0.0129 | 1 | No |
| 10mg/ml 2d vs. 5mg/ml 4d | 1.67E-01 | 1.29E-02 | 0.99 | No |

Figure 2.3 DMPA

One Way Analysis of Variance

Data source: Data 1 in DMPA viability

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.414)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 0d | 3 | 0 | 100 | 8.822 | 5.093 |
| NA 1d | 3 | 0 | 144.245 | 6.41 | 3.701 |
| 0.5mg/ml 1d | 3 | 0 | 2.691 | 1.186 | 0.685 |
| 1mg/ml 1d | 3 | 0 | 4.634 | 2.883 | 1.665 |
| 2mg/ml 1d | 3 | 0 | 4.933 | 0.777 | 0.448 |
| 5mg/ml 1d | 3 | 0 | 5.082 | 1.698 | 0.98 |
| NA 2d | 3 | 0 | 211.211 | 7.883 | 4.551 |
| 0.5mg/ml 2d | 3 | 0 | 4.185 | 1.812 | 1.046 |
| 1mg/ml 2d | 3 | 0 | 6.278 | 2.497 | 1.442 |
| 2mg/ml 2d | 3 | 0 | 5.83 | 1.186 | 0.685 |
| 5mg/ml 2d | 3 | 0 | 3.737 | 1.37 | 0.791 |
| NA 3d | 3 | 0 | 274.29 | 13.069 | 7.545 |
| 0.5mg/ml 3d | 3 | 0 | 4.036 | 0 | 0 |
| 1mg/ml 3d | 3 | 0 | 2.541 | 0.518 | 0.299 |
| 2mg/ml 3d | 3 | 0 | 1.046 | 1.812 | 1.046 |
| 5mg/ml 3d | 3 | 0 | 0.598 | 1.036 | 0.598 |
| NA 4d | 3 | 0 | 386.846 | 32.77 | 18.92 |
| 0.5mg/ml 4d | 3 | 0 | 2.84 | 1.867 | 1.078 |
| 1mg/ml 4d | 3 | 0 | 5.082 | 2.55 | 1.472 |
| 2mg/ml 4d | 3 | 0 | 4.783 | 0.933 | 0.539 |
| 5mg/ml 4d | 3 | 0 | 2.242 | 2.055 | 1.186 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 20 | 703765.9 | 35188.29 | 502.197 | <0.001 |
| Residual | 42 | 2942.884 | 70.069 | | |
| Total | 62 | 706708.8 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| NA 4d vs. 5mg/ml 3d | 386.248 | 56.513 | <0.001 | Yes |
| NA 4d vs. 2mg/ml 3d | 385.8 | 56.448 | <0.001 | Yes |
| NA 4d vs. 5mg/ml 4d | 384.604 | 56.273 | <0.001 | Yes |
| NA 4d vs. 1mg/ml 3d | 384.305 | 56.229 | <0.001 | Yes |
| NA 4d vs. 0.5mg/ml 1d | 384.155 | 56.207 | <0.001 | Yes |
| NA 4d vs. 0.5mg/ml 4d | 384.006 | 56.185 | <0.001 | Yes |
| NA 4d vs. 5mg/ml 2d | 383.109 | 56.054 | <0.001 | Yes |
| NA 4d vs. 0.5mg/ml 3d | 382.81 | 56.01 | <0.001 | Yes |
| NA 4d vs. 0.5mg/ml 2d | 382.661 | 55.988 | <0.001 | Yes |
| NA 4d vs. 1mg/ml 1d | 382.212 | 55.923 | <0.001 | Yes |
| NA 4d vs. 2mg/ml 4d | 382.063 | 55.901 | <0.001 | Yes |
| NA 4d vs. 2mg/ml 1d | 381.913 | 55.879 | <0.001 | Yes |
| NA 4d vs. 1mg/ml 4d | 381.764 | 55.857 | <0.001 | Yes |
| NA 4d vs. 5mg/ml 1d | 381.764 | 55.857 | <0.001 | Yes |
| NA 4d vs. 2mg/ml 2d | 381.016 | 55.748 | <0.001 | Yes |
| NA 4d vs. 1mg/ml 2d | 380.568 | 55.682 | <0.001 | Yes |
| NA 4d vs. 0d | 286.846 | 41.969 | <0.001 | Yes |
| NA 3d vs. 5mg/ml 3d | 273.692 | 40.045 | <0.001 | Yes |
| NA 3d vs. 2mg/ml 3d | 273.244 | 39.979 | <0.001 | Yes |
| NA 3d vs. 5mg/ml 4d | 272.048 | 39.804 | <0.001 | Yes |
| NA 3d vs. 1mg/ml 3d | 271.749 | 39.76 | <0.001 | Yes |
| NA 3d vs. 0.5mg/ml 1d | 271.599 | 39.739 | <0.001 | Yes |
| NA 3d vs. 0.5mg/ml 4d | 271.45 | 39.717 | <0.001 | Yes |
| NA 3d vs. 5mg/ml 2d | 270.553 | 39.586 | <0.001 | Yes |
| NA 3d vs. 0.5mg/ml 3d | 270.254 | 39.542 | <0.001 | Yes |
| NA 3d vs. 0.5mg/ml 2d | 270.105 | 39.52 | <0.001 | Yes |
| NA 3d vs. 1mg/ml 1d | 269.656 | 39.454 | <0.001 | Yes |
| NA 3d vs. 2mg/ml 4d | 269.507 | 39.432 | <0.001 | Yes |
| NA 3d vs. 2mg/ml 1d | 269.357 | 39.411 | <0.001 | Yes |
| NA 3d vs. 5mg/ml 1d | 269.208 | 39.389 | <0.001 | Yes |
| NA 3d vs. 1mg/ml 4d | 269.208 | 39.389 | <0.001 | Yes |
| NA 3d vs. 2mg/ml 2d | 268.46 | 39.279 | <0.001 | Yes |
| NA 3d vs. 1mg/ml 2d | 268.012 | 39.214 | <0.001 | Yes |
| NA 4d vs. NA 1d | 242.601 | 35.496 | <0.001 | Yes |
| NA 2d vs. 5mg/ml 3d | 210.613 | 30.815 | <0.001 | Yes |
| NA 2d vs. 2mg/ml 3d | 210.164 | 30.75 | <0.001 | Yes |
| NA 2d vs. 5mg/ml 4d | 208.969 | 30.575 | <0.001 | Yes |
| NA 2d vs. 1mg/ml 3d | 208.67 | 30.531 | <0.001 | Yes |
| NA 2d vs. 0.5mg/ml 1d | 208.52 | 30.509 | <0.001 | Yes |
| NA 2d vs. 0.5mg/ml 4d | 208.371 | 30.487 | <0.001 | Yes |
| NA 2d vs. 5mg/ml 2d | 207.474 | 30.356 | <0.001 | Yes |
| NA 2d vs. 0.5mg/ml 3d | 207.175 | 30.312 | <0.001 | Yes |
| NA 2d vs. 0.5mg/ml 2d | 207.025 | 30.291 | <0.001 | Yes |
| NA 2d vs. 1mg/ml 1d | 206.577 | 30.225 | <0.001 | Yes |
| NA 2d vs. 2mg/ml 4d | 206.428 | 30.203 | <0.001 | Yes |
| NA 2d vs. 2mg/ml 1d | 206.278 | 30.181 | <0.001 | Yes |
| NA 2d vs. 5mg/ml 1d | 206.129 | 30.159 | <0.001 | Yes |
| NA 2d vs. 1mg/ml 4d | 206.129 | 30.159 | <0.001 | Yes |
| NA 2d vs. 2mg/ml 2d | 205.381 | 30.05 | <0.001 | Yes |

| | | | | |
|---|---|---|---|---|
| NA 2d vs. 1mg/ml 2d | 204.933 | 29.984 | <0.001 | Yes |
| NA 4d vs. NA 2d | 175.635 | 25.698 | <0.001 | Yes |
| NA 3d vs. 0d | 174.29 | 25.501 | <0.001 | Yes |
| NA 1d vs. 5mg/ml 3d | 143.647 | 21.017 | <0.001 | Yes |
| NA 1d vs. 2mg/ml 3d | 143.199 | 20.952 | <0.001 | Yes |
| NA 1d vs. 5mg/ml 4d | 142.003 | 20.777 | <0.001 | Yes |
| NA 1d vs. 1mg/ml 3d | 141.704 | 20.733 | <0.001 | Yes |
| NA 1d vs. 0.5mg/ml 1d | 141.555 | 20.711 | <0.001 | Yes |
| NA 1d vs. 0.5mg/ml 4d | 141.405 | 20.689 | <0.001 | Yes |
| NA 1d vs. 5mg/ml 2d | 140.508 | 20.558 | <0.001 | Yes |
| NA 1d vs. 0.5mg/ml 3d | 140.209 | 20.514 | <0.001 | Yes |
| NA 1d vs. 0.5mg/ml 2d | 140.06 | 20.493 | <0.001 | Yes |
| NA 1d vs. 1mg/ml 1d | 139.611 | 20.427 | <0.001 | Yes |
| NA 1d vs. 2mg/ml 4d | 139.462 | 20.405 | <0.001 | Yes |
| NA 1d vs. 2mg/ml 1d | 139.312 | 20.383 | <0.001 | Yes |
| NA 1d vs. 5mg/ml 1d | 139.163 | 20.361 | <0.001 | Yes |
| NA 1d vs. 1mg/ml 4d | 139.163 | 20.361 | <0.001 | Yes |
| NA 1d vs. 2mg/ml 2d | 138.416 | 20.252 | <0.001 | Yes |
| NA 1d vs. 1mg/ml 2d | 137.967 | 20.186 | <0.001 | Yes |
| NA 3d vs. NA 1d | 130.045 | 19.027 | <0.001 | Yes |
| NA 4d vs. NA 3d | 112.556 | 16.468 | <0.001 | Yes |
| NA 2d vs. 0d | 111.211 | 16.272 | <0.001 | Yes |
| 0d vs. 5mg/ml 3d | 99.402 | 14.544 | <0.001 | Yes |
| 0d vs. 2mg/ml 3d | 98.954 | 14.478 | <0.001 | Yes |
| 0d vs. 5mg/ml 4d | 97.758 | 14.303 | <0.001 | Yes |
| 0d vs. 1mg/ml 3d | 97.459 | 14.26 | <0.001 | Yes |
| 0d vs. 0.5mg/ml 1d | 97.309 | 14.238 | <0.001 | Yes |
| 0d vs. 0.5mg/ml 4d | 97.16 | 14.216 | <0.001 | Yes |
| 0d vs. 5mg/ml 2d | 96.263 | 14.085 | <0.001 | Yes |
| 0d vs. 0.5mg/ml 3d | 95.964 | 14.041 | <0.001 | Yes |
| 0d vs. 0.5mg/ml 2d | 95.815 | 14.019 | <0.001 | Yes |
| 0d vs. 1mg/ml 1d | 95.366 | 13.953 | <0.001 | Yes |
| 0d vs. 2mg/ml 4d | 95.217 | 13.931 | <0.001 | Yes |
| 0d vs. 2mg/ml 1d | 95.067 | 13.91 | <0.001 | Yes |
| 0d vs. 5mg/ml 1d | 94.918 | 13.888 | <0.001 | Yes |
| 0d vs. 1mg/ml 4d | 94.918 | 13.888 | <0.001 | Yes |
| 0d vs. 2mg/ml 2d | 94.17 | 13.778 | <0.001 | Yes |
| 0d vs. 1mg/ml 2d | 93.722 | 13.713 | <0.001 | Yes |
| NA 2d vs. NA 1d | 66.966 | 9.798 | <0.001 | Yes |
| NA 3d vs. NA 2d | 63.079 | 9.229 | <0.001 | Yes |
| NA 1d vs. 0d | 44.245 | 6.474 | <0.001 | Yes |
| 1mg/ml 2d vs. 5mg/ml 3d | 5.68 | 0.831 | 1 | No |
| 1mg/ml 2d vs. 2mg/ml 3d | 5.232 | 0.765 | 1 | No |
| 2mg/ml 2d vs. 5mg/ml 3d | 5.232 | 0.765 | 1 | No |
| 2mg/ml 2d vs. 2mg/ml 3d | 4.783 | 0.7 | 1 | No |
| 1mg/ml 4d vs. 5mg/ml 3d | 4.484 | 0.656 | 1 | No |
| 5mg/ml 1d vs. 5mg/ml 3d | 4.484 | 0.656 | 1 | No |
| 2mg/ml 1d vs. 5mg/ml 3d | 4.335 | 0.634 | 1 | No |
| 2mg/ml 4d vs. 5mg/ml 3d | 4.185 | 0.612 | 1 | No |

| | | | | |
|---|---|---|---|---|
| 5mg/ml 1d vs. 2mg/ml 3d | 4.036 | 0.591 | 1 | No |
| 1mg/ml 2d vs. 5mg/ml 4d | 4.036 | 0.591 | 1 | No |
| 1mg/ml 4d vs. 2mg/ml 3d | 4.036 | 0.591 | 1 | No |
| 1mg/ml 1d vs. 5mg/ml 3d | 4.036 | 0.591 | 1 | No |
| 2mg/ml 1d vs. 2mg/ml 3d | 3.886 | 0.569 | 1 | No |
| 2mg/ml 4d vs. 2mg/ml 3d | 3.737 | 0.547 | 1 | No |
| 1mg/ml 2d vs. 1mg/ml 3d | 3.737 | 0.547 | 1 | No |
| 0.5mg/ml 2d vs. 5mg/ml 3d | 3.587 | 0.525 | 1 | No |
| 2mg/ml 2d vs. 5mg/ml 4d | 3.587 | 0.525 | 1 | No |
| 1mg/ml 1d vs. 2mg/ml 3d | 3.587 | 0.525 | 1 | No |
| 1mg/ml 2d vs. 0.5mg/ml 1d | 3.587 | 0.525 | 1 | No |
| 0.5mg/ml 3d vs. 5mg/ml 3d | 3.438 | 0.503 | 1 | No |
| 1mg/ml 2d vs. 0.5mg/ml 4d | 3.438 | 0.503 | 1 | No |
| 2mg/ml 2d vs. 1mg/ml 3d | 3.288 | 0.481 | 1 | No |
| 2mg/ml 2d vs. 0.5mg/ml 1d | 3.139 | 0.459 | 1 | No |
| 0.5mg/ml 2d vs. 2mg/ml 3d | 3.139 | 0.459 | 1 | No |
| 5mg/ml 2d vs. 5mg/ml 3d | 3.139 | 0.459 | 1 | No |
| 2mg/ml 2d vs. 0.5mg/ml 4d | 2.99 | 0.437 | 1 | No |
| 0.5mg/ml 3d vs. 2mg/ml 3d | 2.99 | 0.437 | 1 | No |
| 5mg/ml 1d vs. 5mg/ml 4d | 2.84 | 0.416 | 1 | No |
| 1mg/ml 4d vs. 5mg/ml 4d | 2.84 | 0.416 | 1 | No |
| 2mg/ml 1d vs. 5mg/ml 4d | 2.691 | 0.394 | 1 | No |
| 5mg/ml 2d vs. 2mg/ml 3d | 2.691 | 0.394 | 1 | No |
| 1mg/ml 4d vs. 1mg/ml 3d | 2.541 | 0.372 | 1 | No |
| 2mg/ml 4d vs. 5mg/ml 4d | 2.541 | 0.372 | 1 | No |
| 1mg/ml 2d vs. 5mg/ml 2d | 2.541 | 0.372 | 1 | No |
| 5mg/ml 1d vs. 1mg/ml 3d | 2.541 | 0.372 | 1 | No |
| 1mg/ml 4d vs. 0.5mg/ml 1d | 2.392 | 0.35 | 1 | No |
| 1mg/ml 1d vs. 5mg/ml 4d | 2.392 | 0.35 | 1 | No |
| 2mg/ml 1d vs. 1mg/ml 3d | 2.392 | 0.35 | 1 | No |
| 5mg/ml 1d vs. 0.5mg/ml 1d | 2.392 | 0.35 | 1 | No |
| 2mg/ml 4d vs. 1mg/ml 3d | 2.242 | 0.328 | 1 | No |
| 0.5mg/ml 4d vs. 5mg/ml 3d | 2.242 | 0.328 | 1 | No |
| 2mg/ml 1d vs. 0.5mg/ml 1d | 2.242 | 0.328 | 1 | No |
| 1mg/ml 2d vs. 0.5mg/ml 3d | 2.242 | 0.328 | 1 | No |
| 1mg/ml 4d vs. 0.5mg/ml 4d | 2.242 | 0.328 | 1 | No |
| 5mg/ml 1d vs. 0.5mg/ml 4d | 2.242 | 0.328 | 1 | No |
| 1mg/ml 1d vs. 1mg/ml 3d | 2.093 | 0.306 | 1 | No |
| 1mg/ml 2d vs. 0.5mg/ml 2d | 2.093 | 0.306 | 1 | No |
| 2mg/ml 1d vs. 0.5mg/ml 4d | 2.093 | 0.306 | 1 | No |
| 2mg/ml 4d vs. 0.5mg/ml 1d | 2.093 | 0.306 | 1 | No |
| 2mg/ml 2d vs. 5mg/ml 2d | 2.093 | 0.306 | 1 | No |
| 0.5mg/ml 1d vs. 5mg/ml 3d | 2.093 | 0.306 | 1 | No |
| 0.5mg/ml 2d vs. 5mg/ml 4d | 1.943 | 0.284 | 1 | No |
| 1mg/ml 1d vs. 0.5mg/ml 1d | 1.943 | 0.284 | 1 | No |
| 2mg/ml 4d vs. 0.5mg/ml 4d | 1.943 | 0.284 | 1 | No |
| 1mg/ml 3d vs. 5mg/ml 3d | 1.943 | 0.284 | 1 | No |
| 0.5mg/ml 3d vs. 5mg/ml 4d | 1.794 | 0.262 | 1 | No |
| 0.5mg/ml 4d vs. 2mg/ml 3d | 1.794 | 0.262 | 1 | No |

| | | | |
|---|---|---|---|
| 1mg/ml 1d vs. 0.5mg/ml 4d | 1.794 | 0.262 | 1 No |
| 2mg/ml 2d vs. 0.5mg/ml 3d | 1.794 | 0.262 | 1 No |
| 5mg/ml 4d vs. 5mg/ml 3d | 1.644 | 0.241 | 1 No |
| 1mg/ml 2d vs. 1mg/ml 1d | 1.644 | 0.241 | 1 No |
| 0.5mg/ml 2d vs. 1mg/ml 3d | 1.644 | 0.241 | 1 No |
| 0.5mg/ml 1d vs. 2mg/ml 3d | 1.644 | 0.241 | 1 No |
| 2mg/ml 2d vs. 0.5mg/ml 2d | 1.644 | 0.241 | 1 No |
| 0.5mg/ml 2d vs. 0.5mg/ml 1d | 1.495 | 0.219 | 1 No |
| 1mg/ml 2d vs. 2mg/ml 4d | 1.495 | 0.219 | 1 No |
| 5mg/ml 2d vs. 5mg/ml 4d | 1.495 | 0.219 | 1 No |
| 1mg/ml 3d vs. 2mg/ml 3d | 1.495 | 0.219 | 1 No |
| 0.5mg/ml 3d vs. 1mg/ml 3d | 1.495 | 0.219 | 1 No |
| 0.5mg/ml 2d vs. 0.5mg/ml 4d | 1.345 | 0.197 | 1 No |
| 1mg/ml 2d vs. 2mg/ml 1d | 1.345 | 0.197 | 1 No |
| 5mg/ml 1d vs. 5mg/ml 2d | 1.345 | 0.197 | 1 No |
| 1mg/ml 4d vs. 5mg/ml 2d | 1.345 | 0.197 | 1 No |
| 0.5mg/ml 3d vs. 0.5mg/ml 1d | 1.345 | 0.197 | 1 No |
| 5mg/ml 4d vs. 2mg/ml 3d | 1.196 | 0.175 | 1 No |
| 5mg/ml 2d vs. 1mg/ml 3d | 1.196 | 0.175 | 1 No |
| 2mg/ml 2d vs. 1mg/ml 1d | 1.196 | 0.175 | 1 No |
| 1mg/ml 2d vs. 5mg/ml 1d | 1.196 | 0.175 | 1 No |
| 0.5mg/ml 3d vs. 0.5mg/ml 4d | 1.196 | 0.175 | 1 No |
| 2mg/ml 1d vs. 5mg/ml 2d | 1.196 | 0.175 | 1 No |
| 1mg/ml 2d vs. 1mg/ml 4d | 1.196 | 0.175 | 1 No |
| 2mg/ml 2d vs. 2mg/ml 4d | 1.046 | 0.153 | 1 No |
| 2mg/ml 4d vs. 5mg/ml 2d | 1.046 | 0.153 | 1 No |
| 5mg/ml 1d vs. 0.5mg/ml 3d | 1.046 | 0.153 | 1 No |
| 5mg/ml 2d vs. 0.5mg/ml 1d | 1.046 | 0.153 | 1 No |
| 1mg/ml 4d vs. 0.5mg/ml 3d | 1.046 | 0.153 | 1 No |
| 2mg/ml 1d vs. 0.5mg/ml 3d | 0.897 | 0.131 | 1 No |
| 1mg/ml 1d vs. 5mg/ml 2d | 0.897 | 0.131 | 1 No |
| 5mg/ml 1d vs. 0.5mg/ml 2d | 0.897 | 0.131 | 1 No |
| 1mg/ml 4d vs. 0.5mg/ml 2d | 0.897 | 0.131 | 1 No |
| 5mg/ml 2d vs. 0.5mg/ml 4d | 0.897 | 0.131 | 1 No |
| 2mg/ml 2d vs. 2mg/ml 1d | 0.897 | 0.131 | 1 No |
| 2mg/ml 1d vs. 0.5mg/ml 2d | 0.747 | 0.109 | 1 No |
| 2mg/ml 2d vs. 5mg/ml 1d | 0.747 | 0.109 | 1 No |
| 2mg/ml 4d vs. 0.5mg/ml 3d | 0.747 | 0.109 | 1 No |
| 2mg/ml 2d vs. 1mg/ml 4d | 0.747 | 0.109 | 1 No |
| 0.5mg/ml 4d vs. 5mg/ml 4d | 0.598 | 0.0875 | 1 No |
| 1mg/ml 1d vs. 0.5mg/ml 3d | 0.598 | 0.0875 | 1 No |
| 2mg/ml 4d vs. 0.5mg/ml 2d | 0.598 | 0.0875 | 1 No |
| 0.5mg/ml 1d vs. 5mg/ml 4d | 0.448 | 0.0656 | 1 No |
| 1mg/ml 2d vs. 2mg/ml 2d | 0.448 | 0.0656 | 1 No |
| 2mg/ml 3d vs. 5mg/ml 3d | 0.448 | 0.0656 | 1 No |
| 1mg/ml 4d vs. 1mg/ml 1d | 0.448 | 0.0656 | 1 No |
| 1mg/ml 1d vs. 0.5mg/ml 2d | 0.448 | 0.0656 | 1 No |
| 5mg/ml 1d vs. 1mg/ml 1d | 0.448 | 0.0656 | 1 No |
| 0.5mg/ml 2d vs. 5mg/ml 2d | 0.448 | 0.0656 | 1 No |

| | | | | |
|---|---|---|---|---|
| 1mg/ml 4d vs. 2mg/ml 4d | 0.299 | 0.0437 | 1 | No |
| 5mg/ml 1d vs. 2mg/ml 4d | 0.299 | 0.0437 | 1 | No |
| 1mg/ml 3d vs. 5mg/ml 4d | 0.299 | 0.0437 | 1 | No |
| 0.5mg/ml 3d vs. 5mg/ml 2d | 0.299 | 0.0437 | 1 | No |
| 2mg/ml 1d vs. 1mg/ml 1d | 0.299 | 0.0437 | 1 | No |
| 0.5mg/ml 4d vs. 1mg/ml 3d | 0.299 | 0.0437 | 1 | No |
| 2mg/ml 1d vs. 2mg/ml 4d | 0.149 | 0.0219 | 1 | No |
| 5mg/ml 1d vs. 2mg/ml 1d | 0.149 | 0.0219 | 1 | No |
| 1mg/ml 4d vs. 2mg/ml 1d | 0.149 | 0.0219 | 1 | No |
| 2mg/ml 4d vs. 1mg/ml 1d | 0.149 | 0.0219 | 1 | No |
| 0.5mg/ml 2d vs. 0.5mg/ml 3d | 0.149 | 0.0219 | 1 | No |
| 0.5mg/ml 1d vs. 1mg/ml 3d | 0.149 | 0.0219 | 1 | No |
| 0.5mg/ml 4d vs. 0.5mg/ml 1d | 0.149 | 0.0219 | 1 | No |
| 5mg/ml 1d vs. 1mg/ml 4d | 3.55E-15 | 5.20E-16 | 1 | No |

Figure 2.4 eosin Y - p-AKT

One Way Analysis of Variance

Data source: Data 1 in eosin Y WB

Normality Test (Shapiro-Wilk) Passed (P = 0.794)

Equal Variance Test: Passed (P = 0.631)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 3 | 0 | 1 | 0.0346 | 0.02 |
| 2.5ul/ml | 3 | 0 | 0.915 | 0.194 | 0.112 |
| 5ul/ml | 3 | 0 | 0.873 | 0.236 | 0.136 |
| 10ul/ml | 3 | 0 | 0.836 | 0.1 | 0.058 |
| 25ul/ml | 3 | 0 | 0.952 | 0.187 | 0.108 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 4 | 0.0496 | 0.0124 | 0.444 | 0.774 |
| Residual | 10 | 0.279 | 0.0279 | | |
| Total | 14 | 0.329 | | | |

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.774).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists.
Negative results should be interpreted cautiously.

Figure 2.4 eosin Y- AKT1

Normality Test (Shapiro-Wilk) Passed (P = 0.472)

Equal Variance Test: Passed (P = 0.708)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 3 | 0 | 1 | 0.0354 | 0.0204 |
| 2.5ul/ml | 3 | 0 | 1.014 | 0.0962 | 0.0555 |
| 5ul/ml | 3 | 0 | 1.077 | 0.0439 | 0.0254 |
| 10ul/ml | 3 | 0 | 1.029 | 0.0569 | 0.0329 |
| 25ul/ml | 3 | 0 | 0.936 | 0.0658 | 0.038 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 4 | 0.0314 | 0.00785 | 1.963 | 0.176 |
| Residual | 10 | 0.04 | 0.004 | | |

| | | |
|---|---|---|
| Total | 14 | 0.0714 |

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.176).

Power of performed test with alpha = 0.050: 0.205

The power of the performed test (0.205) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists.
Negative results should be interpreted cautiously.

Figure 2.5 Irgacure2959 - p-AKT

One Way Analysis of Variance

Data source: Data 1 in Irgacure 2959 WB

Normality Test (Shapiro-Wilk) Passed (P = 0.117)

Equal Variance Test: Passed (P = 0.423)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 3 | 0 | 1 | 0.0254 | 0.0147 |
| 0mg/ml | 3 | 0 | 1.01 | 0.0214 | 0.0123 |
| 1mg/ml | 3 | 0 | 0.882 | 0.22 | 0.127 |
| 2mg/ml | 3 | 0 | 0.843 | 0.381 | 0.22 |
| 5mg/ml | 3 | 0 | 0.481 | 0.261 | 0.151 |
| 10mg/ml | 3 | 0 | 0.0884 | 0.0716 | 0.0414 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 5 | 1.979 | 0.396 | 8.842 | 0.001 |
| Residual | 12 | 0.537 | 0.0448 | | |
| Total | 17 | 2.516 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.001).

Power of performed test with alpha = 0.050: 0.985

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| 0mg/ml vs. 10mg/ml | 0.921 | 5.333 | 0.003 | Yes |
| NA vs. 10mg/ml | 0.912 | 5.276 | 0.003 | Yes |
| 1mg/ml vs. 10mg/ml | 0.793 | 4.591 | 0.008 | Yes |
| 2mg/ml vs. 10mg/ml | 0.754 | 4.367 | 0.011 | Yes |
| 0mg/ml vs. 5mg/ml | 0.529 | 3.063 | 0.103 | No |
| NA vs. 5mg/ml | 0.519 | 3.007 | 0.104 | No |
| 1mg/ml vs. 5mg/ml | 0.401 | 2.322 | 0.299 | No |
| 5mg/ml vs. 10mg/ml | 0.392 | 2.27 | 0.293 | No |
| 2mg/ml vs. 5mg/ml | 0.362 | 2.097 | 0.341 | No |
| 0mg/ml vs. 2mg/ml | 0.167 | 0.966 | 0.927 | No |
| NA vs. 2mg/ml | 0.157 | 0.91 | 0.909 | No |
| 0mg/ml vs. 1mg/ml | 0.128 | 0.741 | 0.923 | No |
| NA vs. 1mg/ml | 0.118 | 0.685 | 0.88 | No |
| 1mg/ml vs. 2mg/ml | 0.0388 | 0.225 | 0.97 | No |
| 0mg/ml vs. NA | 0.00971 | 0.0562 | 0.956 | No |

Figure 2.5 Iragcure2959 - AKT1

Normality Test (Shapiro-Wilk) Passed (P = 0.104)

Equal Variance Test: Passed (P = 0.073)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 3 | 0 | 1 | 0.0289 | 0.0167 |
| 0mg/ml | 3 | 0 | 1.092 | 0.131 | 0.0757 |
| 1mg/ml | 3 | 0 | 1.157 | 0.0318 | 0.0183 |
| 2mg/ml | 3 | 0 | 1.178 | 0.149 | 0.0861 |
| 5mg/ml | 3 | 0 | 1.084 | 0.0288 | 0.0166 |
| 10mg/ml | 3 | 0 | 0.89 | 0.0418 | 0.0242 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 5 | 0.171 | 0.0342 | 4.684 | 0.013 |
| Residual | 12 | 0.0877 | 0.00731 | | |
| Total | 17 | 0.259 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.013).

Power of performed test with alpha = 0.050: 0.765

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| 2mg/ml vs. 10mg/ml | 0.288 | 4.121 | 0.021 | Yes |
| 1mg/ml vs. 10mg/ml | 0.267 | 3.822 | 0.033 | Yes |
| 0mg/ml vs. 10mg/ml | 0.202 | 2.895 | 0.161 | No |
| 5mg/ml vs. 10mg/ml | 0.193 | 2.772 | 0.185 | No |
| 2mg/ml vs. NA | 0.178 | 2.548 | 0.248 | No |
| 1mg/ml vs. NA | 0.157 | 2.249 | 0.363 | No |
| NA vs. 10mg/ml | 0.11 | 1.573 | 0.747 | No |
| 2mg/ml vs. 5mg/ml | 0.0942 | 1.35 | 0.836 | No |
| 0mg/ml vs. NA | 0.0923 | 1.321 | 0.81 | No |
| 2mg/ml vs. 0mg/ml | 0.0856 | 1.226 | 0.813 | No |
| 5mg/ml vs. NA | 0.0836 | 1.198 | 0.769 | No |
| 1mg/ml vs. 5mg/ml | 0.0734 | 1.051 | 0.779 | No |
| 1mg/ml vs. 0mg/ml | 0.0648 | 0.928 | 0.752 | No |
| 2mg/ml vs. 1mg/ml | 0.0208 | 0.299 | 0.947 | No |
| 0mg/ml vs. 5mg/ml | 0.0086 | 0.123 | 0.904 | No |

Figure 2.6 DMPA - p-AKT

One Way Analysis of Variance

Data source: Data 1 in DMPA WB

Normality Test (Shapiro-Wilk) Passed (P = 0.349)

Equal Variance Test: Passed (P = 0.794)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 3 | 0 | 1 | 0.0897 | 0.0518 |
| 0mg/ml | 3 | 0 | 0.962 | 0.121 | 0.0697 |
| 0.5mg/ml | 3 | 0 | 0.959 | 0.275 | 0.159 |
| 1mg/ml | 3 | 0 | 0.539 | 0.134 | 0.0772 |
| 2mg/ml | 3 | 0 | 0.271 | 0.0474 | 0.0273 |
| 5mg/ml | 3 | 0 | 0.213 | 0.0753 | 0.0435 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 5 | 1.987 | 0.397 | 19.224 | <0.001 |
| Residual | 12 | 0.248 | 0.0207 | | |
| Total | 17 | 2.235 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| NA vs. 5mg/ml | 0.787 | 6.703 | <0.001 | Yes |
| 0mg/ml vs. 5mg/ml | 0.749 | 6.378 | <0.001 | Yes |
| 0.5mg/ml vs. 5mg/ml | 0.746 | 6.358 | <0.001 | Yes |
| NA vs. 2mg/ml | 0.729 | 6.212 | <0.001 | Yes |
| 0mg/ml vs. 2mg/ml | 0.691 | 5.888 | <0.001 | Yes |
| 0.5mg/ml vs. 2mg/ml | 0.689 | 5.867 | <0.001 | Yes |
| NA vs. 1mg/ml | 0.461 | 3.93 | 0.018 | Yes |
| 0mg/ml vs. 1mg/ml | 0.423 | 3.605 | 0.029 | Yes |
| 0.5mg/ml vs. 1mg/ml | 0.421 | 3.585 | 0.026 | Yes |
| 1mg/ml vs. 5mg/ml | 0.326 | 2.773 | 0.097 | No |
| 1mg/ml vs. 2mg/ml | 0.268 | 2.283 | 0.191 | No |
| 2mg/ml vs. 5mg/ml | 0.0576 | 0.49 | 0.982 | No |
| NA vs. 0.5mg/ml | 0.0405 | 0.345 | 0.982 | No |
| NA vs. 0mg/ml | 0.0381 | 0.325 | 0.938 | No |
| 0mg/ml vs. 0.5mg/ml | 0.00239 | 0.0203 | 0.984 | No |

Figure 2.6 DMPA - AKT1

Normality Test (Shapiro-Wilk) Passed (P = 0.256)

Equal Variance Test: Passed (P = 0.728)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 3 | 0 | 1 | 0.0322 | 0.0186 |
| 0mg/ml | 3 | 0 | 1.031 | 0.0703 | 0.0406 |
| 0.5mg/ml | 3 | 0 | 1.125 | 0.14 | 0.0809 |
| 1mg/ml | 3 | 0 | 1.168 | 0.0917 | 0.0529 |
| 2mg/ml | 3 | 0 | 1.223 | 0.111 | 0.064 |
| 5mg/ml | 3 | 0 | 1.131 | 0.211 | 0.122 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 5 | 0.106 | 0.0212 | 1.395 | 0.294 |
| Residual | 12 | 0.182 | 0.0152 | | |
| Total | 17 | 0.288 | | | |

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.294).

Power of performed test with alpha = 0.050: 0.115

The power of the performed test (0.115) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists.
Negative results should be interpreted cautiously.

Figure 2.8 eosin Y - UV

One Way Analysis of Variance

Data source: Data 1 in eosin Y UV viability

Normality Test (Shapiro-Wilk) Passed (P = 0.424)

Equal Variance Test: Passed (P = 0.485)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 0d | 3 | 0 | 99.649 | 12.734 | 7.352 |
| NA 1d | 3 | 0 | 143.86 | 19.702 | 11.375 |
| 2.5ul/ml 1d | 3 | 0 | 92.982 | 2.191 | 1.265 |
| 5ul/ml 1d | 3 | 0 | 69.474 | 14.24 | 8.221 |
| 10ul/ml 1d | 3 | 0 | 60.702 | 11.643 | 6.722 |
| NA 2d | 3 | 0 | 187.719 | 16.787 | 9.692 |
| 2.5ul/ml 2d | 3 | 0 | 67.719 | 5.986 | 3.456 |
| 5ul/ml 2d | 3 | 0 | 63.86 | 7.468 | 4.312 |
| 10ul/ml 2d | 3 | 0 | 49.474 | 5.57 | 3.216 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 8 | 49745.6 | 6218.2 | 43.12 | <0.001 |
| Residual | 18 | 2595.753 | 144.208 | | |
| Total | 26 | 52341.36 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | | | | |
|---|---|---|---|---|
| NA 2d vs. 10ul/ml 2d | 138.246 | 14.099 | <0.001 | Yes |
| NA 2d vs. 10ul/ml 1d | 127.018 | 12.954 | <0.001 | Yes |
| NA 2d vs. 5ul/ml 2d | 123.86 | 12.632 | <0.001 | Yes |
| NA 2d vs. 2.5ul/ml 2d | 120 | 12.239 | <0.001 | Yes |
| NA 2d vs. 5ul/ml 1d | 118.246 | 12.06 | <0.001 | Yes |
| NA 2d vs. 2.5ul/ml 1d | 94.737 | 9.662 | <0.001 | Yes |
| NA 1d vs. 10ul/ml 2d | 94.386 | 9.626 | <0.001 | Yes |
| NA 2d vs. 0d | 88.07 | 8.982 | <0.001 | Yes |
| NA 1d vs. 10ul/ml 1d | 83.158 | 8.481 | <0.001 | Yes |
| NA 1d vs. 5ul/ml 2d | 80 | 8.159 | <0.001 | Yes |
| NA 1d vs. 2.5ul/ml 2d | 76.14 | 7.765 | <0.001 | Yes |
| NA 1d vs. 5ul/ml 1d | 74.386 | 7.586 | <0.001 | Yes |

| | | | | |
|---|---|---|---|---|
| NA 1d vs. 2.5ul/ml 1d | 50.877 | 5.189 | 0.001 | Yes |
| 0d vs. 10ul/ml 2d | 50.175 | 5.117 | 0.002 | Yes |
| NA 1d vs. 0d | 44.211 | 4.509 | 0.006 | Yes |
| NA 2d vs. NA 1d | 43.86 | 4.473 | 0.006 | Yes |
| 2.5ul/ml 1d vs. 10ul/ml 2d | 43.509 | 4.437 | 0.006 | Yes |
| 0d vs. 10ul/ml 1d | 38.947 | 3.972 | 0.017 | Yes |
| 0d vs. 5ul/ml 2d | 35.789 | 3.65 | 0.032 | Yes |
| 2.5ul/ml 1d vs. 10ul/ml 1d | 32.281 | 3.292 | 0.067 | No |
| 0d vs. 2.5ul/ml 2d | 31.93 | 3.256 | 0.068 | No |
| 0d vs. 5ul/ml 1d | 30.175 | 3.078 | 0.093 | No |
| 2.5ul/ml 1d vs. 5ul/ml 2d | 29.123 | 2.97 | 0.109 | No |
| 2.5ul/ml 1d vs. 2.5ul/ml 2d | 25.263 | 2.577 | 0.221 | No |
| 2.5ul/ml 1d vs. 5ul/ml 1d | 23.509 | 2.398 | 0.285 | No |
| 5ul/ml 1d vs. 10ul/ml 2d | 20 | 2.04 | 0.472 | No |
| 2.5ul/ml 2d vs. 10ul/ml 2d | 18.246 | 1.861 | 0.562 | No |
| 5ul/ml 2d vs. 10ul/ml 2d | 14.386 | 1.467 | 0.791 | No |
| 10ul/ml 1d vs. 10ul/ml 2d | 11.228 | 1.145 | 0.917 | No |
| 5ul/ml 1d vs. 10ul/ml 1d | 8.772 | 0.895 | 0.966 | No |
| 2.5ul/ml 2d vs. 10ul/ml 1d | 7.018 | 0.716 | 0.981 | No |
| 0d vs. 2.5ul/ml 1d | 6.667 | 0.68 | 0.97 | No |
| 5ul/ml 1d vs. 5ul/ml 2d | 5.614 | 0.573 | 0.967 | No |
| 2.5ul/ml 2d vs. 5ul/ml 2d | 3.86 | 0.394 | 0.973 | No |
| 5ul/ml 2d vs. 10ul/ml 1d | 3.158 | 0.322 | 0.938 | No |
| 5ul/ml 1d vs. 2.5ul/ml 2d | 1.754 | 0.179 | 0.86 | No |

Figure 2.9 Irgacure 2959 - UV

One Way Analysis of Variance

Data source: Data 1 in Irgacure 2959 UV viability

Normality Test (Shapiro-Wilk) Passed (P = 0.708)

Equal Variance Test: Passed (P = 0.345)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 0d | 3 | 0 | 100 | 11.628 | 6.713 |
| NA 1d | 3 | 0 | 144.186 | 20.44 | 11.801 |
| 1mg/ml 1d | 3 | 0 | 78.682 | 16.622 | 9.596 |
| 2mg/ml 1d | 3 | 0 | 65.504 | 20.318 | 11.731 |
| 5mg/ml 1d | 3 | 0 | 3.101 | 2.421 | 1.398 |
| NA 2d | 3 | 0 | 207.364 | 18.544 | 10.706 |
| 1mg/ml 2d | 3 | 0 | 70.155 | 14.724 | 8.501 |
| 2mg/ml 2d | 3 | 0 | 23.256 | 3.076 | 1.776 |
| 5mg/ml 2d | 3 | 0 | 2.326 | 2.326 | 1.343 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 8 | 108435.6 | 13554.45 | 66.899 | <0.001 |
| Residual | 18 | 3647.016 | 202.612 | | |
| Total | 26 | 112082.6 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| NA 2d vs. 5mg/ml 2d | 205.039 | 17.642 | <0.001 | Yes |
| NA 2d vs. 5mg/ml 1d | 204.264 | 17.575 | <0.001 | Yes |
| NA 2d vs. 2mg/ml 2d | 184.109 | 15.841 | <0.001 | Yes |
| NA 2d vs. 2mg/ml 1d | 141.86 | 12.206 | <0.001 | Yes |
| NA 1d vs. 5mg/ml 2d | 141.86 | 12.206 | <0.001 | Yes |
| NA 1d vs. 5mg/ml 1d | 141.085 | 12.139 | <0.001 | Yes |
| NA 2d vs. 1mg/ml 2d | 137.209 | 11.806 | <0.001 | Yes |
| NA 2d vs. 1mg/ml 1d | 128.682 | 11.072 | <0.001 | Yes |
| NA 1d vs. 2mg/ml 2d | 120.93 | 10.405 | <0.001 | Yes |
| NA 2d vs. 0d | 107.364 | 9.238 | <0.001 | Yes |
| 0d vs. 5mg/ml 2d | 97.674 | 8.404 | <0.001 | Yes |
| 0d vs. 5mg/ml 1d | 96.899 | 8.337 | <0.001 | Yes |

| | | | | |
|---|---|---|---|---|
| NA 1d vs. 2mg/ml 1d | 78.682 | 6.77 | <0.001 | Yes |
| 0d vs. 2mg/ml 2d | 76.744 | 6.603 | <0.001 | Yes |
| 1mg/ml 1d vs. 5mg/ml 2d | 76.357 | 6.57 | <0.001 | Yes |
| 1mg/ml 1d vs. 5mg/ml 1d | 75.581 | 6.503 | <0.001 | Yes |
| NA 1d vs. 1mg/ml 2d | 74.031 | 6.37 | <0.001 | Yes |
| 1mg/ml 2d vs. 5mg/ml 2d | 67.829 | 5.836 | <0.001 | Yes |
| 1mg/ml 2d vs. 5mg/ml 1d | 67.054 | 5.77 | <0.001 | Yes |
| NA 1d vs. 1mg/ml 1d | 65.504 | 5.636 | <0.001 | Yes |
| NA 2d vs. NA 1d | 63.178 | 5.436 | <0.001 | Yes |
| 2mg/ml 1d vs. 5mg/ml 2d | 63.178 | 5.436 | <0.001 | Yes |
| 2mg/ml 1d vs. 5mg/ml 1d | 62.403 | 5.369 | <0.001 | Yes |
| 1mg/ml 1d vs. 2mg/ml 2d | 55.426 | 4.769 | 0.002 | Yes |
| 1mg/ml 2d vs. 2mg/ml 2d | 46.899 | 4.035 | 0.009 | Yes |
| NA 1d vs. 0d | 44.186 | 3.802 | 0.014 | Yes |
| 2mg/ml 1d vs. 2mg/ml 2d | 42.248 | 3.635 | 0.019 | Yes |
| 0d vs. 2mg/ml 1d | 34.496 | 2.968 | 0.072 | No |
| 0d vs. 1mg/ml 2d | 29.845 | 2.568 | 0.145 | No |
| 0d vs. 1mg/ml 1d | 21.318 | 1.834 | 0.456 | No |
| 2mg/ml 2d vs. 5mg/ml 2d | 20.93 | 1.801 | 0.426 | No |
| 2mg/ml 2d vs. 5mg/ml 1d | 20.155 | 1.734 | 0.409 | No |
| 1mg/ml 1d vs. 2mg/ml 1d | 13.178 | 1.134 | 0.719 | No |
| 1mg/ml 1d vs. 1mg/ml 2d | 8.527 | 0.734 | 0.853 | No |
| 1mg/ml 2d vs. 2mg/ml 1d | 4.651 | 0.4 | 0.906 | No |
| 5mg/ml 1d vs. 5mg/ml 2d | 7.75E-01 | 6.67E-02 | 0.948 | No |

Figure 2.10 DMPA - UV

One Way Analysis of Variance

Data source: Data 1 in DMPA UV viability

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.291)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 0d | 3 | 0 | 100 | 11.628 | 6.713 |
| NA 1d | 3 | 0 | 144.186 | 20.44 | 11.801 |
| 1mg/ml 1d | 3 | 0 | 9.69 | 2.421 | 1.398 |
| 2mg/ml 1d | 3 | 0 | 9.69 | 3.357 | 1.938 |
| 5mg/ml 1d | 3 | 0 | 11.628 | 5.068 | 2.926 |
| NA 2d | 3 | 0 | 207.364 | 18.544 | 10.706 |
| 1mg/ml 2d | 3 | 0 | 5.814 | 0 | 0 |
| 2mg/ml 2d | 3 | 0 | 8.14 | 2.014 | 1.163 |
| 5mg/ml 2d | 3 | 0 | 7.364 | 4.699 | 2.713 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 8 | 138170.3 | 17271.29 | 160.94 | <0.001 |
| Residual | 18 | 1931.675 | 107.315 | | |
| Total | 26 | 140102 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| NA 2d vs. 1mg/ml 2d | 201.55 | 23.829 | <0.001 | Yes |
| NA 2d vs. 5mg/ml 2d | 200 | 23.645 | <0.001 | Yes |
| NA 2d vs. 2mg/ml 2d | 199.225 | 23.554 | <0.001 | Yes |
| NA 2d vs. 1mg/ml 1d | 197.674 | 23.37 | <0.001 | Yes |
| NA 2d vs. 2mg/ml 1d | 197.674 | 23.37 | <0.001 | Yes |
| NA 2d vs. 5mg/ml 1d | 195.736 | 23.141 | <0.001 | Yes |
| NA 1d vs. 1mg/ml 2d | 138.372 | 16.359 | <0.001 | Yes |
| NA 1d vs. 5mg/ml 2d | 136.822 | 16.176 | <0.001 | Yes |
| NA 1d vs. 2mg/ml 2d | 136.047 | 16.084 | <0.001 | Yes |
| NA 1d vs. 2mg/ml 1d | 134.496 | 15.901 | <0.001 | Yes |
| NA 1d vs. 1mg/ml 1d | 134.496 | 15.901 | <0.001 | Yes |
| NA 1d vs. 5mg/ml 1d | 132.558 | 15.672 | <0.001 | Yes |

| | | | | |
|---|---|---|---|---|
| NA 2d vs. 0d | 107.364 | 12.693 | <0.001 | Yes |
| 0d vs. 1mg/ml 2d | 94.186 | 11.135 | <0.001 | Yes |
| 0d vs. 5mg/ml 2d | 92.636 | 10.952 | <0.001 | Yes |
| 0d vs. 2mg/ml 2d | 91.86 | 10.86 | <0.001 | Yes |
| 0d vs. 2mg/ml 1d | 90.31 | 10.677 | <0.001 | Yes |
| 0d vs. 1mg/ml 1d | 90.31 | 10.677 | <0.001 | Yes |
| 0d vs. 5mg/ml 1d | 88.372 | 10.448 | <0.001 | Yes |
| NA 2d vs. NA 1d | 63.178 | 7.469 | <0.001 | Yes |
| NA 1d vs. 0d | 44.186 | 5.224 | <0.001 | Yes |
| 5mg/ml 1d vs. 1mg/ml 2d | 5.814 | 0.687 | 1 | No |
| 5mg/ml 1d vs. 5mg/ml 2d | 4.264 | 0.504 | 1 | No |
| 1mg/ml 1d vs. 1mg/ml 2d | 3.876 | 0.458 | 1 | No |
| 2mg/ml 1d vs. 1mg/ml 2d | 3.876 | 0.458 | 1 | No |
| 5mg/ml 1d vs. 2mg/ml 2d | 3.488 | 0.412 | 1 | No |
| 1mg/ml 1d vs. 5mg/ml 2d | 2.326 | 0.275 | 1 | No |
| 2mg/ml 1d vs. 5mg/ml 2d | 2.326 | 0.275 | 1 | No |
| 2mg/ml 2d vs. 1mg/ml 2d | 2.326 | 0.275 | 1 | No |
| 5mg/ml 1d vs. 1mg/ml 1d | 1.938 | 0.229 | 1 | No |
| 5mg/ml 1d vs. 2mg/ml 1d | 1.938 | 0.229 | 1 | No |
| 1mg/ml 1d vs. 2mg/ml 2d | 1.55 | 0.183 | 1 | No |
| 2mg/ml 1d vs. 2mg/ml 2d | 1.55 | 0.183 | 1 | No |
| 5mg/ml 2d vs. 1mg/ml 2d | 1.55 | 0.183 | 0.997 | No |
| 2mg/ml 2d vs. 5mg/ml 2d | 0.775 | 0.0916 | 0.995 | No |
| 1mg/ml 1d vs. 2mg/ml 1d | 7.11E-15 | 8.40E-16 | 1 | No |

Figure 2.11 UV- p-AKT

One Way Analysis of Variance

Data source: Data 1 in UV photo WB

Normality Test (Shapiro-Wilk) Passed (P = 0.213)

Equal Variance Test: Passed (P = 0.902)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 3 | 0 | 1 | 0.124 | 0.0718 |
| DMPA 2mg/ml | 3 | 0 | 0.572 | 0.0776 | 0.0448 |
| DMPA 5mg/ml | 3 | 0 | 0.521 | 0.0763 | 0.0441 |
| eosin Y 10ul/ml | 3 | 0 | 0.622 | 0.0811 | 0.0468 |
| eosin Y 25ul/ml | 3 | 0 | 0.271 | 0.061 | 0.0352 |
| I2959 2mg/ml | 3 | 0 | 0.423 | 0.0602 | 0.0347 |
| I2959 5mg/ml | 3 | 0 | 0.197 | 0.0586 | 0.0339 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 6 | 1.256 | 0.209 | 32.823 | <0.001 |
| Residual | 14 | 0.0893 | 0.00638 | | |
| Total | 20 | 1.346 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| NA vs. I2959 5mg/ml | 0.803 | 12.307 | <0.001 | Yes |
| NA vs. eosin Y 25ul/ml | 0.729 | 11.178 | <0.001 | Yes |
| NA vs. I2959 2mg/ml | 0.577 | 8.847 | <0.001 | Yes |
| NA vs. DMPA 5mg/ml | 0.479 | 7.352 | <0.001 | Yes |
| NA vs. DMPA 2mg/ml | 0.428 | 6.563 | <0.001 | Yes |
| eosin Y 10ul vs. I2959 5mg/ml | 0.425 | 6.511 | <0.001 | Yes |
| NA vs. eosin Y 10ul/ml | 0.378 | 5.796 | <0.001 | Yes |
| DMPA 2mg/ml vs. I2959 5mg/ml | 0.375 | 5.744 | <0.001 | Yes |
| eosin Y 10ul vs. eosin Y 25ul | 0.351 | 5.381 | 0.001 | Yes |
| DMPA 5mg/ml vs. I2959 5mg/ml | 0.323 | 4.955 | 0.003 | Yes |
| DMPA 2mg/ml vs. eosin Y 25ul | 0.301 | 4.614 | 0.004 | Yes |
| DMPA 5mg/ml vs. eosin Y 25ul | 0.249 | 3.826 | 0.018 | Yes |
| I2959 2mg/ml vs. I2959 5mg/ml | 0.226 | 3.461 | 0.034 | Yes |
| eosin Y 10ul vs. I2959 2mg/ml | 0.199 | 3.05 | 0.067 | No |

| | | | | |
|---|---|---|---|---|
| I2959 2mg/ml vs. eosin Y 25ul | 0.152 | 2.331 | 0.222 | No |
| DMPA 2mg/ml vs. I2959 2mg/ml | 0.149 | 2.283 | 0.21 | No |
| eosin Y 10ul vs. DMPA 5mg/ml | 0.101 | 1.556 | 0.535 | No |
| DMPA 5mg/ml vs. I2959 2mg/ml | 0.0975 | 1.495 | 0.495 | No |
| eosin Y 25ul vs. I2959 5mg/ml | 0.0737 | 1.13 | 0.623 | No |
| DMPA 2mg/ml vs. DMPA 5mg/n | 0.0514 | 0.789 | 0.69 | No |
| eosin Y 10ul vs. DMPA 2mg/ml | 0.05 | 0.767 | 0.456 | No |

Figure 2.11 UV- AKT1

Normality Test (Shapiro-Wilk) Passed (P = 0.783)

Equal Variance Test: Passed (P = 0.424)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 3 | 0 | 1 | 0.0659 | 0.0381 |
| DMPA 2mg/ml | 3 | 0 | 1.192 | 0.0955 | 0.0551 |
| DMPA 5mg/ml | 3 | 0 | 1.212 | 0.248 | 0.143 |
| eosin Y 10ul/ml | 3 | 0 | 1.208 | 0.129 | 0.0742 |
| eosin Y 25ul/ml | 3 | 0 | 1.307 | 0.133 | 0.0768 |
| I2959 2mg/ml | 3 | 0 | 1.168 | 0.167 | 0.0962 |
| I2959 5mg/ml | 3 | 0 | 1.349 | 0.206 | 0.119 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 6 | 0.225 | 0.0374 | 1.459 | 0.262 |
| Residual | 14 | 0.359 | 0.0256 | | |
| Total | 20 | 0.584 | | | |

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.262).

Power of performed test with alpha = 0.050: 0.138

The power of the performed test (0.138) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists.
Negative results should be interpreted cautiously.

Figure 2.12 viability

One Way Analysis of Variance

Data source: Data 1 in UV time eosin Y viability

Normality Test (Shapiro-Wilk) Passed (P = 0.471)

Equal Variance Test: Passed (P = 0.591)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 3 | 0 | 0.179 | 0.0119 | 0.00689 |
| eosin Y | 3 | 0 | 0.0983 | 0.00777 | 0.00448 |
| 0h UV eosin Y | 3 | 0 | 0.0537 | 0.00503 | 0.00291 |
| 6h UV eosin Y | 3 | 0 | 0.00833 | 0.00306 | 0.00176 |
| 24h UV eosin Y | 3 | 0 | 0.00933 | 0.00321 | 0.00186 |
| 48h UV eosin Y | 3 | 0 | 0.009 | 0.00265 | 0.00153 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 5 | 0.0708 | 0.0142 | 333.427 | <0.001 |
| Residual | 12 | 0.000509 | 4.24E-05 | | |
| Total | 17 | 0.0713 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| NA vs. 6h UV eosin Y | 0.171 | 32.146 | <0.001 | Yes |
| NA vs. 48h UV eosin Y | 0.17 | 32.021 | <0.001 | Yes |
| NA vs. 24h UV eosin Y | 0.17 | 31.958 | <0.001 | Yes |
| NA vs. 0h UV eosin Y | 0.126 | 23.624 | <0.001 | Yes |
| eosin Y vs. 6h UV eosin Y | 0.09 | 16.919 | <0.001 | Yes |
| eosin Y vs. 48h UV eosin Y | 0.0893 | 16.794 | <0.001 | Yes |
| eosin Y vs. 24h UV eosin Y | 0.089 | 16.731 | <0.001 | Yes |
| NA vs. eosin Y | 0.081 | 15.227 | <0.001 | Yes |
| 0h UV eosin vs. 6h UV eosin | 0.0453 | 8.522 | <0.001 | Yes |
| 0h UV eosin vs. 48h UV eosin | 0.0447 | 8.397 | <0.001 | Yes |
| eosin Y vs. 0h UV eosin Y | 0.0447 | 8.397 | <0.001 | Yes |
| 0h UV eosin vs. 24h UV eosin | 0.0443 | 8.334 | <0.001 | Yes |
| 24h UV eosin vs. 6h UV eosin | 0.001 | 0.188 | 0.997 | No |
| 48h UV eosin vs. 6h UV eosin | 0.000667 | 0.125 | 0.99 | No |
| 24h UV eosin vs. 48h UV eosin | 0.000333 | 0.0627 | 0.951 | No |

Figure 2.13 UV - p-AKT

One Way Analysis of Variance

Data source: Data 1 in UV time eosin Y WB

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.186)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 3 | 0 | 1 | 0.0964 | 0.0556 |
| eoain Y | 3 | 0 | 1.043 | 0.115 | 0.0662 |
| eosin Y UV 0h | 3 | 0 | 0.0594 | 0.00737 | 0.00425 |
| eosin Y UV 6h | 3 | 0 | 0.0401 | 0.00549 | 0.00317 |
| eosin Y UV 24h | 3 | 0 | 0.015 | 0.00153 | 0.000882 |
| eosin Y UV 48h | 3 | 0 | 0.0351 | 0.0122 | 0.00704 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 5 | 3.88 | 0.776 | 205.414 | <0.001 |
| Residual | 12 | 0.0453 | 0.00378 | | |
| Total | 17 | 3.925 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| eoain Y vs. eosin Y UV 24h | 1.028 | 20.485 | <0.001 | Yes |
| eoain Y vs. eosin Y UV 48h | 1.008 | 20.085 | <0.001 | Yes |
| eoain Y vs. eosin Y UV 6h | 1.003 | 19.986 | <0.001 | Yes |
| NA vs. eosin Y UV 24h | 0.985 | 19.628 | <0.001 | Yes |
| eoain Y vs. eosin Y UV 0h | 0.984 | 19.6 | <0.001 | Yes |
| NA vs. eosin Y UV 48h | 0.965 | 19.228 | <0.001 | Yes |
| NA vs. eosin Y UV 6h | 0.96 | 19.129 | <0.001 | Yes |
| NA vs. eosin Y UV 0h | 0.941 | 18.744 | <0.001 | Yes |
| eosin Y UV 0 vs. eosin Y UV 2 | 0.0444 | 0.885 | 0.97 | No |
| eoain Y vs. NA | 0.043 | 0.857 | 0.957 | No |
| eosin Y UV 6 vs. eosin Y UV 2 | 0.0251 | 0.5 | 0.993 | No |
| eosin Y UV 0 vs. eosin Y UV 4 | 0.0243 | 0.485 | 0.983 | No |
| eosin Y UV 4 vs. eosin Y UV 2 | 0.0201 | 0.4 | 0.972 | No |
| eosin Y UV 0 vs. eosin Y UV 6 | 0.0193 | 0.385 | 0.914 | No |
| eosin Y UV 6 vs. eosin Y UV 4 | 0.00501 | 0.0998 | 0.922 | No |

Figure 2.13 UV - AKT1

Normality Test (Shapiro-Wilk) Passed (P = 0.268)

Equal Variance Test: Passed (P = 0.857)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 3 | 0 | 1 | 0.0397 | 0.0229 |
| eoain Y | 3 | 0 | 1.116 | 0.0706 | 0.0407 |
| eosin Y UV 0h | 3 | 0 | 1.321 | 0.122 | 0.0704 |
| eosin Y UV 6h | 3 | 0 | 1.399 | 0.145 | 0.0837 |
| eosin Y UV 24h | 3 | 0 | 1.59 | 0.0696 | 0.0402 |
| eosin Y UV 48h | 3 | 0 | 1.504 | 0.0727 | 0.042 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 5 | 0.772 | 0.154 | 17.62 | <0.001 |
| Residual | 12 | 0.105 | 0.00876 | | |
| Total | 17 | 0.877 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| eosin Y UV 24h vs. NA | 0.59 | 7.725 | <0.001 | Yes |
| eosin Y UV 48h vs. NA | 0.504 | 6.595 | <0.001 | Yes |
| eosin Y UV 24h vs. eoain Y | 0.475 | 6.211 | <0.001 | Yes |
| eosin Y UV 6h vs. NA | 0.399 | 5.216 | 0.003 | Yes |
| eosin Y UV 48h vs. eoain Y | 0.388 | 5.082 | 0.003 | Yes |
| eosin Y UV 0h vs. NA | 0.321 | 4.195 | 0.012 | Yes |
| eosin Y UV 6h vs. eoain Y | 0.283 | 3.702 | 0.027 | Yes |
| eosin Y UV 2 vs. eosin Y UV 0 | 0.27 | 3.53 | 0.033 | Yes |
| eosin Y UV 0h vs. eoain Y | 0.205 | 2.681 | 0.132 | No |
| eosin Y UV 2 vs. eosin Y UV 6 | 0.192 | 2.509 | 0.154 | No |
| eosin Y UV 4 vs. eosin Y UV 0 | 0.183 | 2.4 | 0.157 | No |
| eoain Y vs. NA | 0.116 | 1.514 | 0.493 | No |
| eosin Y UV 4 vs. eosin Y UV 6 | 0.105 | 1.379 | 0.474 | No |
| eosin Y UV 2 vs. eosin Y UV 4 | 0.0863 | 1.129 | 0.483 | No |
| eosin Y UV 6 vs. eosin Y UV 0 | 0.078 | 1.021 | 0.327 | No |

Figure 3.2

One Way Analysis of Variance

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 4 | 0 | 100 | 3.136 | 1.568 |
| PEG17.4 | 4 | 0 | 95.716 | 4.984 | 2.492 |
| PEG87 | 4 | 0 | 83.197 | 3.546 | 1.773 |
| PEG174 | 4 | 0 | 70.769 | 2.238 | 1.119 |
| PEG696 | 4 | 0 | 35.156 | 9.383 | 4.691 |
| PEG1739 | 4 | 0 | 8.231 | 0.467 | 0.233 |
| PEG-DA17.4 | 4 | 0 | 70.358 | 1.605 | 0.803 |
| PEG-DA87 | 4 | 0 | 18.78 | 0.589 | 0.295 |
| PEG-DA174 | 4 | 0 | 7.979 | 0.61 | 0.305 |
| PEG-DA696 | 4 | 0 | 8.214 | 0.759 | 0.38 |
| PEG-DA1739 | 4 | 0 | 7.946 | 0.355 | 0.178 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 10 | 58338.12 | 5833.812 | 444.062 | <0.001 |
| Residual | 33 | 433.533 | 13.137 | | |
| Total | 43 | 58771.65 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| NA vs. PEG-DA1739 | 92.054 | 35.917 | <0.001 | Yes |
| NA vs. PEG-DA174 | 92.021 | 35.905 | <0.001 | Yes |
| NA vs. PEG-DA696 | 91.786 | 35.813 | <0.001 | Yes |
| NA vs. PEG1739 | 91.769 | 35.806 | <0.001 | Yes |
| PEG17.4 vs. PEG-DA1739 | 87.769 | 34.245 | <0.001 | Yes |
| PEG17.4 vs. PEG-DA174 | 87.737 | 34.233 | <0.001 | Yes |
| PEG17.4 vs. PEG-DA696 | 87.501 | 34.141 | <0.001 | Yes |
| PEG17.4 vs. PEG1739 | 87.485 | 34.135 | <0.001 | Yes |
| NA vs. PEG-DA87 | 81.22 | 31.69 | <0.001 | Yes |
| PEG17.4 vs. PEG-DA87 | 76.935 | 30.018 | <0.001 | Yes |

| | | | | |
|---|---|---|---|---|
| PEG87 vs. PEG-DA1739 | 75.25 | 29.361 | <0.001 | Yes |
| PEG87 vs. PEG-DA174 | 75.218 | 29.348 | <0.001 | Yes |
| PEG87 vs. PEG-DA696 | 74.982 | 29.256 | <0.001 | Yes |
| PEG87 vs. PEG1739 | 74.966 | 29.25 | <0.001 | Yes |
| NA vs. PEG696 | 64.844 | 25.3 | <0.001 | Yes |
| PEG87 vs. PEG-DA87 | 64.417 | 25.134 | <0.001 | Yes |
| PEG174 vs. PEG-DA1739 | 62.822 | 24.512 | <0.001 | Yes |
| PEG174 vs. PEG-DA174 | 62.79 | 24.499 | <0.001 | Yes |
| PEG174 vs. PEG-DA696 | 62.554 | 24.407 | <0.001 | Yes |
| PEG174 vs. PEG1739 | 62.538 | 24.401 | <0.001 | Yes |
| PEG-DA17.4 vs. PEG-DA1739 | 62.412 | 24.352 | <0.001 | Yes |
| PEG-DA17.4 vs. PEG-DA174 | 62.38 | 24.339 | <0.001 | Yes |
| PEG-DA17.4 vs. PEG-DA696 | 62.144 | 24.247 | <0.001 | Yes |
| PEG-DA17.4 vs. PEG1739 | 62.128 | 24.241 | <0.001 | Yes |
| PEG17.4 vs. PEG696 | 60.559 | 23.629 | <0.001 | Yes |
| PEG174 vs. PEG-DA87 | 51.989 | 20.285 | <0.001 | Yes |
| PEG-DA17.4 vs. PEG-DA87 | 51.578 | 20.125 | <0.001 | Yes |
| PEG87 vs. PEG696 | 48.04 | 18.744 | <0.001 | Yes |
| PEG174 vs. PEG696 | 35.612 | 13.895 | <0.001 | Yes |
| PEG-DA17.4 vs. PEG696 | 35.202 | 13.735 | <0.001 | Yes |
| NA vs. PEG-DA17.4 | 29.642 | 11.565 | <0.001 | Yes |
| NA vs. PEG174 | 29.231 | 11.405 | <0.001 | Yes |
| PEG696 vs. PEG-DA1739 | 27.21 | 10.617 | <0.001 | Yes |
| PEG696 vs. PEG-DA174 | 27.178 | 10.604 | <0.001 | Yes |
| PEG696 vs. PEG-DA696 | 26.942 | 10.512 | <0.001 | Yes |
| PEG696 vs. PEG1739 | 26.926 | 10.506 | <0.001 | Yes |
| PEG17.4 vs. PEG-DA17.4 | 25.357 | 9.894 | <0.001 | Yes |
| PEG17.4 vs. PEG174 | 24.947 | 9.734 | <0.001 | Yes |
| NA vs. PEG87 | 16.803 | 6.556 | <0.001 | Yes |
| PEG696 vs. PEG-DA87 | 16.376 | 6.39 | <0.001 | Yes |
| PEG87 vs. PEG-DA17.4 | 12.838 | 5.009 | <0.001 | Yes |
| PEG17.4 vs. PEG87 | 12.519 | 4.885 | <0.001 | Yes |
| PEG87 vs. PEG174 | 12.428 | 4.849 | <0.001 | Yes |
| PEG-DA87 vs. PEG-DA1739 | 10.834 | 4.227 | 0.002 | Yes |
| PEG-DA87 vs. PEG-DA174 | 10.801 | 4.214 | 0.002 | Yes |
| PEG-DA87 vs. PEG-DA696 | 10.566 | 4.123 | 0.002 | Yes |
| PEG-DA87 vs. PEG1739 | 10.55 | 4.116 | 0.002 | Yes |
| NA vs. PEG17.4 | 4.284 | 1.672 | 0.585 | No |
| PEG174 vs. PEG-DA17.4 | 0.41 | 0.16 | 1 | No |
| PEG1739 vs. PEG-DA1739 | 0.284 | 0.111 | 1 | No |
| PEG-DA696 vs. PEG-DA1739 | 0.268 | 0.105 | 1 | No |
| PEG1739 vs. PEG-DA174 | 0.252 | 0.0983 | 1 | No |
| PEG-DA696 vs. PEG-DA174 | 0.236 | 0.0919 | 1 | No |
| PEG-DA174 vs. PEG-DA1739 | 0.0323 | 0.0126 | 1 | No |
| PEG1739 vs. PEG-DA696 | 0.0163 | 0.00635 | 0.995 | No |

Figure 3.3

One Way Analysis of Variance

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 4 | 0 | 99.725 | 6.073 | 3.037 |
| GF/0PEGDA+5eY | 4 | 0 | 112.4 | 1.146 | 0.573 |
| GF/1PEGDA+5eY | 4 | 0 | 106.95 | 3.648 | 1.824 |
| GF/2PEGDA+5eY | 4 | 0 | 17.336 | 1.563 | 0.781 |
| GF/5PEGDA+5eY | 4 | 0 | 2.95 | 0.265 | 0.132 |
| GF/10PEGDA+5eY | 4 | 0 | 3.325 | 0.222 | 0.111 |
| GF/20PEGDA+5eY | 4 | 0 | 3.95 | 0.3 | 0.15 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 6 | 68751.17 | 11458.53 | 1481.032 | <0.001 |
| Residual | 21 | 162.474 | 7.737 | | |
| Total | 27 | 68913.65 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| GF/0PEGDA+5e vs. GF/5PEGDA+5 | 109.45 | 55.648 | <0.001 | Yes |
| GF/0PEGDA+5e vs. GF/10PEGDA+ | 109.075 | 55.457 | <0.001 | Yes |
| GF/0PEGDA+5e vs. GF/20PEGDA+ | 108.45 | 55.139 | <0.001 | Yes |
| GF/1PEGDA+5e vs. GF/5PEGDA+5 | 104 | 52.877 | <0.001 | Yes |
| GF/1PEGDA+5e vs. GF/10PEGDA+ | 103.625 | 52.686 | <0.001 | Yes |
| GF/1PEGDA+5e vs. GF/20PEGDA+ | 103 | 52.368 | <0.001 | Yes |
| NA vs. GF/5PEGDA+5eY | 96.775 | 49.204 | <0.001 | Yes |
| NA vs. GF/10PEGDA+5eY | 96.4 | 49.013 | <0.001 | Yes |
| NA vs. GF/20PEGDA+5eY | 95.775 | 48.695 | <0.001 | Yes |
| GF/0PEGDA+5e vs. GF/2PEGDA+5 | 95.064 | 48.333 | <0.001 | Yes |
| GF/1PEGDA+5e vs. GF/2PEGDA+5 | 89.614 | 45.562 | <0.001 | Yes |
| NA vs. GF/2PEGDA+5eY | 82.389 | 41.889 | <0.001 | Yes |
| GF/2PEGDA+5e vs. GF/5PEGDA+5 | 14.386 | 7.315 | <0.001 | Yes |
| GF/2PEGDA+5e vs. GF/10PEGDA+ | 14.011 | 7.124 | <0.001 | Yes |

| | | | | |
|---|---|---|---|---|
| GF/2PEGDA+5e vs. GF/20PEGDA+ | 13.386 | 6.806 | <0.001 | Yes |
| GF/0PEGDA+5eY vs. NA | 12.675 | 6.444 | <0.001 | Yes |
| GF/1PEGDA+5eY vs. NA | 7.225 | 3.673 | 0.007 | Yes |
| GF/0PEGDA+5e vs. GF/1PEGDA+5 | 5.45 | 2.771 | 0.045 | Yes |
| GF/20PEGDA+5 vs. GF/5PEGDA+5 | 1 | 0.508 | 0.944 | No |
| GF/20PEGDA+5 vs. GF/10PEGDA+ | 0.625 | 0.318 | 0.939 | No |
| GF/10PEGDA+5 vs. GF/5PEGDA+5 | 0.375 | 0.191 | 0.851 | No |

Figure 3.4

One Way Analysis of Variance

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 0min | 5 | 0 | 100 | 0 | 0 |
| GF-15min | 5 | 0 | 0.15 | 0.077 | 0.0345 |
| GF/0eY-15min | 5 | 0 | 0.124 | 0.063 | 0.0282 |
| GF/2eY-15min | 5 | 0 | 49.38 | 5.372 | 2.402 |
| GF/5eY-15min | 5 | 0 | 75.497 | 2.322 | 1.038 |
| GF/10eY-15min | 5 | 0 | 77.562 | 0.421 | 0.188 |
| GF/2eY-30min | 5 | 0 | 29.828 | 12.544 | 5.61 |
| GF/5eY-30min | 5 | 0 | 65.323 | 6.071 | 2.715 |
| GF/10eY-30min | 5 | 0 | 77.97 | 1.246 | 0.557 |
| GF/2eY-60min | 5 | 0 | 1.688 | 2.433 | 1.088 |
| GF/5eY-60min | 5 | 0 | 45.016 | 9.581 | 4.285 |
| GF/10eY-60min | 5 | 0 | 75.544 | 3.682 | 1.647 |
| GF/2eY-120min | 5 | 0 | 0 | 0 | 0 |
| GF/5eY-120min | 5 | 0 | 23.846 | 11.855 | 5.302 |
| GF/10eY-120min | 5 | 0 | 70.657 | 2.783 | 1.245 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 14 | 83545.78 | 5967.556 | 182.775 | <0.001 |
| Residual | 60 | 1958.988 | 32.65 | | |
| Total | 74 | 85504.77 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| 0min vs. GF/2eY-120min | 100 | 27.671 | <0.001 | Yes |
| 0min vs. GF/0eY-15min | 99.876 | 27.637 | <0.001 | Yes |
| 0min vs. GF-15min | 99.85 | 27.63 | <0.001 | Yes |
| 0min vs. GF/2eY-60min | 98.312 | 27.204 | <0.001 | Yes |
| GF/10eY-30mi vs. GF/2eY-120mi | 77.97 | 21.575 | <0.001 | Yes |
| GF/10eY-30min vs. GF/0eY-15min | 77.846 | 21.541 | <0.001 | Yes |

| | | | | |
|---|---|---|---|---|
| GF/10eY-30min vs. GF-15min | 77.82 | 21.534 | <0.001 | Yes |
| GF/10eY-15mi vs. GF/2eY-120mi | 77.562 | 21.462 | <0.001 | Yes |
| GF/10eY-15min vs. GF/0eY-15min | 77.438 | 21.428 | <0.001 | Yes |
| GF/10eY-15min vs. GF-15min | 77.412 | 21.421 | <0.001 | Yes |
| GF/10eY-30min vs. GF/2eY-60min | 76.282 | 21.108 | <0.001 | Yes |
| 0min vs. GF/5eY-120min | 76.154 | 21.073 | <0.001 | Yes |
| GF/10eY-15min vs. GF/2eY-60min | 75.874 | 20.995 | <0.001 | Yes |
| GF/10eY-60mi vs. GF/2eY-120mi | 75.544 | 20.904 | <0.001 | Yes |
| GF/5eY-15min vs. GF/2eY-120min | 75.497 | 20.891 | <0.001 | Yes |
| GF/10eY-60min vs. GF/0eY-15min | 75.42 | 20.87 | <0.001 | Yes |
| GF/10eY-60min vs. GF-15min | 75.394 | 20.863 | <0.001 | Yes |
| GF/5eY-15min vs. GF/0eY-15min | 75.373 | 20.857 | <0.001 | Yes |
| GF/5eY-15min vs. GF-15min | 75.347 | 20.85 | <0.001 | Yes |
| GF/10eY-60min vs. GF/2eY-60min | 73.856 | 20.437 | <0.001 | Yes |
| GF/5eY-15min vs. GF/2eY-60min | 73.809 | 20.424 | <0.001 | Yes |
| GF/10eY-120m vs. GF/2eY-120mi | 70.657 | 19.552 | <0.001 | Yes |
| GF/10eY-120m vs. GF/0eY-15min | 70.533 | 19.517 | <0.001 | Yes |
| GF/10eY-120min vs. GF-15min | 70.507 | 19.51 | <0.001 | Yes |
| 0min vs. GF/2eY-30min | 70.172 | 19.417 | <0.001 | Yes |
| GF/10eY-120m vs. GF/2eY-60min | 68.969 | 19.085 | <0.001 | Yes |
| GF/5eY-30min vs. GF/2eY-120min | 65.323 | 18.076 | <0.001 | Yes |
| GF/5eY-30min vs. GF/0eY-15min | 65.199 | 18.042 | <0.001 | Yes |
| GF/5eY-30min vs. GF-15min | 65.174 | 18.034 | <0.001 | Yes |
| GF/5eY-30min vs. GF/2eY-60min | 63.636 | 17.609 | <0.001 | Yes |
| 0min vs. GF/5eY-60min | 54.984 | 15.215 | <0.001 | Yes |
| GF/10eY-30mi vs. GF/5eY-120mi | 54.123 | 14.977 | <0.001 | Yes |
| GF/10eY-15mi vs. GF/5eY-120mi | 53.716 | 14.864 | <0.001 | Yes |
| GF/10eY-60mi vs. GF/5eY-120mi | 51.698 | 14.305 | <0.001 | Yes |
| GF/5eY-15min vs. GF/5eY-120min | 51.65 | 14.292 | <0.001 | Yes |
| 0min vs. GF/2eY-15min | 50.62 | 14.007 | <0.001 | Yes |
| GF/2eY-15min vs. GF/2eY-120min | 49.38 | 13.664 | <0.001 | Yes |
| GF/2eY-15min vs. GF/0eY-15min | 49.256 | 13.63 | <0.001 | Yes |
| GF/2eY-15min vs. GF-15min | 49.231 | 13.623 | <0.001 | Yes |
| GF/10eY-30min vs. GF/2eY-30min | 48.141 | 13.321 | <0.001 | Yes |
| GF/10eY-15min vs. GF/2eY-30min | 47.733 | 13.208 | <0.001 | Yes |
| GF/2eY-15min vs. GF/2eY-60min | 47.693 | 13.197 | <0.001 | Yes |
| GF/10eY-120m vs. GF/5eY-120mi | 46.81 | 12.953 | <0.001 | Yes |
| GF/10eY-60min vs. GF/2eY-30min | 45.716 | 12.65 | <0.001 | Yes |
| GF/5eY-15min vs. GF/2eY-30min | 45.668 | 12.637 | <0.001 | Yes |
| GF/5eY-60min vs. GF/2eY-120min | 45.016 | 12.456 | <0.001 | Yes |
| GF/5eY-60min vs. GF/0eY-15min | 44.892 | 12.422 | <0.001 | Yes |
| GF/5eY-60min vs. GF-15min | 44.866 | 12.415 | <0.001 | Yes |
| GF/5eY-60min vs. GF/2eY-60min | 43.328 | 11.989 | <0.001 | Yes |
| GF/5eY-30min vs. GF/5eY-120min | 41.477 | 11.477 | <0.001 | Yes |
| GF/10eY-120m vs. GF/2eY-30min | 40.828 | 11.298 | <0.001 | Yes |
| GF/5eY-30min vs. GF/2eY-30min | 35.495 | 9.822 | <0.001 | Yes |
| 0min vs. GF/5eY-30min | 34.677 | 9.595 | <0.001 | Yes |
| GF/10eY-30min vs. GF/5eY-60min | 32.954 | 9.119 | <0.001 | Yes |
| GF/10eY-15min vs. GF/5eY-60min | 32.546 | 9.006 | <0.001 | Yes |

| | | | | |
|---|---|---|---|---|
| GF/10eY-60min vs. GF/5eY-60min | 30.528 | 8.448 | <0.001 | Yes |
| GF/5eY-15min vs. GF/5eY-60min | 30.481 | 8.435 | <0.001 | Yes |
| GF/2eY-30min vs. GF/2eY-120min | 29.828 | 8.254 | <0.001 | Yes |
| GF/2eY-30min vs. GF/0eY-15min | 29.704 | 8.22 | <0.001 | Yes |
| GF/2eY-30min vs. GF-15min | 29.679 | 8.213 | <0.001 | Yes |
| 0min vs. GF/10eY-120min | 29.343 | 8.12 | <0.001 | Yes |
| GF/10eY-30min vs. GF/2eY-15min | 28.589 | 7.911 | <0.001 | Yes |
| GF/10eY-15min vs. GF/2eY-15min | 28.182 | 7.798 | <0.001 | Yes |
| GF/2eY-30min vs. GF/2eY-60min | 28.141 | 7.787 | <0.001 | Yes |
| GF/10eY-60min vs. GF/2eY-15min | 26.164 | 7.24 | <0.001 | Yes |
| GF/5eY-15min vs. GF/2eY-15min | 26.116 | 7.227 | <0.001 | Yes |
| GF/10eY-120m vs. GF/5eY-60min | 25.641 | 7.095 | <0.001 | Yes |
| GF/2eY-15min vs. GF/5eY-120min | 25.534 | 7.066 | <0.001 | Yes |
| 0min vs. GF/5eY-15min | 24.503 | 6.78 | <0.001 | Yes |
| 0min vs. GF/10eY-60min | 24.456 | 6.767 | <0.001 | Yes |
| GF/5eY-120mi vs. GF/2eY-120mi | 23.846 | 6.599 | <0.001 | Yes |
| GF/5eY-120min vs. GF/0eY-15min | 23.722 | 6.564 | <0.001 | Yes |
| GF/5eY-120min vs. GF-15min | 23.697 | 6.557 | <0.001 | Yes |
| 0min vs. GF/10eY-15min | 22.438 | 6.209 | <0.001 | Yes |
| GF/5eY-120min vs. GF/2eY-60min | 22.159 | 6.132 | <0.001 | Yes |
| 0min vs. GF/10eY-30min | 22.03 | 6.096 | <0.001 | Yes |
| GF/10eY-120m vs. GF/2eY-15min | 21.276 | 5.887 | <0.001 | Yes |
| GF/5eY-60min vs. GF/5eY-120min | 21.169 | 5.858 | <0.001 | Yes |
| GF/5eY-30min vs. GF/5eY-60min | 20.308 | 5.619 | <0.001 | Yes |
| GF/2eY-15min vs. GF/2eY-30min | 19.552 | 5.41 | <0.001 | Yes |
| GF/5eY-30min vs. GF/2eY-15min | 15.943 | 4.412 | 0.001 | Yes |
| GF/5eY-60min vs. GF/2eY-30min | 15.187 | 4.202 | 0.002 | Yes |
| GF/10eY-30min vs. GF/5eY-30min | 12.646 | 3.499 | 0.02 | Yes |
| GF/10eY-15min vs. GF/5eY-30min | 12.239 | 3.387 | 0.027 | Yes |
| GF/10eY-60min vs. GF/5eY-30min | 10.221 | 2.828 | 0.125 | No |
| GF/5eY-15min vs. GF/5eY-30min | 10.173 | 2.815 | 0.124 | No |
| GF/10eY-30mi vs. GF/10eY-120m | 7.313 | 2.024 | 0.603 | No |
| GF/10eY-15mi vs. GF/10eY-120m | 6.905 | 1.911 | 0.677 | No |
| GF/2eY-30min vs. GF/5eY-120min | 5.982 | 1.655 | 0.843 | No |
| GF/10eY-120m vs. GF/5eY-30min | 5.333 | 1.476 | 0.919 | No |
| GF/10eY-60mi vs. GF/10eY-120m | 4.887 | 1.352 | 0.95 | No |
| GF/5eY-15min vs. GF/10eY-120m | 4.84 | 1.339 | 0.943 | No |
| GF/2eY-15min vs. GF/5eY-60min | 4.365 | 1.208 | 0.968 | No |
| GF/10eY-30min vs. GF/5eY-15min | 2.473 | 0.684 | 1 | No |
| GF/10eY-30mi vs. GF/10eY-60mi | 2.425 | 0.671 | 1 | No |
| GF/10eY-15min vs. GF/5eY-15min | 2.065 | 0.571 | 1 | No |
| GF/10eY-15mi vs. GF/10eY-60mi | 2.018 | 0.558 | 1 | No |
| GF/2eY-60min vs. GF/2eY-120min | 1.688 | 0.467 | 1 | No |
| GF/2eY-60min vs. GF/0eY-15min | 1.564 | 0.433 | 1 | No |
| GF/2eY-60min vs. GF-15min | 1.538 | 0.426 | 0.999 | No |
| GF/10eY-30mi vs. GF/10eY-15mi | 0.408 | 0.113 | 1 | No |
| GF-15min vs. GF/2eY-120min | 0.15 | 0.0414 | 1 | No |
| GF/0eY-15min vs. GF/2eY-120min | 0.124 | 0.0343 | 1 | No |
| GF/10eY-60min vs. GF/5eY-15min | 0.0474 | 0.0131 | 1 | No |

| | | | | |
|---|---|---|---|---|
| GF-15min vs. GF/0eY-15min | 0.0257 | 0.0071 | 0.994 | No |

Figure 3.5

One Way Analysis of Variance

Data source: Data 1 in Notebook1

**Thickness (inch)**

Normality Test (Shapiro-Wilk) Passed (P = 0.138)

Equal Variance Test: Failed (P < 0.050)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| GF | 18 | 0 | 0.0177 | 0.00372 | 0.000877 |
| sIPN GF | 12 | 0 | 0.0145 | 0.00166 | 0.000479 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 1 | 7.03E-05 | 7.03E-05 | 7.409 | 0.011 |
| Residual | 28 | 0.000266 | 9.49E-06 | | |
| Total | 29 | 0.000336 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.011).

Power of performed test with alpha = 0.050: 0.691

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| GF vs. sIPN GF | 0.00312 | 2.722 | 0.011 | Yes |

**Peak Load (N)**

Normality Test (Shapiro-Wilk) Passed (P = 0.954)

Equal Variance Test: Passed (P = 0.936)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| GF | 18 | 0 | 2.414 | 1.23 | 0.29 |
| sIPN GF | 12 | 0 | 15.74 | 1.428 | 0.412 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 1 | 1278.752 | 1278.752 | 743.72 | <0.001 |
| Residual | 28 | 48.143 | 1.719 | | |
| Total | 29 | 1326.896 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| sIPN GF vs. GF | 13.327 | 27.271 | <0.001 | Yes |

**Peak Stress (Mpa)**

Normality Test (Shapiro-Wilk) Passed (P = 0.094)

Equal Variance Test: Passed (P = 0.729)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| GF | 18 | 0 | 2.037 | 1.095 | 0.258 |
| sIPN GF | 12 | 0 | 16.067 | 1.474 | 0.425 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 1 | 1417.271 | 1417.271 | 896.475 | <0.001 |
| Residual | 28 | 44.266 | 1.581 | | |
| Total | 29 | 1461.538 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| sIPN GF vs. GF | 14.03 | 29.941 | <0.001 | Yes |

**Modulus (Mpa)**

Normality Test (Shapiro-Wilk) Passed (P = 0.073)

Equal Variance Test: Passed (P = 0.443)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| GF | 18 | 0 | 135.485 | 53.042 | 12.502 |
| sIPN GF | 12 | 0 | 676.871 | 74.447 | 21.491 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 1 | 2110312 | 2110312 | 543.128 | <0.001 |
| Residual | 28 | 108793.4 | 3885.477 | | |
| Total | 29 | 2219105 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| sIPN GF vs. GF | 541.386 | 23.305 | <0.001 | Yes |

**Strain At Break (mm/mm)**

Normality Test (Shapiro-Wilk) Passed (P = 0.833)

Equal Variance Test: Passed (P = 0.491)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| GF | 18 | 0 | 0.0208 | 0.00772 | 0.00182 |
| sIPN GF | 12 | 0 | 0.0497 | 0.00925 | 0.00267 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 1 | 0.00602 | 0.00602 | 86.326 | <0.001 |
| Residual | 28 | 0.00195 | 6.97E-05 | | |
| Total | 29 | 0.00797 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| sIPN GF vs. GF | 0.0289 | 9.291 | <0.001 | Yes |

**Energy To Break (N*mm)**

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| GF | 18 | 0 | 0.227 | 0.17 | 0.04 |
| sIPN GF | 12 | 0 | 3.884 | 1.179 | 0.34 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 1 | 96.258 | 96.258 | 170.807 | <0.001 |
| Residual | 28 | 15.779 | 0.564 | | |
| Total | 29 | 112.037 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| sIPN GF vs. GF | 3.656 | 13.069 | <0.001 | Yes |

Figure 3.7

One Way Analysis of Variance

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.473)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| GF | 60 | 0 | 3.154 | 1.244 | 0.161 |
| sIPN GF | 60 | 0 | 4.868 | 1.522 | 0.197 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 1 | 88.121 | 88.121 | 45.594 | <0.001 |
| Residual | 118 | 228.061 | 1.933 | | |
| Total | 119 | 316.182 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| sIPN GF vs. GF | 1.714 | 6.752 | <0.001 | Yes |

Figure 3.9

One Way Analysis of Variance

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.592)

Equal Variance Test: Passed (P = 0.417)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| sIPN GIF 15min | 3 | 0 | 44.792 | 1.96 | 1.132 |
| sIPN GIF 30min | 3 | 0 | 48.371 | 1.974 | 1.14 |
| sIPN GIF 60min | 3 | 0 | 53.276 | 1.889 | 1.09 |
| sIPN GIF 90min | 3 | 0 | 56.532 | 2.657 | 1.534 |
| sIPN GIF 120min | 3 | 0 | 58.556 | 4.108 | 2.372 |
| sIPN GIF 240min | 3 | 0 | 64.451 | 3.007 | 1.736 |
| GIF 15min | 3 | 0 | 56.304 | 0.827 | 0.478 |
| GIF 30min | 3 | 0 | 65.122 | 0.652 | 0.376 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 7 | 1058.221 | 151.174 | 26.642 | <0.001 |
| Residual | 16 | 90.79 | 5.674 | | |
| Total | 23 | 1149.011 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| GIF 30min vs. sIPN GIF 15min | 20.33 | 10.453 | <0.001 | Yes |
| sIPN GIF 240 vs. sIPN GIF 15m | 19.659 | 10.108 | <0.001 | Yes |
| GIF 30min vs. sIPN GIF 30min | 16.751 | 8.612 | <0.001 | Yes |
| sIPN GIF 240 vs. sIPN GIF 30m | 16.08 | 8.268 | <0.001 | Yes |
| sIPN GIF 120 vs. sIPN GIF 15m | 13.764 | 7.077 | <0.001 | Yes |
| GIF 30min vs. sIPN GIF 60min | 11.846 | 6.09 | <0.001 | Yes |
| sIPN GIF 90m vs. sIPN GIF 15m | 11.74 | 6.036 | <0.001 | Yes |
| GIF 15min vs. sIPN GIF 15min | 11.512 | 5.919 | <0.001 | Yes |
| sIPN GIF 240 vs. sIPN GIF 60m | 11.175 | 5.746 | <0.001 | Yes |
| sIPN GIF 120 vs. sIPN GIF 30m | 10.185 | 5.237 | 0.002 | Yes |
| GIF 30min vs. GIF 15min | 8.818 | 4.534 | 0.006 | Yes |
| GIF 30min vs. sIPN GIF 90min | 8.59 | 4.416 | 0.007 | Yes |
| sIPN GIF 60m vs. sIPN GIF 15m | 8.484 | 4.362 | 0.008 | Yes |

| | | | | |
|---|---|---|---|---|
| sIPN GIF 90m vs. sIPN GIF 30m | 8.161 | 4.196 | 0.01 | Yes |
| sIPN GIF 240min vs. GIF 15min | 8.147 | 4.189 | 0.01 | Yes |
| GIF 15min vs. sIPN GIF 30min | 7.933 | 4.079 | 0.011 | Yes |
| sIPN GIF 240 vs. sIPN GIF 90m | 7.919 | 4.071 | 0.011 | Yes |
| GIF 30min vs. sIPN GIF 120min | 6.566 | 3.376 | 0.042 | Yes |
| sIPN GIF 240 vs. sIPN GIF 120 | 5.895 | 3.031 | 0.077 | No |
| sIPN GIF 120 vs. sIPN GIF 60m | 5.28 | 2.715 | 0.13 | No |
| sIPN GIF 60m vs. sIPN GIF 30m | 4.905 | 2.522 | 0.167 | No |
| sIPN GIF 30m vs. sIPN GIF 15m | 3.579 | 1.84 | 0.46 | No |
| sIPN GIF 90m vs. sIPN GIF 60m | 3.256 | 1.674 | 0.515 | No |
| GIF 15min vs. sIPN GIF 60min | 3.028 | 1.557 | 0.527 | No |
| sIPN GIF 120min vs. GIF 15min | 2.252 | 1.158 | 0.707 | No |
| sIPN GIF 120 vs. sIPN GIF 90m | 2.024 | 1.04 | 0.677 | No |
| GIF 30min vs. sIPN GIF 240min | 0.671 | 0.345 | 0.93 | No |
| sIPN GIF 90min vs. GIF 15min | 0.228 | 0.117 | 0.908 | No |

Figure 3.11

One Way Analysis of Variance

Data source: Data 1 in Notebook1

**p-AKT**

Normality Test (Shapiro-Wilk) Passed (P = 0.143)

Equal Variance Test: Failed (P < 0.050)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 4 | 0 | 1 | 0 | 0 |
| Insulin | 4 | 0 | 6.207 | 0.128 | 0.0641 |
| GF | 4 | 0 | 1.855 | 0.687 | 0.343 |
| sIPN GF | 4 | 0 | 1.512 | 0.647 | 0.323 |
| GIF | 4 | 0 | 6.326 | 0.443 | 0.222 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 4 | 112.608 | 28.152 | 127.609 | <0.001 |
| Residual | 15 | 3.309 | 0.221 | | |
| Total | 19 | 115.917 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| GIF vs. NA | 5.326 | 16.037 | <0.001 | Yes |
| Insulin vs. NA | 5.207 | 15.678 | <0.001 | Yes |
| GIF vs. sIPN GF | 4.815 | 14.496 | <0.001 | Yes |
| Insulin vs. sIPN GF | 4.695 | 14.137 | <0.001 | Yes |
| GIF vs. GF | 4.471 | 13.462 | <0.001 | Yes |
| Insulin vs. GF | 4.352 | 13.102 | <0.001 | Yes |
| GF vs. NA | 0.855 | 2.576 | 0.082 | No |
| sIPN GF vs. NA | 0.512 | 1.541 | 0.373 | No |
| GF vs. sIPN GF | 0.344 | 1.035 | 0.534 | No |
| GIF vs. Insulin | 0.119 | 0.359 | 0.724 | No |

**AKT1**

| | | |
|---|---|---|
| Normality Test (Shapiro-Wilk) | Passed | (P = 0.489) |
| Equal Variance Test: | Passed | (P = 0.155) |

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 4 | 0 | 1 | 0 | 0 |
| Insulin | 4 | 0 | 0.889 | 0.126 | 0.063 |
| GF | 4 | 0 | 0.967 | 0.143 | 0.0716 |
| sIPN GF | 4 | 0 | 1.04 | 0.112 | 0.0561 |
| GIF | 4 | 0 | 0.924 | 0.175 | 0.0874 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 4 | 0.0576 | 0.0144 | 0.905 | 0.486 |
| Residual | 15 | 0.239 | 0.0159 | | |
| Total | 19 | 0.296 | | | |

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.486).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists.
Negative results should be interpreted cautiously.

Figure 3.12

One Way Analysis of Variance

Data source: Data 1 in Notebook1

**p-AKT**

Normality Test (Shapiro-Wilk) Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 3 | 0 | 1 | 0 | 0 |
| GIF 30min | 3 | 0 | 6.052 | 0.275 | 0.159 |
| sIPN GIF 15min | 3 | 0 | 3.218 | 0.659 | 0.38 |
| sIPN GIF 30min | 3 | 0 | 3.712 | 0.967 | 0.559 |
| sIPN GIF 60min | 3 | 0 | 4.386 | 0.757 | 0.437 |
| sIPN GIF 120min | 3 | 0 | 5.838 | 0.668 | 0.386 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 5 | 52.264 | 10.453 | 25.442 | <0.001 |
| Residual | 12 | 4.93 | 0.411 | | |
| Total | 17 | 57.194 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| GIF 30min vs. NA | 5.052 | 9.653 | <0.001 | Yes |
| sIPN GIF 120min vs. NA | 4.838 | 9.243 | <0.001 | Yes |
| sIPN GIF 60min vs. NA | 3.386 | 6.469 | <0.001 | Yes |
| GIF 30min vs. sIPN GIF 15min | 2.833 | 5.414 | 0.002 | Yes |
| sIPN GIF 30min vs. NA | 2.712 | 5.183 | 0.003 | Yes |
| sIPN GIF 120 vs. sIPN GIF 15m | 2.619 | 5.004 | 0.003 | Yes |
| GIF 30min vs. sIPN GIF 30min | 2.339 | 4.47 | 0.007 | Yes |
| sIPN GIF 15min vs. NA | 2.218 | 4.239 | 0.009 | Yes |
| sIPN GIF 120 vs. sIPN GIF 30m | 2.125 | 4.061 | 0.011 | Yes |
| GIF 30min vs. sIPN GIF 60min | 1.666 | 3.184 | 0.046 | Yes |
| sIPN GIF 120 vs. sIPN GIF 60m | 1.452 | 2.775 | 0.081 | No |
| sIPN GIF 60m vs. sIPN GIF 15m | 1.167 | 2.23 | 0.17 | No |
| sIPN GIF 60m vs. sIPN GIF 30m | 0.673 | 1.286 | 0.53 | No |

| | | | | |
|---|---|---|---|---|
| sIPN GIF 30m vs. sIPN GIF 15m | 0.494 | 0.944 | 0.595 | No |
| GIF 30min vs. sIPN GIF 120min | 0.214 | 0.409 | 0.69 | No |

**AKT1**

Normality Test (Shapiro-Wilk) Passed (P = 0.168)

Equal Variance Test: Passed (P = 0.064)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| NA | 3 | 0 | 1 | 0 | 0 |
| GIF 30min | 3 | 0 | 0.928 | 0.0657 | 0.038 |
| sIPN GIF 15min | 3 | 0 | 1.104 | 0.109 | 0.0629 |
| sIPN GIF 30min | 3 | 0 | 1.156 | 0.0959 | 0.0554 |
| sIPN GIF 60min | 3 | 0 | 1.075 | 0.144 | 0.0834 |
| sIPN GIF 120min | 3 | 0 | 1.033 | 0.268 | 0.155 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 5 | 0.0976 | 0.0195 | 0.99 | 0.463 |
| Residual | 12 | 0.236 | 0.0197 | | |
| Total | 17 | 0.334 | | | |

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.463).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists.
Negative results should be interpreted cautiously.

Figure 3.14

One Way Analysis of Variance

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.070)

Equal Variance Test: Failed (P < 0.050)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| 0 min | 8 | 0 | 100 | 0 | 0 |
| 15 min | 8 | 0 | 74.875 | 2.961 | 1.047 |
| 30 min | 8 | 0 | 54.625 | 15.716 | 5.556 |
| 60 min | 8 | 0 | 39.938 | 12.339 | 4.362 |
| 120 min | 8 | 0 | 35.438 | 17.514 | 6.192 |
| 240 min | 8 | 0 | 8.813 | 6.948 | 2.457 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 5 | 40949.3 | 8189.859 | 64.401 | <0.001 |
| Residual | 42 | 5341.156 | 127.17 | | |
| Total | 47 | 46290.45 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| 0 min vs. 240 min | 91.188 | 16.172 | <0.001 | Yes |
| 15 min vs. 240 min | 66.063 | 11.716 | <0.001 | Yes |
| 0 min vs. 120 min | 64.563 | 11.45 | <0.001 | Yes |
| 0 min vs. 60 min | 60.063 | 10.652 | <0.001 | Yes |
| 30 min vs. 240 min | 45.813 | 8.125 | <0.001 | Yes |
| 0 min vs. 30 min | 45.375 | 8.047 | <0.001 | Yes |
| 15 min vs. 120 min | 39.438 | 6.994 | <0.001 | Yes |
| 15 min vs. 60 min | 34.938 | 6.196 | <0.001 | Yes |
| 60 min vs. 240 min | 31.125 | 5.52 | <0.001 | Yes |
| 120 min vs. 240 min | 26.625 | 4.722 | <0.001 | Yes |
| 0 min vs. 15 min | 25.125 | 4.456 | <0.001 | Yes |
| 15 min vs. 30 min | 20.25 | 3.591 | 0.003 | Yes |
| 30 min vs. 120 min | 19.188 | 3.403 | 0.004 | Yes |
| 30 min vs. 60 min | 14.688 | 2.605 | 0.025 | Yes |
| 60 min vs. 120 min | 4.5 | 0.798 | 0.429 | No |

Figure 3.16

One Way Analysis of Variance

Data source: Data 1 in Notebook1

Normality Test (Shapiro-Wilk) Passed (P = 0.326)

Equal Variance Test: Passed (P = 0.393)

| Group Name | N | Missing | Mean | Std Dev | SEM |
|---|---|---|---|---|---|
| sIPN GIF 15min | 3 | 0 | 0.666 | 0.0144 | 0.00829 |
| sIPN GIF 30min | 3 | 0 | 0.712 | 0.0066 | 0.00381 |
| sIPN GIF 60min | 3 | 0 | 0.868 | 0.037 | 0.0213 |
| sIPN GIF 120min | 3 | 0 | 1.612 | 0.0551 | 0.0318 |
| sIPN GIF 240min | 3 | 0 | 1.798 | 0.0419 | 0.0242 |
| Insulin 15min | 3 | 0 | 0.666 | 0.0611 | 0.0353 |
| Insulin 30min | 3 | 0 | 0.75 | 0.00287 | 0.00166 |
| Insulin 60min | 3 | 0 | 0.879 | 0.0349 | 0.0202 |
| Insulin 120min | 3 | 0 | 1.507 | 0.0811 | 0.0468 |
| Insulin 240min | 3 | 0 | 1.563 | 0.0743 | 0.0429 |

| Source of Variation | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Between Groups | 9 | 5.646 | 0.627 | 267.426 | <0.001 |
| Residual | 20 | 0.0469 | 0.00235 | | |
| Total | 29 | 5.692 | | | |

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor:

| Comparison | Diff of Me | t | P | P<0.050 |
|---|---|---|---|---|
| sIPN GIF 240 vs. sIPN GIF 15m | 1.132 | 28.624 | <0.001 | Yes |
| sIPN GIF 240 vs. Insulin 15mi | 1.132 | 28.624 | <0.001 | Yes |
| sIPN GIF 240 vs. sIPN GIF 30m | 1.086 | 27.471 | <0.001 | Yes |
| sIPN GIF 240 vs. Insulin 30mi | 1.049 | 26.52 | <0.001 | Yes |
| sIPN GIF 120 vs. sIPN GIF 15m | 0.945 | 23.907 | <0.001 | Yes |
| sIPN GIF 120 vs. Insulin 15mi | 0.945 | 23.907 | <0.001 | Yes |
| sIPN GIF 240 vs. sIPN GIF 60m | 0.93 | 23.514 | <0.001 | Yes |
| sIPN GIF 240 vs. Insulin 60mi | 0.919 | 23.245 | <0.001 | Yes |
| sIPN GIF 120 vs. sIPN GIF 30m | 0.9 | 22.754 | <0.001 | Yes |
| Insulin 240m vs. sIPN GIF 15m | 0.897 | 22.684 | <0.001 | Yes |
| Insulin 240m vs. Insulin 15mi | 0.897 | 22.684 | <0.001 | Yes |

| | | | | |
|---|---|---|---|---|
| sIPN GIF 120 vs. Insulin 30mi | 0.862 | 21.804 | <0.001 | Yes |
| Insulin 240m vs. sIPN GIF 30m | 0.851 | 21.531 | <0.001 | Yes |
| Insulin 120m vs. sIPN GIF 15m | 0.841 | 21.262 | <0.001 | Yes |
| Insulin 120m vs. Insulin 15mi | 0.841 | 21.262 | <0.001 | Yes |
| Insulin 240m vs. Insulin 30mi | 0.814 | 20.581 | <0.001 | Yes |
| Insulin 120m vs. sIPN GIF 30m | 0.795 | 20.109 | <0.001 | Yes |
| Insulin 120m vs. Insulin 30mi | 0.758 | 19.159 | <0.001 | Yes |
| sIPN GIF 120 vs. sIPN GIF 60m | 0.743 | 18.797 | <0.001 | Yes |
| sIPN GIF 120 vs. Insulin 60mi | 0.733 | 18.528 | <0.001 | Yes |
| Insulin 240m vs. sIPN GIF 60m | 0.695 | 17.575 | <0.001 | Yes |
| Insulin 240m vs. Insulin 60mi | 0.684 | 17.305 | <0.001 | Yes |
| Insulin 120m vs. sIPN GIF 60m | 0.639 | 16.152 | <0.001 | Yes |
| Insulin 120m vs. Insulin 60mi | 0.628 | 15.883 | <0.001 | Yes |
| sIPN GIF 240 vs. Insulin 120m | 0.291 | 7.362 | <0.001 | Yes |
| sIPN GIF 240 vs. Insulin 240m | 0.235 | 5.94 | <0.001 | Yes |
| Insulin 60mi vs. sIPN GIF 15m | 0.213 | 5.379 | <0.001 | Yes |
| Insulin 60mi vs. Insulin 15mi | 0.213 | 5.379 | <0.001 | Yes |
| sIPN GIF 60m vs. sIPN GIF 15m | 0.202 | 5.11 | <0.001 | Yes |
| sIPN GIF 60m vs. Insulin 15mi | 0.202 | 5.11 | <0.001 | Yes |
| sIPN GIF 240 vs. sIPN GIF 120 | 0.187 | 4.717 | 0.002 | Yes |
| Insulin 60mi vs. sIPN GIF 30m | 0.167 | 4.226 | 0.006 | Yes |
| sIPN GIF 60m vs. sIPN GIF 30m | 0.156 | 3.957 | 0.01 | Yes |
| Insulin 60mi vs. Insulin 30mi | 0.13 | 3.275 | 0.044 | Yes |
| sIPN GIF 60m vs. Insulin 30mi | 0.119 | 3.006 | 0.074 | No |
| sIPN GIF 120 vs. Insulin 120m | 0.105 | 2.645 | 0.145 | No |
| Insulin 30mi vs. sIPN GIF 15m | 0.0832 | 2.103 | 0.359 | No |
| Insulin 30mi vs. Insulin 15mi | 0.0832 | 2.103 | 0.327 | No |
| Insulin 240m vs. Insulin 120m | 0.0562 | 1.422 | 0.729 | No |
| sIPN GIF 120 vs. Insulin 240m | 0.0484 | 1.223 | 0.801 | No |
| sIPN GIF 30m vs. sIPN GIF 15m | 0.0456 | 1.153 | 0.782 | No |
| sIPN GIF 30m vs. Insulin 15mi | 0.0456 | 1.153 | 0.704 | No |
| Insulin 30mi vs. sIPN GIF 30m | 0.0376 | 0.95 | 0.73 | No |
| Insulin 60mi vs. sIPN GIF 60m | 0.0106 | 0.269 | 0.956 | No |
| Insulin 15mi vs. sIPN GIF 15m | 3.33E-16 | 8.42E-15 | 1 | No |