BRANCHED CHAIN AMINO ACID SUPPLEMENTATION MODULATES THE EFFECT OF INFLAMMATORY MEDIATORS ON THE FUNCTION OF A HEPATOMA CELL LINE

By

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ABSTRACT OF THESIS

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Severe trauma injuries often lead to a prolonged inflammatory response associated with metabolic abnormalities. These abnormalities lead to persistent skeletal muscle proteolysis and rapid loss of lean body mass, which may lead to multiple organ dysfunction and death. The liver plays an important role in this systemic response to injury by modulating the inflammatory processes, immune functions, and metabolic pathways. The acute phase of the response is characterized by the release of inflammatory mediators, including proinflammatory cytokines and reactive oxygen species (ROS), as well as a change in the protein secretion pattern and increased amino acid utilization by the liver. When these changes persist for long periods of time, they likely contribute to the body's negative nitrogen balance as well as loss of lean body mass (LBM). Among all proteins secreted by the liver, albumin is the most abundant one and its secretion rate significantly decreases in inflammatory states, and results in a lower circulating concentration in patients. Albumin provides the critical colloid osmotic pressure

to regulate the passage of water and diffusible solutes through the capillaries. Increased utilization of amino acids in trauma is thought to be important for defense against diseases; however, it reduces body stores of proteins and free amino acids to supply amino acid needs for tissue repair, acute phase protein production, and gluconeogenesis. Nutritional supplementation is currently being used to alleviate endogenous nitrogen depletion and to maximize protein synthesis for optimal wound healing and immune function. Among nutritional supplements, there has been increased interest in using branched chain amino acids (BCAAs), and clinical studies suggest that BCAAs can enhance liver function in inflammatory states. The mechanism whereby BCAAs impact the liver function during inflammation is unclear. The purpose of this research is to investigate the effect of proinflammatory mediators (more specifically the cytokines interleukin-1ß & interleukin-6) and reactive oxygen species H_2O_2, and BCAAs on a cell culture model of liver consisting of HepG2/C3A hepatoma cells. It was found that these mediators reversibly suppressed albumin and urea production in a dose-dependent manner, and also decreased the amount of the intracellular antioxidant reduced glutathione. BCAA supplementation mitigated the effect of these inflammatory mediators; however, this effect was maximized at a relatively low concentration of BCAAs, above which no further benefit was observed. Therefore, BCAAs are potentially beneficial to support liver function during inflammation.

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DEDICATIONS

To my parents and my brother

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CHAPTER 1

1. INTRODUCTION

1.1 Motivation

Severe trauma injuries lead to significant patient morbidity and mortality (Herndon D.N, 2001). Severe trauma injuries carry a significant risk of infection that causes a more prolonged inflammatory response, resulting in metabolic and catabolic abnormalities. These abnormalities lead to the rapid loss of lean body mass (LBM) and an increase in energy demands due to the body's response to the injury. Moreover, the wound consumes energy and protein due to the increase in local metabolic activity of inflammatory cells and production of new tissue (MacKay D, 2003). The hypermetabolism causes a significant alteration in the utilization of amino acids as well as a negative nitrogen balance in the whole body (Pawlik T.M, 2000).

Severe trauma injuries are often associated with bacterial infections followed by a persistent inflammatory response. These conditions initiate changes in the immune system and result in local and systemic inflammation, which can progress to multiple organ-dysfunction (Oppeltz R.F, 2010) and death. The liver plays an important role in this context. The liver is responsible for the production of acute-phase proteins and the regulation of the systemic inflammatory response (Price L.A, 2007). Following trauma injuries, the acute phase response induces the liver to synthesize and release proteins that exert effects on a variety of other tissues (Kushner I, 1982). In this situation, hepatic protein synthesis is shifted from hepatic constitutive proteins to acute phase proteins (Jeschke M.G, 2009). The

acute phase proteins act as mediators of the inflammatory process, functioning as transport proteins, and participating in wound healing (Jeschke M.G, 1999).

Amino acids are important biological molecules with variety of side-chains, which permits a variety range of chemical reactions. A deficiency of amino acids impairs proteins synthesis, and as a result weaken the immune functions and increase the risk of infection (Li P, 2007). Amino acids also play an important role in immune responses, and adequate dietary intake of all amino acids is necessary to protect the host from a variety of diseases. Branched chain amino acid (BCAA) supplementation has been used to improve energy metabolism, nitrogen metabolism, carbohydrate metabolism, insulin resistance, liver disease, serum albumin levels, and the quality of serum albumin (Plauth M, 2011).

1.2 Project Scope

The object of this project is to focus on the effect of inflammatory mediators, more specifically the proinflammatory cytokines interleukin-1 ß and interleukine-6 (IL-1ß & IL-6) and reactive oxygen species (ROS), on the liver following trauma injury, as well as the effect of BCAAs as nutritional supplements. The second chapter briefly reviews the studies available in the literature regarding the effect of these inflammatory mediators' and their role in metabolic flux alteration in trauma injury. Since this project is performed based on *in vitro* experiments, background information about the hepatoma (HepG2/C3A) cell line that was used here (and widely in general) to investigate the effect of stress mediators on liver is provided as well. Chapters 3 through 8 generally cover the following topics: effect of the culture medium on the HepG2 cell line, exposure of HepG2 cells to different

concentrations of cytokines (IL-1ß and IL-6) and hydrogen peroxide (used as source of ROS), and effect of BCAAs (valine, leucine, and isoleucine) as nutritional supplements to overcome the metabolic changes effected by these inflammatory mediators.

CHAPTER 2

LITERATURE REVIEW

The liver plays an important role in trauma injuries by modulating the acute phase response, immune functions, metabolic pathways, and the inflammatory processes (Jeschke M.G, 2007). Severe trauma injury causes liver damage, induces hepatocyte apoptosis and necrosis, and as a result impair hepatic metabolism and hepatic protein synthesis (Jeschke M.G, 2001, 2007). Trauma also causes an increase in cytokine levels (including IL-1ß & IL-6) in sreum and liver. It has been suggested that the elevation of proinflammatory cytokines (IL-1ß & IL-6) (Jeschke M.G., 2007, Kao C.C, 2000) induces apoptosis in affected tissues. Severe trauma injuries evoke an immediate systemic response, which leads to protein malnutrition and increases in metabolic rate and nitrogen loss (Parihar A, 2008).

2.1. Hepatocellular Carcinoma (HepG2/C3A)

HepG2/C3A cells are cancer cells with characteristics of both hepatocytes and tumor cells. They are a subtype of HepG2 cells that are contact-inhibited in culture (unlike other HepG2 lines) and exhibit many of the characteristics of normal hepatocytes including production of serum proteins (Knowles B.B, 1980, Kelly J.H, 1989). HepG2/C3A cells were selected for “high albumin production, and ability to grow in glucose deficient medium” (Kelly J.H, 1994).

These cells display liver specific biological activity. They can be easily maintained, cultured, and used as a relevant model for *in vitro* studies (Wilkening S, 2003). Studies have shown that the phenotype of this cell line evolves from exhibiting features characteristic of fetal to adult liver cells as they reach confluence in culture (Kelly J.H, 1989, Kamiya A, 2002). Increased cell-cell interactions in this hepatoma cell line *in vitro* are thought to result in a more differentiated pattern of gene expression, and the phenotypic changes due to this interaction resemble hepatic differentiation and regeneration (Butura A, 2004). At low density (<200,000 cells/cm^2), they exhibit several characteristics of fetal liver cells (Kelly J.H, 1989). They have a rapid division characterized by a doubling time of 24 h, with a reduced synthesis of albumin. At higher density (>10^6 cells/cm^2) when the culture becomes confluent, growth slows with a doubling time of over about 200 h, with a four-fold higher level of albumin production (Kelly J.H, 1989). When a high-density culture is trypsinized and re-plated to a lower density, the cells again enter a rapid division cycle.

It has been reported that the human hepatoma cell line HepG2 possesses major neutral amino acid transport systems (Goenner S, 1997) and the specific hepatic system N[1] (Goenner S, 1992). Prior studies have shown that IL-1ß and IL-6 have no effect on glutamine uptake by HepG2 cells after various incubation times and at different concentrations (ranging from 0.1 – 5000U/ml) (Goenner S, 1997).

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[1] System N is the principal mediator to transport glutamine and consists of SN1 and SN2 proteins. The proteins are coupled to Na^+ and H^+ gradients to mediate the transport process in which Na^+ and glutamine move in one direction and H^+ moves in the opposite direction (Umapathy NS, 2008). Glutamine transport system N is similar in normal human liver and human HepG2 cells, but only partially mediates glutamine uptake (Bode B.P, 1999).

2.2. Inflammatory Response in Severe Trauma Injuries

Inflammation is the essential part of the body's immune response to remove harmful stimuli such as damaged cells or invading pathogens. Local and systemic inflammatory responses to trauma injury are extremely complex. Studies have shown that liver function and integrity are important for patient survival by modulating the inflammatory processes, immune functions, the acute phase response, and metabolic pathways. The acute phase response is a series of actions to activate repair processes, inhibit organ damage (Haycock J.W, 1997, Jeschke M.G, 2004), and relaease proinflammatory cytokines including IL-6 and IL-1 (Jeschke M.G, 2004, Haycock J.W, 1997). Clinical studies suggest that an "exaggerated" acute phase response may be dangerous and lead to multi-organ dysfunction, hypermetabolism, hypercatabolism, morbidity, and mortality (Tracey K.J, 1987, Haycock J.W, 1997, Bode J.G, 2012, Miert V, 1995, Heinrich P.C, 1990).

2.2.1. Cytokines

Cytokines are non-antibody proteins that act as mediators. They are secreted by specific cells of the immune system to mediate and regulate immunity and inflammation. There are different mediators secreted by activation of leukocytes (Gruys E, 2005) including monocytes, macrophages, and neutrophil granulocytes (Bode J.G, 2001). Leukocytes are the first cells attracted to the site of injury and therefore candidates for the local synthesis and release of these mediators (Heinrich P.C, 1990). Monocytes and macrophages are the main source of inflammatory mediators in the family of cytokines and chemokines (Bode J.G, 2001). Cytokines that possess proinflammatory properties,

including IL-1α/ß and IL-6, are responsible for muscle catabolism, proliferation of fibroblasts, and macrophages (Heinrich P.C, 1990, Miert V, 1995, Späte U, 2004, Neuvians T.P, 2004).

In the hepatic acute phase reaction, IL-1ß and IL-6 are responsible for the activation of hepatocyte receptors as well as the regulation and synthesis of varying acute phase proteins (Gruys E, 2005, Bode J.G, 2012). IL-1β induces other cytokines including IL-6 and inflammatory mediators such as nitric oxide (Black S, 2004, Gruys E, 2005). IL-1 along with TNF-α also stimulate fibroblasts, endothelial cells, and keratinocytes to synthesize IL-6 and amplify its biological effects (Heinrich P.C, 1990). IL-6 is the most effective mediator to activate acute phase protein synthesis in human hepatocytes, and plays a key role in the stimulation of hepatic glutamine transport following trauma injuries (Pawlik T.M, 2003).

2.2.2 Acute phase proteins

The liver is the primary organ for synthesizing acute phase proteins (Miller L.L, 1951, Bode J.G, 2012). There are varieties of plasma proteins identified as acute phase proteins, based on changes in circulating concentration of at least 25% after an inflammatory stimulus (Black S, 2004). Cytokine stimulation therefore dramatically changes the pattern of protein synthesis by the hepatocytes and results in an increase or decrease of different blood proteins.

Acute phase proteins can be divided into two groups. ***Group I*** includes complement C3, C-reactive protein (CRP), and α1-acid glycoprotein, which are induced by IL-1α/ß, TNF-α/ß (Baumann H, 1994, Haycock J.W, 1997, Bode J.G , 2012). ***Group II*** includes fibrinogens,

haptoglobin, α1-antichymotrypsin, and α 1-antitrypsin, which are induced by IL-6-type cytokines (Bode J.G, 2012). As a result of the acute phase response, expression of some of these proteins (albumin, transferrin, retinol-binding protein, etc) is reduced (Heinrich P.C, 1990, Heinrich P.C, 1998, Jeschke M.G, 2007), and those are referred to as *negative acute phase proteins*. In contrast, expression of some of the other proteins (C-reactive protien (CRP), fibrinogen, haptoglobin, α2-macroglobulin, etc) is increased (Gruys E, 2005, Jeschke M.G , 2007), and those are called *positive acute phase proteins*.

Albumin makes up 55-60% of the serum proteins (Gosling P, 1995), and is only produced by liver (Busher J.T, 1990). By making up more than half of the total protein in serum, albumin is the major protein providing the critical colloid osmotic or oncotic pressure that regulates passage of water and diffusible solutes through the capillaries (Busher J.T, 1990).

Serum albumin concentration depends on its rates of synthesis and degradation as well as its distribution between the intravascular and extravascular compartments. Albumin synthesis rate can be influenced by nutrition and cytokines produced during the inflammatory response (Busher J.T, 1990, Jeschke M.G 2007, 2008). A decrease in albumin synthesis can also be caused by intestinal malabsorption syndromes, protein-calorie malnutrition, and end-stage liver disease (Busher J.T, 1990) [2]. John and Miller (John D.W, 1969) demonstrated the major role of amino acids in accelerating albumin synthesis.

[2] The only clinical situation that causes an elevation in serum albumin is acute dehydration (Busher J.T, 1990)

However, recent studies suggest that decreased albumin synthesis is not the only mechanism responsible for persistent hypoalbuminemia that follows trauma injury (Hiyama D.T, 1991). Hypoalbuminemia in trauma injury may also result from dilution due to fluid resuscitation (Pulimood T.B, 2000), transcapillary leak, and large volume body fluid losses (Martini W.Z, 2010).

2.3. Metabolic Response in Trauma Injuries

Trauma injuries are followed by hypermetabolism and hypercatabolism. A severe trauma injury leads to metabolic disturbances, including extensive fluid redistribution (Deitch E.A, 1995, De-Souza D.A, 1998). It also causes an increase in secretion of inflammatory mediators and reactive oxygen species (ROS) (Tredget E.E, 1992, Jeschke M.G, 2004). The result of these metabolic alterations is the increase in glucose and oxidative metabolism, net nitrogen loss, gluconeogenesis, ureagenesis, alteration of lipid metabolism, proteolysis, and acceleration of amino acid flux, primarily glutamine and alanine (Burdge J.J, 1986).

Glucose is the main energy substrate in patients with trauma injuries. Metabolic interrelations among glucose, protein, urea, and fatty acids make glucose as a central controlling factor in patient's metabolic state (Wolf R.R, 1985). Serum glucose increases through hepatic gluconeogenesis to meet the increased demand from wounds. The liver is responsible for most glucose production under normal conditions as well as in abnormal metabolic circumstances (during acidosis and prolonged starvation), although; the kidney can also contribute some amount (Cano N, 2002). Glucose production in the liver is derived

from stored glycogen or by “de novo” synthesis, a.k.a. gluconeogenesis. The main gluconeogenic substrates are glycerol, alanine, and lactate (Williams F.N, 2011), the latter being the most important one. It is derived from plasma glucose via glycolysis and re-synthesis of glucose from lactate takes place within the Cori cycle (Wolf R.R, 1985), in which, muscle glycogen is broken down to glucose and metabolized to lactic acid. In the liver, lactate acid is then converted to glucose to be either stored as glycogen or served as an energy source in the muscle.

Gluconeogenesis increases in response to severe injury and leads to the “diabetes of stress” (Demling R.H, 2000). Another study has also suggested that the elevation of glucose may result from the increase in proteolysis (Flakoll P.J, 1993). Inflammatory cytokines, including IL-6, promote hyperglycemia in liver by modifying the signaling properties of insulin receptor substrate (Fan J, 1996, Sell H, 2006). Insulin is the most important hormone to control glucose metabolism in body. Insulin released in trauma patients is about twice normal in response to a glucose load (Galster A.D, 1984, Cree M.G, 2007); althought, the glucose level in plasma is often elevated, which indicates the development of insulin resistance (Cree M.G, 2007).

Glutamine, is one of the most abundant free amino acids. It is a major gluconeogenic substrate and the primary extrahepatic ammonia shuttle (Bode B.P, 1999). Severe trauma injury associated with a hypermetabolic state, results in an increase in muscle proteolysis, glutamine efflux (Baquet A, 1991), and a net negative nitrogen balance (Rennie M.J, 1985, Burdge J.J, 1986) that can be monitored by hepatic urea production

(Tredget E.E, 1992, Cynober L, 1989). The glutamine released from skeletal muscle plays an important role in hepatic metabolism and serves as a substrate for gluconeogenesis and ureagenesis (Häussinger D, 1986, Nurjhan N, 1995). The uptake of glutamine across the plasma membrane of hepatocytes occurs via a Na^+-dependent amino acid transporter known as "System N", for its selectivity for glutamine, histidine, and asparagine[3] (Pawlik T.M, 2000). Trauma injury stimulates glutamine transport rates, and the maximum transport stimulation is proportional to the size of the injury (Lohmann R, 1998).

Proteins make up 20% of the total body weight and "each protein molecule has a functional role in homeostasis" (Demling R.H, 2000). The major defect in protein metabolism due to the severe injuries is proteolysis or protein breakdown (Wolfe R, 1983, Bessey P, 1989), which leads to decreased muscle strength, and markedly contributes to mortality. Large quantities of amino acids, such as leucine, glutamine, and alanine are oxidized (Demling R.H, 2000). A study reported a marked depletion of nonessential amino acids in muscle following injury, which is primarily due to a 50% reduction of glutamine (Askanazi J, 1980); however, asparagine, aspartate, glutamate, isoleucine, and valine are converted into glutamine, which is then released into the circulation (Stinnett J.D, 1982). Glutamine and alanine supply energy to the liver (Soeters P.B, 2004) by removal of nitrogen and converting to glucose. The changes in BCAAs (valine, leucine, and isoleucine) concentrations are less reported because they are mostly catabolized in extrahepatic tissues (Holecek M, 2010). In a catabolic situation, amino acids are redistributed from

[3] Amino acids bearing nitrogenous side-chain

muscle to the liver and stimulate hepatic protein synthesis and secretion of acute phase proteins (Roth E, 2011).

Branched chain amino acids (BCAAs), namely valine, leucine, and isoleucine, are essential substrates for protein synthesis, biochemical regulation, and as precursors in complex metabolic reactions (Stinnett J.D, 1982). Although, the largest free amino acid pool can be found in skeletal muscle, BCAAs only account for 0.1 g (0.6-1.2 mmol)/kg of muscle (Rennie M.J, 1996). The pool size of free BCAAs is quite small compared to the total BCAA content in muscle proteins. This is important because free BCAAs, especially leucine, promote protein synthesis and inhibit protein degradation (Bolster D.R, 2004). BCAAs as nutritional supplements have been used to reduce catabolism in trauma injuries (Wang X.Y, 2003).

Most amino acids are oxidized primarily in the liver, however the liver cannot directly catabolize BCAAs and they oxidized in skeletal muscle cells (Shimomura Y, 2006), which are known as the major site of BCAA metabolism (both BCAA transamination and oxidation) in humans (Elia M, 1983, Suryawan A, 1998). BCAA catabolism is the result of distribution, activity, and expression of the two enzymes known as branched chain α-keto acid dehydrogenase (BCKDH) and branched chain aminotransferase (BCAT). Branched chain α-keto acids (BCKAs) including α-ketoisovaleric acid (KIV), α-ketoisocaproic acid (KIC), and α-keto-ß-methylvaleric acid (KMV) can substitute for the corresponding essential branched chain amino acids: valine, leucine, and isoleucine respectively. The liver has very active system for the degradation of BCKAs derived from the corresponding BCAAs (Harper A.E,

1984). There are only two metabolic pathways available for BCKA including *I)* oxidative decarboxylation followed by degradation to CO_2 and water for energy purposes, *II)* conversion via transamination into the corresponding BCAAs, which can then be incorporated into tissue proteins (Khatra B.S, 1977) (Fig-1).

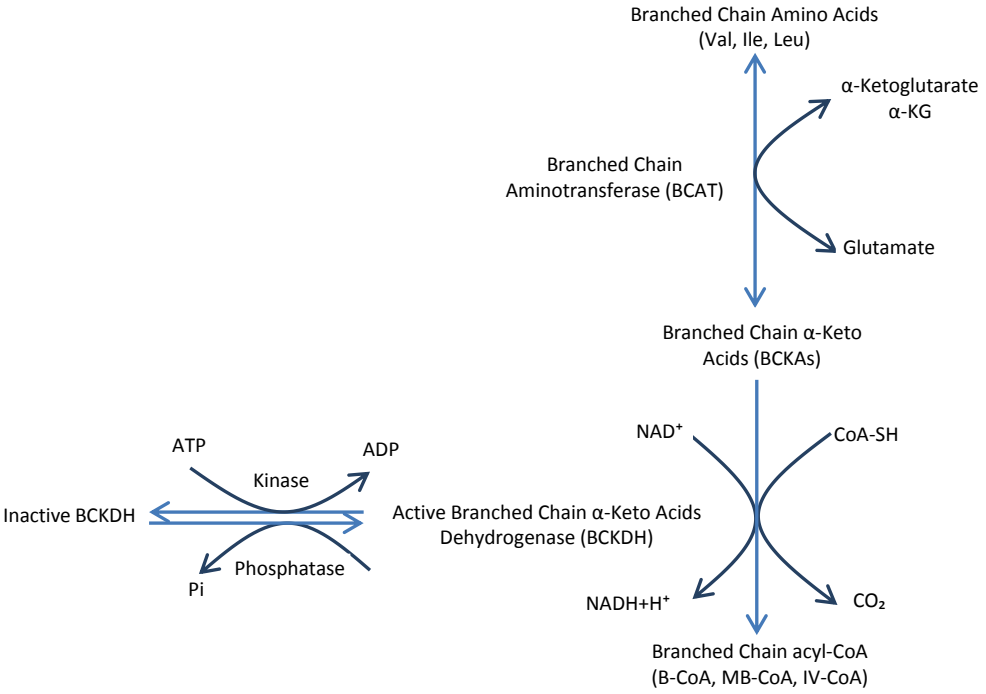

Figure 1: BCAAs Catabolism: The first two steps of the catabolic pathway and regulation of BCKDH complex.[4]

The first two steps in BCAAs catabolism are the same for the three BCAAs. BCAT catalyzes transamination of BCAAs, which is a reversible reaction to form BCKAs; and the BCKDH complex catalyzes the irreversible reaction of oxidative decarboxylation of BCKAs

[4] Modified from (Shimomura Y, 2006)

(Shimomura Y, 2006). The BCKDH complex is regulated by a phosphorylation / dephosphorylation cycle (Shimomura Y, 2006).

Human recombinant tumor necrosis factor-α (TNF) and IL-1ß or α increase muscle BCKDH activation to two to four-fold (Nawabi m.d, 1990). Therefore, protein catabolic states in sepsis and trauma can be associated with acceleration in oxidation of BCAAs. A clinical study in 1983 (Pelosi G, 1983) suggested that amino acid solutions enriched in BCAAs inhibit muscle catabolism and promote protein synthesis in trauma patients. BCAA supplementation in cirrhosis patients improves production of albumin in a dose-dependent manner, although; the promotion of albumin synthesis appears to depend mainly on leucine (Ijichi C, 2003). Several essential amino acids including leucine promote protein synthesis through a key transcription factor known as mammalian target of rapamycin (mTOR) (Kimball S.R, 2001, Avruch J, 2009). An animal study showed that severe injury leads to a decrease in the phosphorylation of several proteins involved in the regulation of protein synthesis, including mTOR, and leucine reversed these alterations and normalized protein synthesis (Cynober L, 2006)[5]. In liver, BCAAs enhance the translation of a particular set of mRNA and so up-regulate the capacity of the tissue to synthesize protein (Kimball S.R, 2001). Leucine is a precursor for sterol biosynthesis in adipose tissue and muscle (Rosenthal J, 1974), an inhibitor of urea production in liver (Mourão JM, 1975), and stimulates insulin secretion (Milner R.D, 1970).

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[5] It has been reported that rapamycin, a specific mammalian target of rapamycin (mTOR) inhibitor, inhibits the activity of leucine for albumin synthesis by half (Ijichi C, 2003).

2.4. Oxidative Stress in Trauma Injuries

Oxidative stress occurs when production of ROS is increased and cells cannot detoxify them and/or repair the resulting damage. Oxidative stress plays a pivotal role in many diseases including cancer, inflammation, liver disease, infection etc. (Townsend D.M, 2003).

The liver is one of the major organs to eliminate toxics compounds from the body. It converts them into a less harmful chemical through oxidation, reduction, and hydrolysis reactions. During this process, free radicals are produced; they are highly reactive and tend to react with other molecules to pair the unpaired electron. The most common radicals in biological systems are superoxide anion ($O_2\bullet^-$), hydroxyl radical ($OH\bullet$), and hydrogen peroxide (H_2O_2). Mitochondria reduce molecular oxygen to water during oxidative phosphorylation (ADP→ATP) and giving rise to intermediate ROS:

$$O_2 \rightarrow O_2\bullet^- \rightarrow H_2O_2 \rightarrow OH\bullet \rightarrow H_2O$$

Molecular oxygen → Superoxide anion radical → Hydrogen peroxide → Hydroxyl radical → Water

Free radicals react with molecular components and generate secondary radicals that interact with other molecules and propagate a chain reaction (Closa D, 2004). Increasing in the level of ROS beyond a certain physiological range will cause oxidative damage to tissues followed by mortality (Rodriguez P.G, 2008, Parihar A, 2008).

Xanthine oxidase is another enzyme system generating ROS in cells. Hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\bullet$) are byproducts of xanthine oxidase (Ward P.A, 1990). H_2O_2 can be produced by many reactions in the body and can be converted to hydroxyl

radical. Hydroxyl radical is the most reactive, and thus most damaging radical, although the one with the shortest life. It causes oxidative damage in a variety of biomolecules including proteins, lipids, and DNA. Hydrogen peroxide and superoxide are known as cellular messengers to activate cell motility, cytokine action, angiogenesis, as well as wound healing (Guo S, 2010).

Antioxidant enzymes such as glutathione reductase / glutathione peroxidase (GR/GPx) and superoxide dismutase (SOD) regulate the potential effects of free radicals (Comhair SA, 2002, Closa D, 2004, Parihar A, 2008) and protect tissues against oxidative stress. However, oxidative stress would occurs if the production of oxygen radicals exceeds the scavenging capacity of these enzymes (Closa D, 2004).

Glutathione (GSH) is the major intracellular oxidative stress regulator and mostly synthesised and exported by the liver. Glutathione is composed of the three amino acids glycine, cysteine, and glutamate. The extracellular concentration of GSH is relatively low (2-20 µmol/L in plasma) (Jones D.P, 2002). Glutathione has an effective role in many cellular reactions by scavenging free radicals directly and indirectly through enzymatic reactions; and so glutathione deficiency increases the risk of oxidative stress. Most of the glutathione within cells exists in reduced form (GSH) and a small percentage of the glutathione is the oxidized form (GSSG), which is a dimer of two peptide elements connected by a disulfide bond between the cysteines present in each molecule (Fang Y.Z, 2002). Accumulation of oxidized glutathione (GSSG) is an indicator of oxidative stress. The intracellular GSH:GSSG ratio, which indicates the cellular redox state,

increases by the reduction of GSSG to GSH to provide enough antioxidant (Parihar A, 2008)[6].

Proinflammatory cytokines alter the expression of some glutathione-requiring enzymes including glutathione transferases (GSTs) (Whalen R, 2004). Studies have shown decreased expression of GST-μ and α isoforms in rat hepatocytes by IL-6 (Voss SH, 2002), GST2 and GSTM1 by IL-1ß (Maheo K, 1997), GSTA1 in human intestinal epithelial cells (CAco-2) by IL-1ß (Romero L, 2002), and aslo increased expression of murine GSTA4 in cultured mouse hepatocytes by TNF-α and IL-6 (Desmots F, 2002).

Cysteine is a sulfur containing amino acid, which plays an important role to maintain and support immune functions by modulating the actions of oxidant stress. However, its intracellular pool is relatively small compared to the metabolically active pool of GSH in cells (Chung T.K, 1990). Cysteine is the rate-limiting amino acid in GSH biosynthesis (A, 1984) due to its instability, and the low solubility of cystine (the oxidized cysteine form). A study has shown under conditions of low cysteine availability, the synthesis of GSH is decreased (Grimble R.F, 2006). Transport of cysteine and cystine in mammalian cells are done by distinct membrane carriers (LU S.C, 2009); it is noteworthy that hepatocytes cannot transport cystine (Wu G, 2004).

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[6] The GSH:GSSG ratioin normal condition is >10 (OW G, 1999).

CHAPTER 3

THE METABOLIC RESPONSE OF HEPG2 CELLS EXPOSED TO REDUCED SERUM-SUPPLEMENTED MEDIUM

3.1. INTRODUCTION

Studies have shown that the hepatic response to severe injuries is characterized by a significant up-regulation of glucose and amino acid turnover (Lee K, 2003, Lee K B. F., 2000). Alteration in glucose metabolism leads to hyperglycemia due to increased gluconeogenesis (Gore D.C, 2002) that mostly happens in the liver to supply energy for wound healing. The major substrates for gluconeogenesis are lactate produced by the wound and amino acids derived from muscle proteolysis (Yu Y.M, 1999, Yarmush D.M, 1999). An animal study suggested that some of the glucose from gluconeogenesis may be diverted into pentose phosphate pathway to produce antioxidants (Lee K B. F., 2000) and so glucose output does not necessarily reflects the extent of gluconeogenesis.

Gluconeogenesis and glycolysis regulate the production of urea as well as glutamine and lipid metabolism (Iyer V.V, 2010). In mammalian cells in culture, glucose and glutamine supply most of the carbon, nitrogen, and energy to support cell growth and proliferation (Vander Heiden M.G, 2009). Most cancer cells including HepG2/C3A rely on aerobic glycolysis to generate energy for cellular processes (Vander Heiden M.G, 2009). Iyer (Iyer V.V, 2010) showed HepG2 cells consume less glucose in high glucose medium (3mg/ml) compared to medium with lower glucose (1mg/ml).

The purpose of this experiment is to identify a suitable medium for HepG2/C3A cells so that they proliferate less and show stable viability over time by evaluating the metabolic

responses in two different media (DMEM and Williams' E). Both media have widely been used in *in vitro* studies. The media were purchased from "Life Technology/Invitrogen" and the concentration of amino acids and vitamins in DMEM is about twice more than in Williams' E medium. The details of the composition of each media can be found in Appendix 1.

3.2. Materials and Methods

Media

All media were purchased from Invitrogen.

- Dulbecco's Modified Eagle Medium (DMEM-HG):

- High Glucose (4.5 g/L)
- High L-Glutamine (0.584 g/L)

-Williams' Medium E (WE):

- Low Glucose (2 g/L)
- No L-Glutamine

- Dulbecco's Modified Eagle Medium (DMEM-LG):

- Low Glucose (1 g/L)
- No L-Glutamine

All media were supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2% penicillin-streptomycin (Invitrogen). DMEM-LG and WE were also supplemented with 2 mM L-Glutamine.

Cell Culture

HepG2/C3A cells from ATCC were maintained in DMEM-HG in T-75 plates. At passage 12, cells were harvested and cultured in 6-well plates at 200,000 cells/cm² in DMEM-HG in a total of eight plates. The following day, after 24 h, media were changed and each plate was treated with either DMEM-LG or WE. The media were changed every 24 h for 8 days, and supernatants were collected and stored in -80°C for further analysis. Cells were counted every 24 h for both treatments using a hemocytometer[7].

3.3. Data Analysis

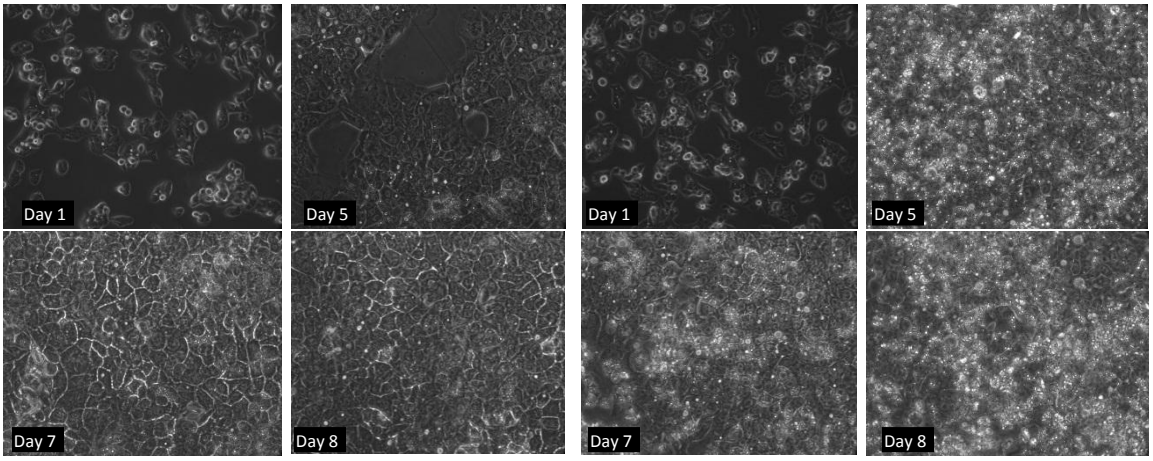
The following extracellular metabolites were detected and quantified using commercial kits: glycerol, triglycerides (TG), glucose, and glutamine (from Sigma); ß-hydroxybutyrate and urea (from Fisher Scientific); lactate (from Trinity Biotech); albumin was measured using enzyme-linked immunosorbent assay (ELISA) (from Bethyl Lab)[8]. The levels of 13 amino acids (Asp, Arg, Ser, Thr, Ala, Gly, Pro, Phen, Tyr, Leu, Isl, Met, Val) were measured using HPLC. The level of production of each component was statistically compared using the data analysis software "KaleidaGraph".

3.4. Results and Discussion

HepG2/C3A cells were cultured for 8 days in two different media, DMEM-LG and WE. The concentration of most of the amino acids and vitamins in WE is less than DMEM-LG (almost half of the DMEM-LG), although the concentration of glucose in WE is two

[7] The protocol can be found in Appendix-2
[8] The protocol of all kits can be found in Appendix-3

times more than that in DMEM-LG. Cell numbers and metabolites were measured every day for 8 days. It was observed that cell density in DMEM-LG was more uniform and stable compared to WE (Fig-2).



a)HepG2 cells cultured in DMEM-LG **b) HepG2 cells cultured in Williams' E**

Figure 2: Cell density in both media as a function of time; a)HepG2 cells cultured in DMEM-LG; b) HepG2 cells cultured in Williams' E

Cell numbers were determined using a hemocytometer and with the trypan blue staining method. Cell density was more stable in DMEM-LG with less number of dead cells compared to WE (Fig-3). The number of dead cells in both media was negligible for the first three days and gradually started to increase on day four. The number of dead cells in WE exceeded the live cells at each time point for the last 4 days (Fig-3-a). The number of dead cells in DMEM-LG was more stable in time and also less than number of the live cells at all time points (Fig-3-b). The number of live cells in WE appeared to be less stable over time compared to DMEM-LG, although the differences were not significant ($p>0.05$) (Fig-3-c). There were more dead cells in WE at each time point compared to DMEM-LG and the differences were statistically significant ($p<0.05$) (Fig-3-d).

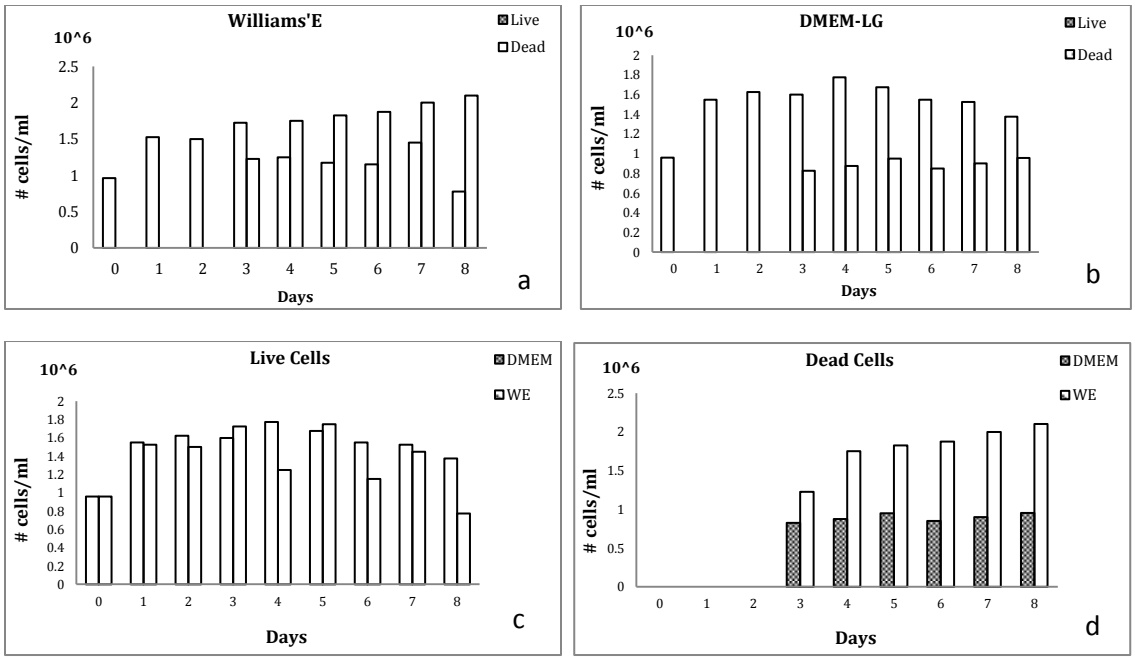


Figure 3: Cell numbers in both media (DMEM & WE). a) The number of live and dead cells in WE in time. b) The number of live and dead cells in DMEM-LG in time. c) Comparing the number of live cells in the two media (p=0.08) d) Comparing the number of dead cells in the two media (p=0.006)

The extracellular metabolites including albumin, urea, glutamine, glycerol, triglyceride, lactate, and ß-hydroxybutyrate were quantified for both conditions using commercial kits (Fig-4). The extracellular metabolites were only measured for the first four days since the number of dead cells after day four were significant for WE. There were statistically significant differences in albumin secretion ($p<0.05$), glucose consumption ($p<<0.05$), and production of triglyceride ($p<<0.05$) between the two media (Fig-4). A previous study had showed glucose consumption flux for HepG2 cells was higher in low glucose medium (Iyer VV, 2010). This experiment also showed the cells cultured in low glucose medium (DMEM-LG) consumed more glucose. The albumin secretion in cells grown in DMEM, with higher concentration of amino acids, was more than those cultured in WE. The concentration of

glutamine in both media was 2 mM and with the exception of day 2, the consumption was almost at the same level in both media (P=0.148).

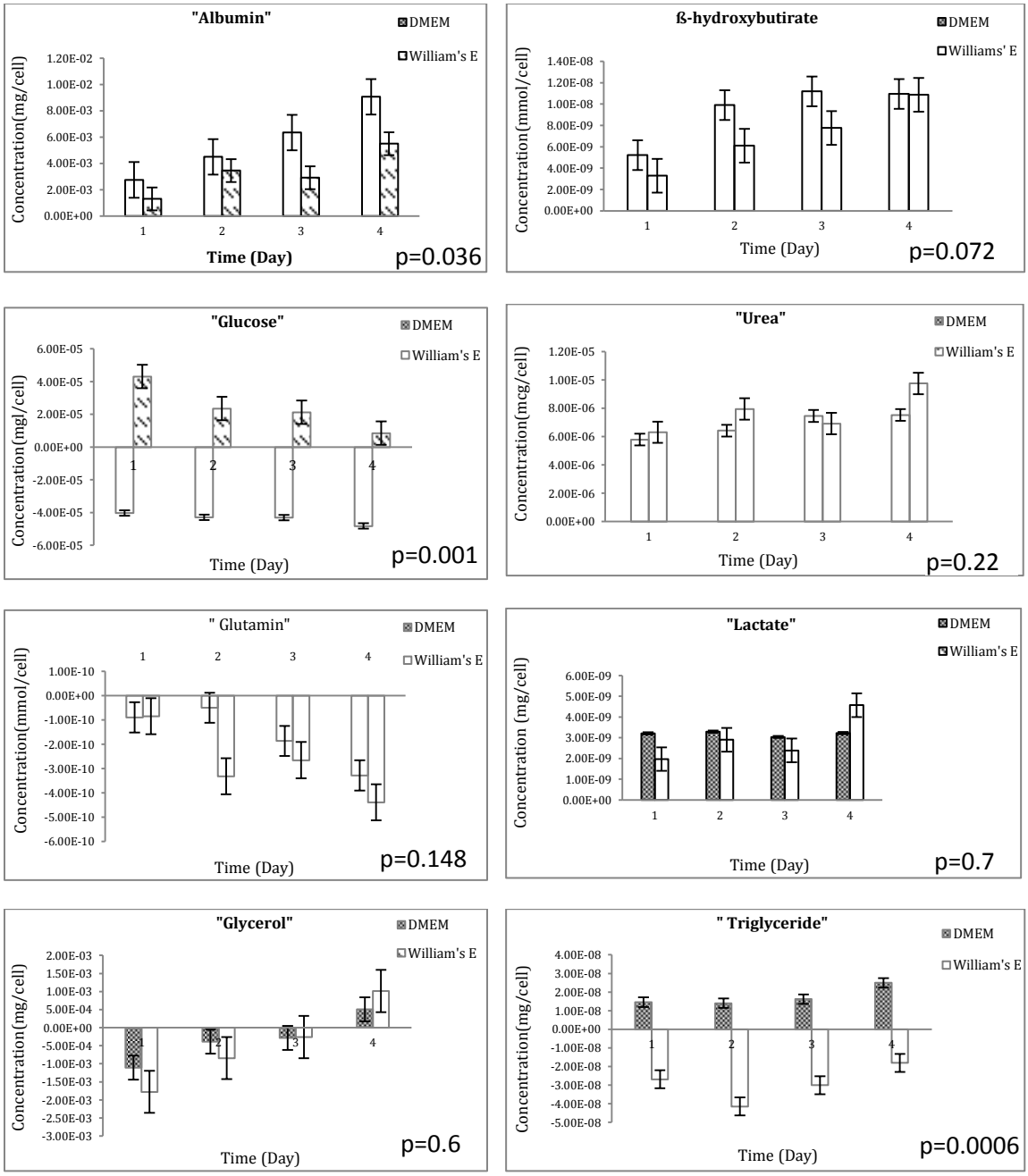


Figure 4: Production/consumption of albumin, lactate, ß-hydroxybutyrate, glucose, glutamine, glycerol, triglyceride, and urea for both media (DMEM & WE).

With the exception of tyrosine, asparagine, and phenylalanine, there were no statistically significant differences in amino acid metabolism in both media (p>0.05) (Fig-5 & 6), even though, the level of amino acids in WE was about half of the DMEM-LG.

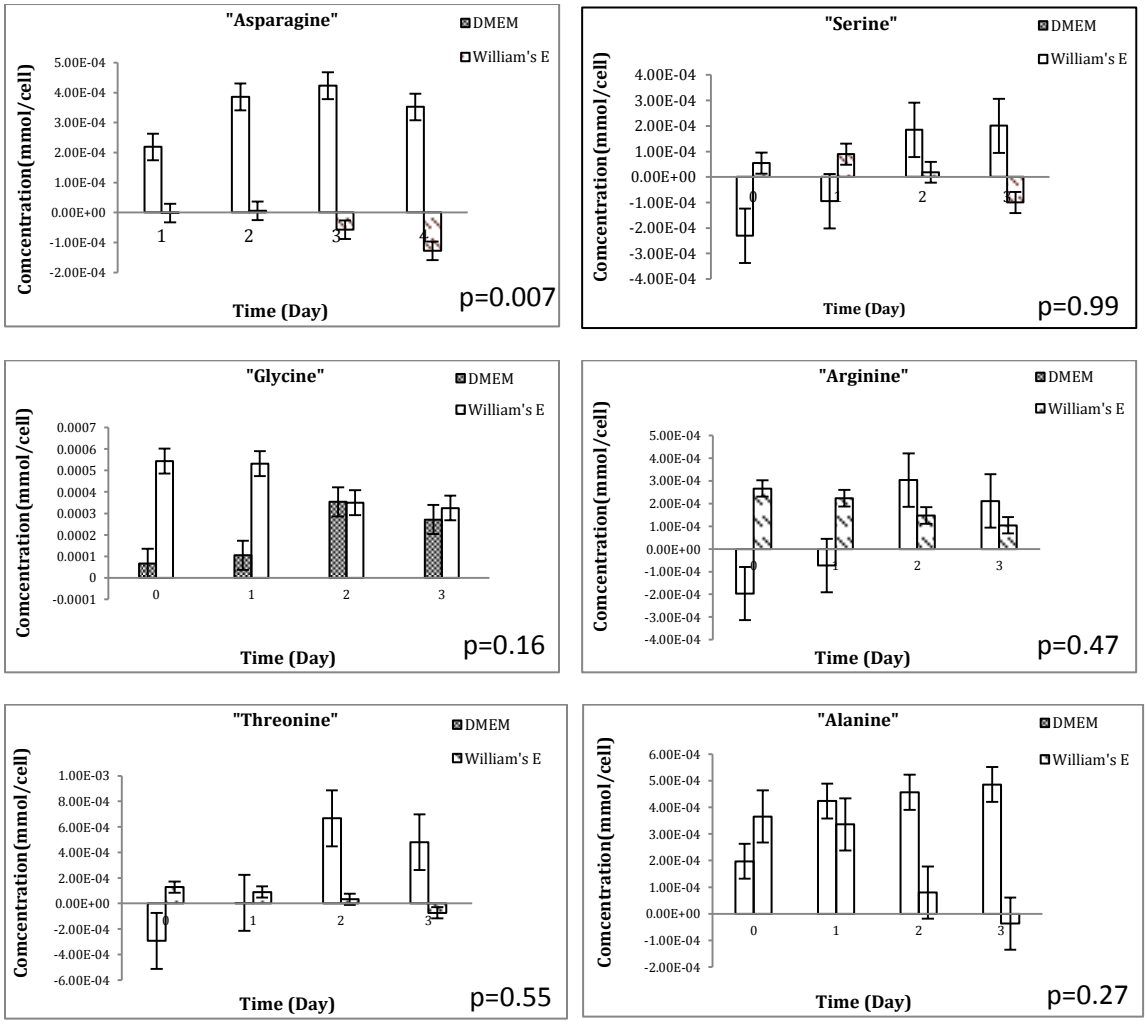


Figure 5: Amino acid metabolism for both media. The p value for each amino acid is denoted on each plot. With the exception of asparagine with p value <0.05, there were statistically significant differences between the two media (DMEM & WE) (to be continued on the next page)

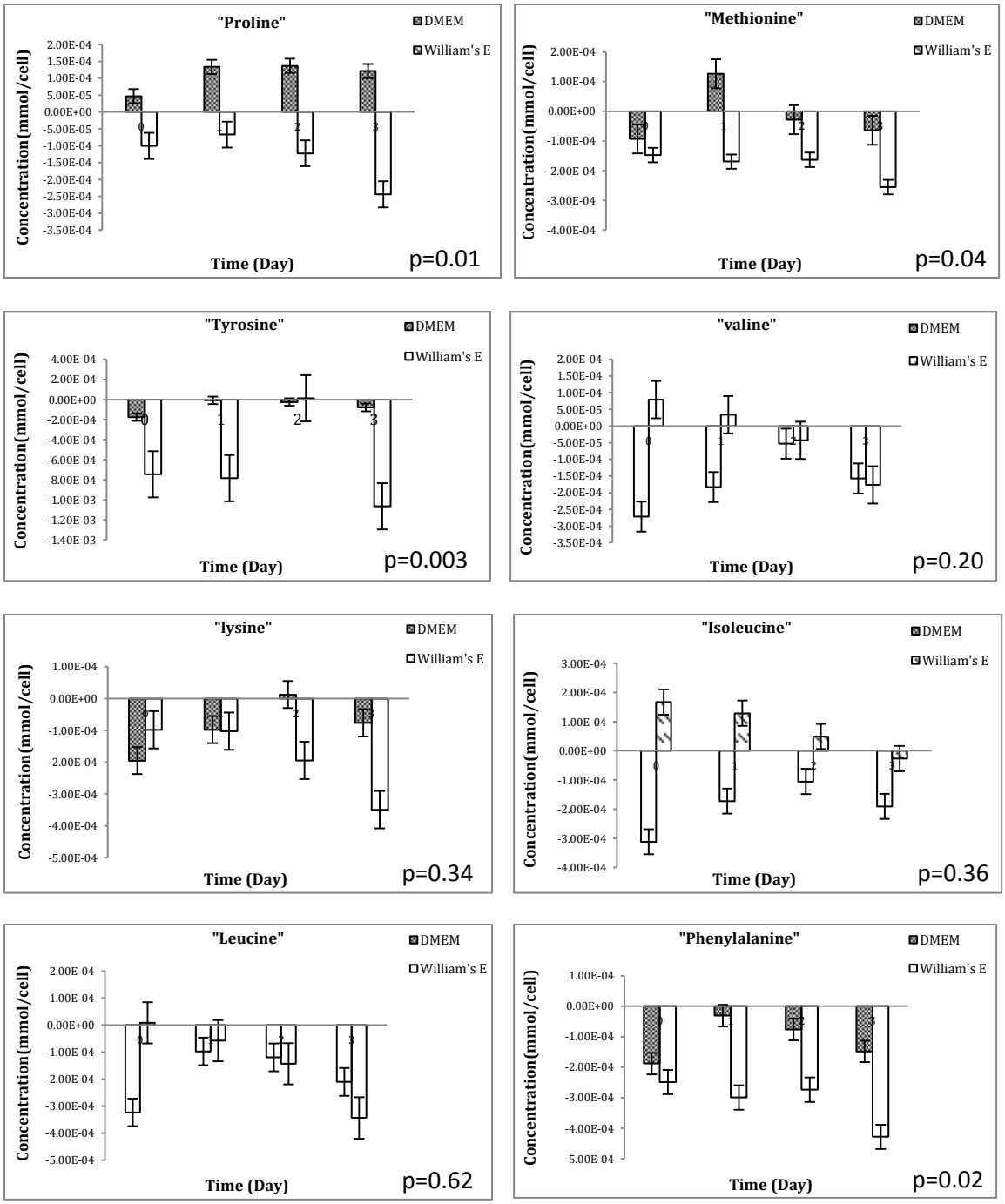


Figure 6: Amino acid metabolism for both media. The p value for each amino acid is denoted on each plot. With the exception of tyrosine, proline, methionine, and phenylalanine with p value <0.05, there were statistically significant differences between the two media (DMEM & WE).

Glutamine and glucose comprise the major sources of energy substrates for immune cells in injured patients (Deitch E.A, 1995). Since the purpose of this study is to investigate the

effect of cytokines in trauma injuries, the production of albumin is also important. Albumin synthesis is important to maintain physiological homeostasis, and its concentration is used as an indicator for nutritional status in patients. The increase in secretion of mediators including IL-1ß and IL-6 leads to a decrease in secretion of constitutive hepatic proteins including albumin (Kang Y.H, 2002). Studies have shown that administration of amino acids increases albumin and skeletal muscle protein synthesis (Hellstern G, 2002, Davis T.A, 2002). Based on these results, I decided to use the DMEM based medium with higher concentration of amino acids, glucose (4500 mg/L or 25 mM), and 2 mM glutamine, supplemented with 10% FBS and 2% penicillin-streptomycin for further experiments.

CHAPTER 4

THE EFFECT OF PROINFLAMMATORY CYTOKINES (IL-1β & IL-6) ON PRODUCTION OF ALBUMIN AND UREA IN HEPG2 CELLS

4.1. INTRODUCTION

The acute phase response can be described as series of actions in response to tissue injury, inflammation, or infection. Trauma injuries result in an increase in infection, which leads to increased morbidity and mortality. Following the acute phase response, the synthesis and plasma levels of some proteins, notably albumin, are decreased (Kang Y.H, 2002). Acute phase proteins are synthesized almost exclusively in the liver and they play an important role to restore homeostasis in case of infection or inflammation. Cytokines are important mediators during the acute phase response. Three cytokines including IL-1ß, IL-6, and TNFα are involved in the response to infection. A clinical study showed that IL-1ß and IL-6 concentration was elevated in the plasma of patients with thermal injury compared to unburned subjects during the first week after injury and declined over time (Drost A.C, 1993).

A stable long-term HepG2 cell culture under well defined conditions can be used to investigate the effects of inflammatory mediators on liver function in injured patients. The purpose of this experiment is to explore the overall effects of cytokines including IL-1ß and IL-6 on HepG2 cells and their metabolic response. For this purpose I used different concentration of each cytokines, ranging from 0.1 ng/ml to 10 ng/ml, and the combination of both at 10 ng/ml of each.

4.2. Materials and Methods

- HepG2/C3A cells from ATCC
- DMEM with 4.5 g/L glucose and no glutamine (Cat# 10313) from Invitrogen
- Recombinant human Il-6 (Cat# 206-IL) from R&D System
- Recombinant human IL-1ß/IL-1F2 (Cat # 201-LB) from R&D System
- PBS from Invitrogen
- L-glutamine from Sigma

The medium was supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2% penicillin-streptomycin (Invitrogen), and 2 mM glutamine.

Cell Culture

HepG2/C3A cells were maintained in medium in T-75 plates. At passage 12, cells were harvested and cultured in 24-well plates at 200,000 cells/cm² in a total number of three plates. The medium was replaced every 24 h with fresh medium for four days until the cells reach confluence. On day four, the medium was replaced with fresh media containing various concentrations of cytokines, more specifically 0.1, 1, and 10 ng/mL of each and the combination of both cytokines as 10/10 ng/mL (Fig-7). The human cytokines were IL-1ß and IL-6 reconstituted in PBS containing at least 0.1% bovine serum albumin (as suggested by R&D System). Reconstituted cytokines were stored at 2-8°C to avoid loss of activity. There would be no detectable loss of activity for up to one month under this condition (R&D Systems). The media was changed every 24 h for 6 days, and supernatants were collected and stored in -80°C for further analysis of albumin and urea. The cells adhered to the wells were released with trypsin-EDTA after day 6 and counted using a hemocytometer.

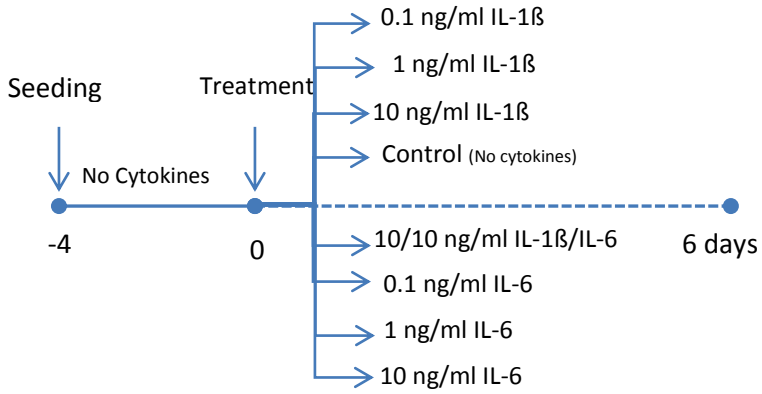


Figure 7: Experimental design. There were 8 different treatments. On day zero, the medium was replaced with fresh media containing various concentrations of each cytokine including 0.1, 1, and 10 ng/mL of each and a combination of both cytokines as 10/10 ng/mL.

4.3. Data Analysis

The following extracellular metabolites were quantified using commercial kits: urea (from Fisher Scientific); and albumin was measured by ELISA (from Bethyl Lab). The concentrations of each were calculated as the average of three samples per day. After 6 days the cells adhering to the wells were counted. The level of production of each component was statistically compared for each condition using the data analysis software "KaleidaGraph".

4.4. Results and Discussion

HepG2/C3A cells were cultured for 6 days under inflammatory condition by adding two cytokines (IL-1ß and IL-6) to the medium. Below are plots (Fig-8 & 9 & 10) showing the results for the production of urea and albumin under each condition.

The results showed IL-6 suppressed albumin and urea production (Fig-8, Table-1), which is a common acute phase response in liver.

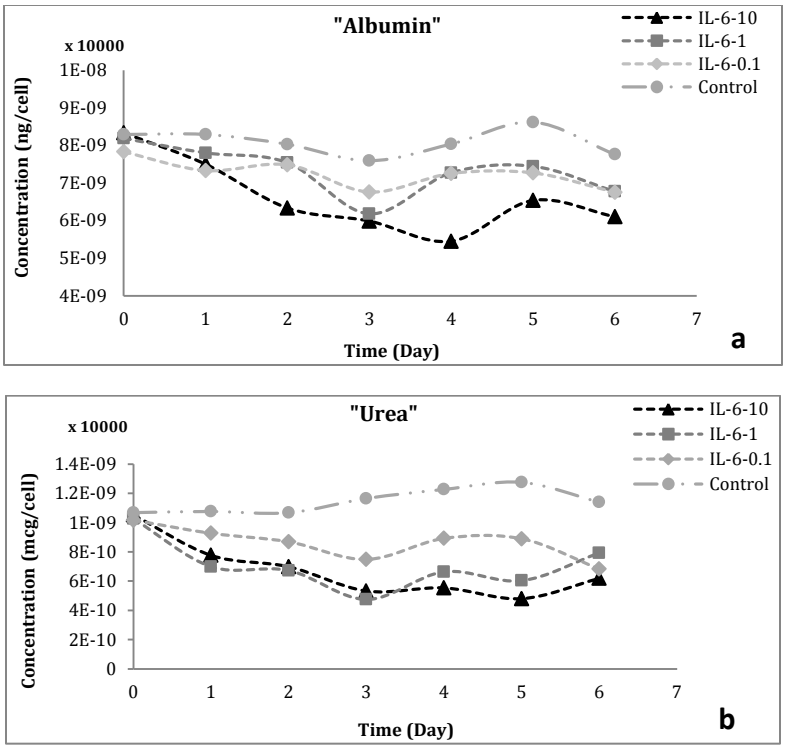


Figure 8: The effect of IL-6 for 6 days on a) albumin and b) urea production. HepG2 cells were cultured for 6 days in the media supplemented with 10, 1, and 0.1 ng/ml of IL-6. Data are the average of three samples at each time point.

Cytokine	Albumin %	p value	Urea %	p value
IL-6-10	-18.42%	< 0.0001	-46.30%	< 0.0001
IL-6-1	-9.66%	0.0338	-42.27%	< 0.0001
IL-6-0.1	-10.46%	0.0278	-30.11%	< 0.0001

Table 1: Statistical analysis of the effect of IL-6 on HepG2 cells compared to control condition for the period of 6 days. % represents decrease (-) or increase (+) compared to the control.

Albumin secretion was decreased to about 18% at 10 ng/ml, 10% at 1 ng/ml, and 10% at 0.1 ng/ml of IL-6 compared to the cytokine-free control. The p value for 10, 1, and 0.1 ng/ml of IL-6 was less than 0.05 compared to control (Table-1). Urea synthesis was decreased about 46% at 10 ng/ml, 42% at 1 ng/ml, and 30% at 0.1 ng/ml of IL-6 compared to control. The p value for all three concentrations was less than 0.05 compared to control (Table-1). Based on the statistical analysis, urea and albumin secretion were significantly decreased by IL-6 in a dose-dependent manner.

IL-1ß alone suppressed albumin and urea synthesis too (Fig-9, Table-2).

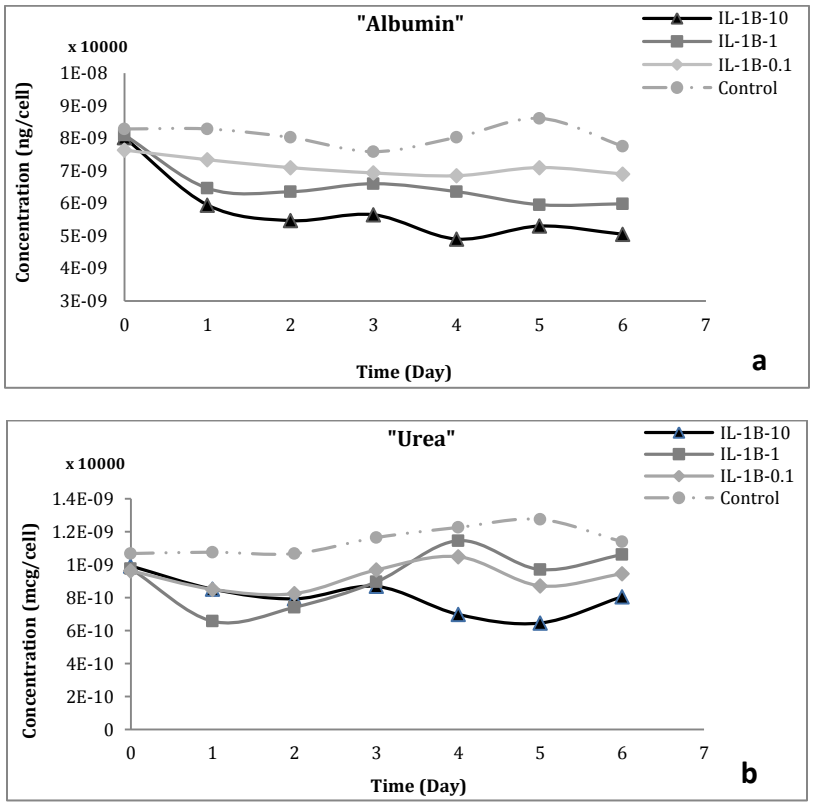


Figure 9: The effect of IL-1ß for 6 days on a) albumin and b) urea production. HepG2 cells were cultured for 6 days in media supplemented with 10, 1, and 0.1 ng/ml IL-1ß. Data are the average of three samples at each time point.

Cytokine	Albumin %	p value	Urea %	p value
IL-1B-10	-28.75%	< 0.0001	-33.29%	0.0001
IL-1B-1	-18.93%	< 0.0001	-21.23%	0.0092
IL-1B-0.1	-11.82%	< 0.0001	-21.30%	0.0112

Table 2: Statistical analysis of the effect of IL-1ß on HepG2 cells compared to the control condition for the period of 6 days. % represents decrease (-) or increase (+) compared to the control.

Albumin production was decreased to about 29% at 10 ng/ml, 19% at 1 ng/ml, and 12% at 0.1 ng/ml of IL-1ß compared to the control. The p value for all concentrations of IL-1ß was less than 0.05 (Table-2). Urea production was decreased to about 33% at 10 ng/ml, 21% at 1 ng/ml and 0.1 ng/ml of IL-1ß. The p value for all three concentrations of IL-1ß was less

than 0.05 (Table-2). Based on this statistical analysis, albumin and urea secretion were significantly decreased by IL-1ß in a dose-dependent manner.

The combination of both IL-1ß and IL-6 also suppressed albumin and urea secretion (Fig-10, Table-3).

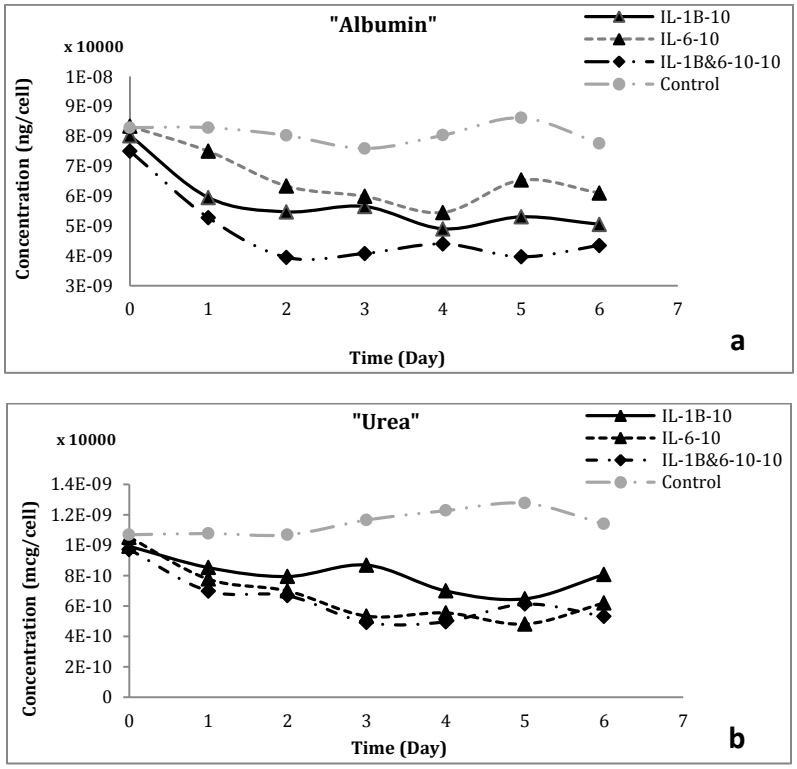


Figure 10: **The effect of the combination of both IL-1ß and IL-6 for 6 days on a) albumin and b) urea production. HepG2 cells were cultured for 6 days in medium supplemented with 10 ng/ml of each cytokine. Data are the average of three samples at each time point.**

Cytokine	Albumin %	p value	Urea %	p value
IL-1B & IL-6: 10&10	-48.89%	< 0.0001	-49.27%	< 0.0001
IL-1B-10	-28.75%	< 0.0001	-33.29%	< 0.0001
IL-6-10	-18.42%	< 0.0001	-46.30%	< 0.0001

Table 3: Statistical analysis of the effect of both cytokines on HepG2 cells compared to the control condition for the period of 6 days. % represents decrease (-) or increase (+) compared to the control.

Albumin secretion was decreased to about 49%, and urea secretion was decreased to

about 46%. Based on the statistical analysis, albumin and urea secretion was significantly altered by the combination of both cytokines ($p < 0.001$) (Table-3).

In this experiment, the effects of two proinflammatory cytokines, IL-1ß and IL-6, on HepG2 cells was studied. Cells were exposed to different concentrations of each cytokine (0.1, 1, 10 ng/ml), and a combination of both at 10 ng/ml of each. The effect was monitored for 6 days for albumin and urea synthesis. This study showed the effect of both IL-1ß and IL-6 on albumin and urea synthesis varies almost in a dose-dependent manner. IL-1ß and IL-6 alone inhibited urea and albumin secretion almost in a dose dependent manner. However, IL-1ß showed a dominant effect on IL-6 for albumin secretion (Fig-9-a). The albumin secretion for the cells treated with both cytokines was less than when those were treated by each one alone ($p < 0.0001$). IL-1ß exhibited lower albumin production compared to IL-6, and IL-6 exhibited lower urea production compared to IL-1ß. After early suppression of albumin and urea production by IL-1ß, the rate was almost steady over the course of treatment. The combination of both cytokines inhibited the production of both urea and albumin to a greater extent than each cytokine alone.

Based on these results, I decided to use 10 ng/ml of each cytokine, either alone or in combination for the following experiments to investigate the effect of branched chain amino acids (BCAAs) on albumin secretion in the context of an inflammatory response.

CHAPTER 5

BRANCHED CHAIN AMINO ACID (BCAA) SUPPLEMENTATION ENHANCES THE ALBUMIN SECRETION IN HEPG2 CELLS TREATED WITH IL-1ß & IL-6

5.1. INTRODUCTION

The acute phase response is responsible for tissue and organ damage as well as activating the repair processes and wound healing. The critical step in this situation is the interaction between the liver and the injured tissue. The liver is responsible for modulation of systemic inflammatory response as well as production of acute phase proteins. Albumin is one of the negative acute phase proteins, which in the previous experiment showed that its expression is decreased in this situation. Albumin performs important metabolic functions, including maintaining the colloid pressure of plasma (Doweiko J.P, 1991), and transporting drugs and bilirubin. The concentration of serum albumin depends on the numerous processes, including albumin synthesis, catabolism and distribution (Rothschild M.A, 1992). It is one of the common factors to evaluate the nutritional status in patients. Studies showed supplementation of BCAAs improves hypoalbuminemia in patients with liver disease (Marchesini G, 2005, Charlton M, 2006). A study showed if polypyrimidine-tract-binding protein (PTB), which is an RNA-binding protein that acts as a splicing regulator, binds to rat albumin mRNA, it would mitigate the albumin mRNA translation (Kuwahata M, 2008). It was found that rat livers with acute liver failure has more albumin mRNA-PTB complex compared to the normal rats and the level of this complex can be decreased with BCAAs (Kuwahata M, 2004). An *in vitro* study showed addition of leucine to amino acid-free medium in HepG2 cells increases the production of albumin in a dose-

dependent manner (Kuwahata M, 2008). Leucine improves albumin secretion by regulation of PTB (Kuwahata M, 2008); although, it would reduce the circulation of concentration of isoleucine and valine in a food-deprived rat (Anthony J.C, 2000). As a result, it is necessary to provide a mixture of all three amino acids to improve hypoalbuminemia in patients.

The purpose of this study is to investigate the effect of BCAAs on albumin and urea secreted by HepG2 cells treated with proinflammatory cytokines.

5.2. Materials and Methods

- HepG2/C3A cells from ATCC
- DMEM with 4.5 g/L glucose, no glutamine (Cat# 10313) from Invitrogen
- Customized DMEM (C-DMEM) with 4.5 g/L glucose, no glutamine, and no BCAAs from Invitrogen
- Recombinant human Il-6 (Cat# 206-IL) from R&D System
- Recombinant human IL-1ß/IL-1F2 (Cat # 201-LB) from R&D System
- PBS from Invitrogen
- L-glutamine from Sigma
- L-leucine, 98.5-101.01% from Sigma
- L-isoleucine, 98.5-101.01% from Sigma
- L-valine, 98.5-101.01% from Sigma

The medium was supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2% penicillin-streptomycin (Invitrogen), and 2 mM glutamine.

Cell Culture

HepG2/C3A cells were maintained in medium in T-75 plates. At passage 20, cells were harvested and cultured in 24-well plates at 200,000 cells/cm². The medium was replaced every 24 hrs with fresh medium for four days until the cells reach confluence. On day four, the cells were divided into three groups; *1)* cells were cultured in BCAA-free

medium (F-BCAAs); *2)* cells were cultured in medium supplemented with 0.8mM of BCAAs (0.8-BCAAs); and *3)* again cells were cultured in F- BCAAs for 24 h and then the medium was replaced with medium supplemented with 2 mM BCAAs (2-BCAAs) (Fig-11). For all three groups there were four subgroups including medium with 10 ng/ml of IL-1ß; medium with 10 ng/ml of IL-6; medium with combination of both IL-1ß and IL-6, each 10 ng/ml; and medium without cytokines as control. As a result, there were 12 different treatments.

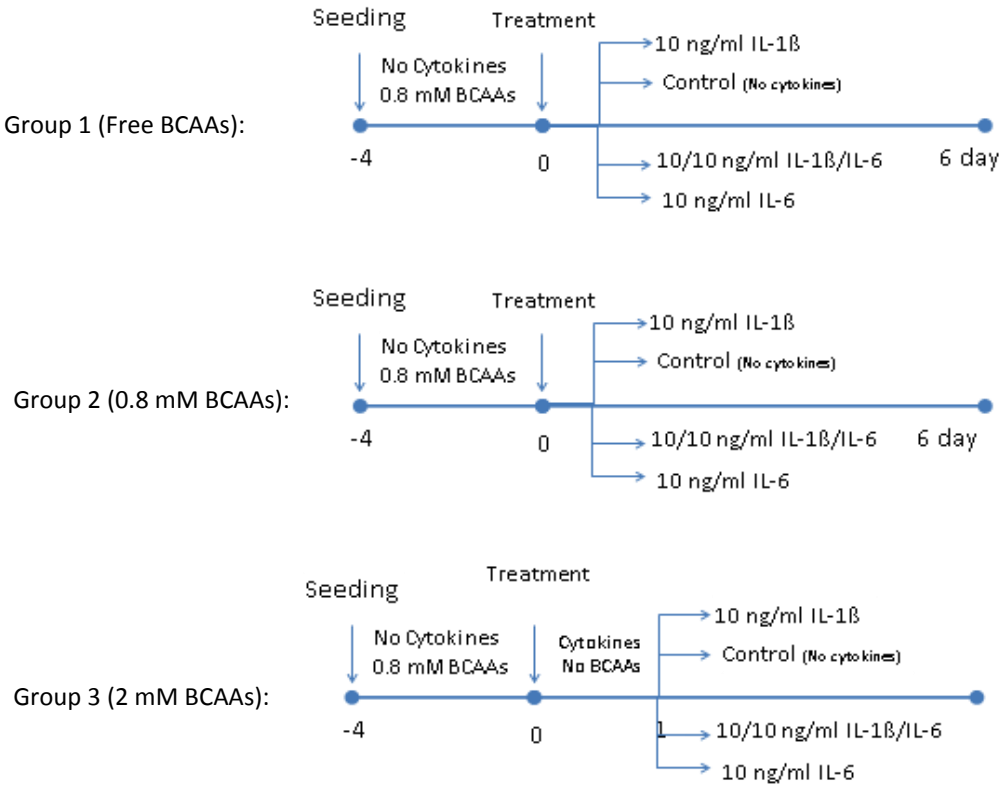


Figure 11: Experimental design for the total three groups. Each group was divided into 4 sub-groups including: medium with 10 ng/ml of IL-1ß; medium with 10 ng/ml of IL-6; medium with combination of both IL-1ß and IL-6, each 10 ng/ml; and cytokines-free medium.

IL-1ß and IL-6 were reconstituted in PBS containing at least 0.1% bovine serum albumin

(as suggested by R&D System). Reconstituted cytokines were stored at 2-8°C to avoid loss of cytokine activity. There would be no detectable loss of activity for up to one month under this condition (R&D System). The media was changed with fresh medium containing either of cytokines or both, as wells as cytokines-free medium every 24 h for 6 days, and supernatants were collected and stored in -80°C for further analysis of albumin and urea. The cells adhered to the wells were released with trypsin-EDTA after day 6 and counted using a hemocytometer.

5.3. Data Analysis

The following extracellular metabolites were quantified using commercial kits: urea (from Fisher Scientific); and albumin was measured using ELISA (from Bethyl Lab). The concentration of each was calculated as the average of three cultures per cells every day. The level of production of each component was statistically compared for all groups and sub-groups using the data analysis software “KaleidaGraph“.

5.4. Results and Discussion

On day zero, the cells were divided into three groups. The first group was treated with medium supplemented with 0.8 mM of BCAAs and cytokines (IL-1ß and IL-6), and groups two and three were treated with BCAA-free medium supplemented with cytokines. On day one, the third group was treated with medium supplemented with 2 mM BCAAs and cytokines (Fig-11).

Albumin

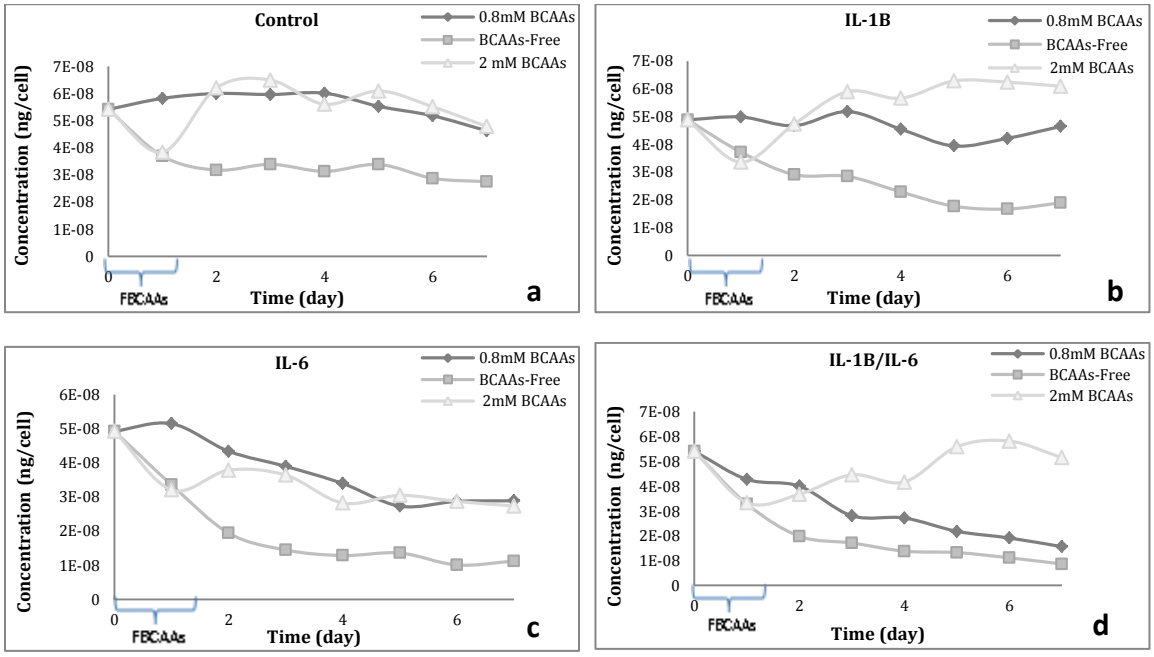


Figure 12: albumin secretion. Comparing albumin secretion in all three groups for a) control conditions; b) 10 ng/ml IL-1ß; c) 10 ng/ml IL-6; and d) combination of both cytokines as 10 ng/ml of each. Data are the average of three samples at each time point.

Comparing the metabolic response in cells grown in BCAAs-free (F-BCAAs) medium with either of the other two groups showed that BCAAs enhance production of albumin. At day 2, there was a jump in albumin and urea synthesis for the third group in all four conditions including IL-1ß, IL-6, combination of both cytokines, and control (Fig-12 & 13).

There were not statistically differences between group two (0.8 mM BCAAs) and group three (2 mM BCAAs) for albumin and urea synthesis in cells treated with either cytokine or the combination of both ($p > 0.05$) (Fig-12 & Fig-13), although; there were significant differences between albumin and urea secretion in cells cultured in BCAA-free medium and either of the other two groups ($p << 0.05$).

Urea

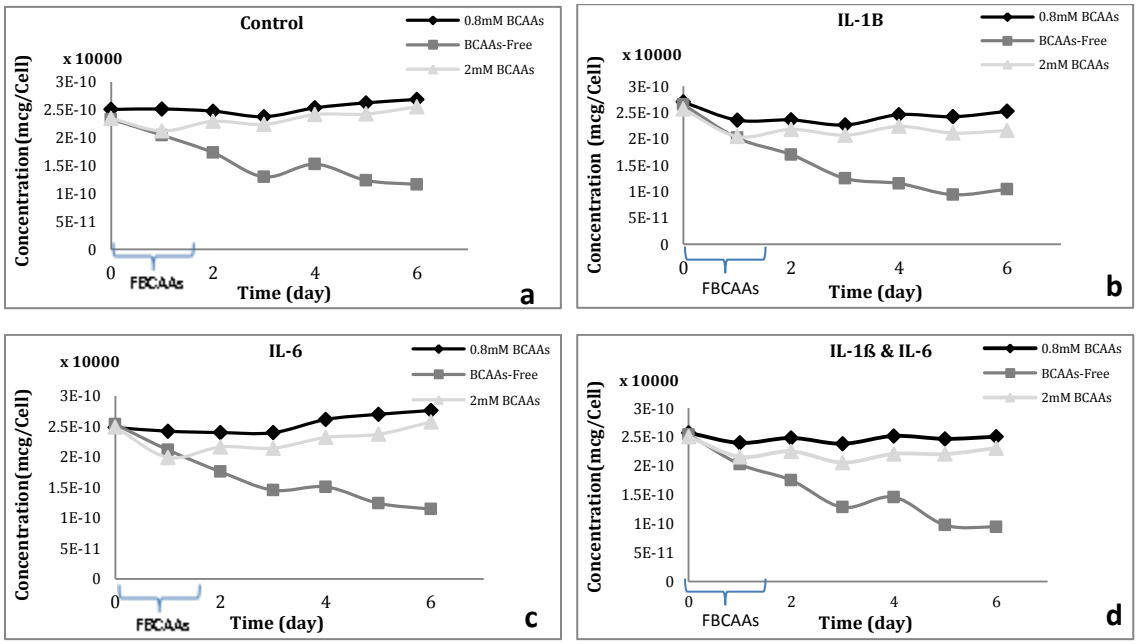


Figure 13: urea production. Comparing urea production in all three groups for a) control conditions; b) 10 ng/ml IL-1ß; c) 10 ng/ml IL-6; and d) combination of both cytokines as 10 ng/ml of each. Data are the average of three samples at each time point.

IL-1ß and IL-6 decrease albumin and urea production in rat hepatocyte culture (Kang Y.H, 2002). In this experiment, as it was observed in the previous experiment, IL-1ß and IL-6 suppressed albumin and urea production. IL-6 caused a steady decrease in production of albumin in all three groups, which is significant compared to control ($P << 0.05$).

IL-1ß alone caused significant changes in albumin secretion for group one (Fig-12- b). In the third group (2mM-BCAAs), albumin secretion was gradually increased and stayed almost constant over time (Fig-12-b).

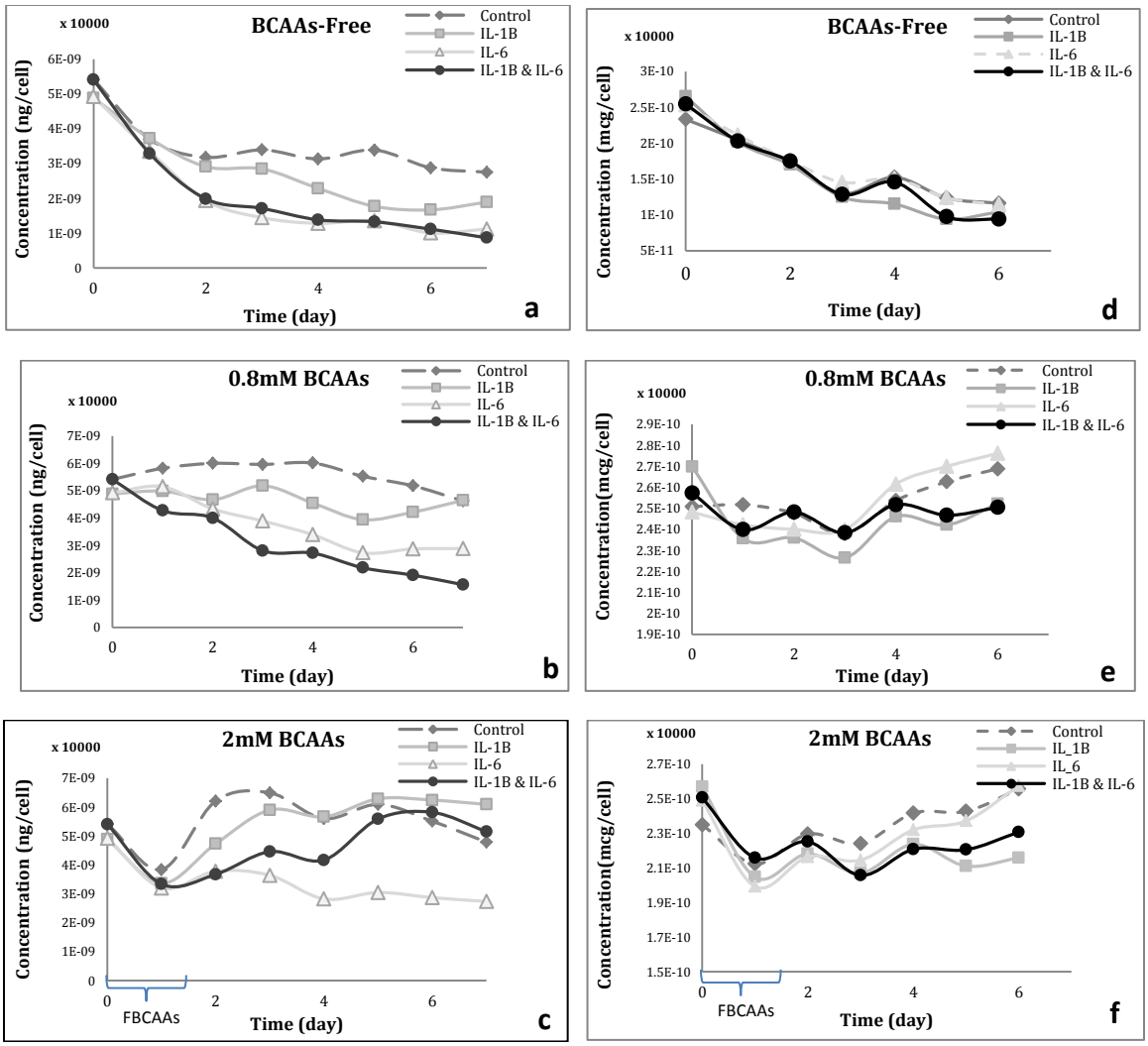


Figure 14: The effect of BCAA supplementation on in HepG2 cells treated with proinflammatory cytokines for 6 days. Data are the average of three samples at each time point.

Albumin production in the third group (2mM BCAAs) treated with a combination of both cytokines almost recovered after day 4 (Fig-14-c).

As a result, proinflammatory cytokines (IL-1ß & IL-6) alone or together, suppress albumin and urea production in the absence of BCAAs as well as the lower

concentration of BCAAs. Higher concentration of BCAAs in presence of IL-1ß alone or combined with IL-6 enhanced albumin production. After early suppression of albumin production by IL-1ß and combined with IL-6, the rate recovered during the course of treatment. Urea production significantly decreased in the presence of both cytokines alone or together in the absence of BCAAs, and no significant changes in urea production was observed at high or low concentration of BCAAs.

Although, no statistically significant alteration in urea secretion was observed in cells grown in 0.8 mM BCAAs and 2 mM BCAAs with or without cytokines, further experimentation is required to investigate whether the excess BCAAs would recover this alteration in cytokine-free media.

CHAPTER 6

REACTIVE OXYGEN SPECIES (H_2O_2) MODIFY ALBUMIN SYNTHESIS IN HEPG2 CELLS

6.1. INTRODUCTION

A severe trauma injury is often followed by secretion of inflammatory mediators and leads to pathophysiological effects on tissues. Inflammatory mediators cause the release of reactive oxidants including ROS (Machida Y, 2002, Lee K, 2003), which play a major role to induce a hypermetabolic stress state in the liver following the injury. The excess ROS, like H_2O_2, affects cellular functions in the liver. This can alter gene expression through the activation and transcription factors leading to up-regulation of cytokines, chemokines, adhesion molecules, and survival genes (Gutierrez-Ruiz M.C, 2001). H_2O_2 causes autoxidation of biological molecules such as proteins (Lee K, 2003, Baskaran H, 2000). Albumin is one of the proteins in plasma whose concentration decreases in severe trauma injuries due to the inflammatory response. In this study HepG2 cells were treated with different concentrations of H_2O_2 to investigate the effect oxidative stress on cell viability as well as production of albumin.

6.2. Materials and Methods

- HepG2/C3A cells from ATCC
- DMEM with 4.5 g/L glucose, no glutamine, no sodium pyruvate, and no BCAAs from Irvine Scientific
- DMEM with 4.5 g/L glucose, no glutamine, and no BCAAs from Invitrogen
- Hydrogen peroxide 30% (W/W) from Sigma
- PBS from Invitrogen
- L-glutamine from Sigma
- Sodium pyruvate from Sigma
- L-leucine, 98.5-101.01% from Sigma

- L-isoleucine, 98.5-101.01% from Sigma
- L-valine, 98.5-101.01% from Sigma

The media was supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2% penicillin-streptomycin (Invitrogen), and 2 mM glutamine. The medium from Irvine Scientific also was supplemented with 0.227 mM sodium pyruvate.

In this experiment the medium from Invitrogen was replaced with the same medium (DMEM) from Irvine Scientific without glutamine, and BCAAs. To clarify the accuracy of the experiment compared to previous experiments, both media were used in parallel to investigate the effect of H_2O_2 on albumin production as well as the cell viability.

Cell Culture

The HepG2/C3A cells were divided into two groups. Group one was cultured in medium from Invitrogen (G-DMEM) and group two was cultured in medium from Irvine Scientific (I-DMEM) supplemented with 0.8 mM BCAAs (same concentration as G-DMEM). The cells were maintained in a T-75 plate. At passage 15, cells were harvested and cultured in 24-well plates at 200,000 cells/cm². The media was replaced every 24 h with fresh media for four days until the cells reach confluence. On day 4, the cells in both groups were treated with different concentrations of H_2O_2 including 1, 2, 3 mM. The plan was to treat all cells with H_2O_2 for 3 days and then return to H_2O_2 free media for another 3 days to determine whether the effect of H_2O_2 is reversible (Fig-15).

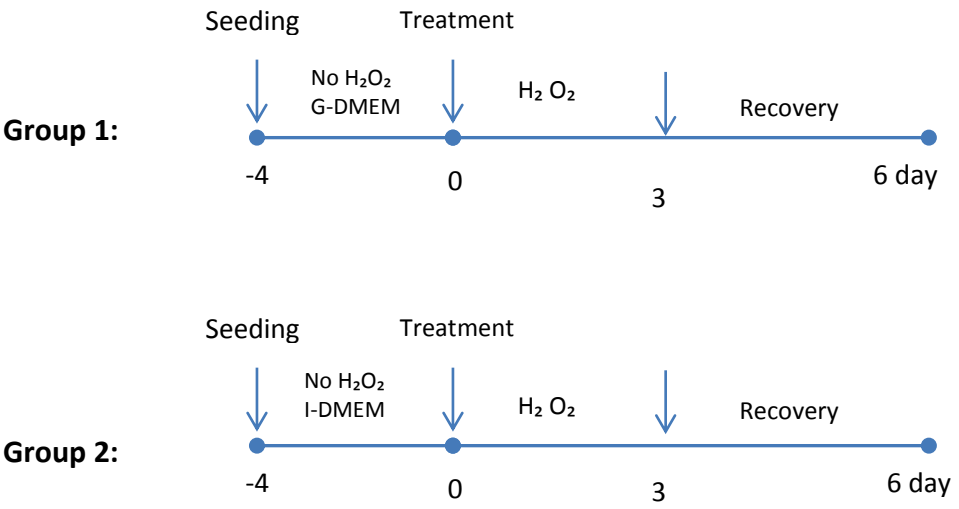


Figure 15: Experimental design to expose HepG2 cells to H_2O_2.

The media was changed every 24 h for 6 days, and supernatants were collected and stored in -80°C for further analysis. The cells were also counted every 24 h for both treatments using a hemocytometer.

However, only viability of the cells treated with 1 mM H_2O_2 were stable after day 2 for both media, therefore the recovery step only applied for the cells treated with 1 mM H_2O_2.

6.3. Data Analysis

Both groups were treated with H_2O_2 for 3 days following 3 days recovery. Only albumin as extracellular metabolite was quantified using ELISA (from Bethyl Lab) for both groups exposed to 1 mM H_2O_2. The concentration of albumin was calculated as the average of three samples per cell every day. The cell viability was monitored every 24 h

using a light microscope, and the cell viability was measured using a hemocytometer and with the trypan blue staining method. The level of production of albumin was statistically compared for each condition using the data analysis software “KaleidaGraph“.

6.4. Results and Discussion

HepG2/C3A cells were cultured for 3 days with media supplemented with different concentrations of H_2O_2 including 1, 2, 3 mM. The cells treated with concentrations higher than 1 mM H_2O_2 did not show stable viability after day 2 and the experiment was only continued with 1 mM H_2O_2. The cell numbers and albumin production were measured every day. For the first two days, the cell viability was monitored using a light microscope (Fig-16).

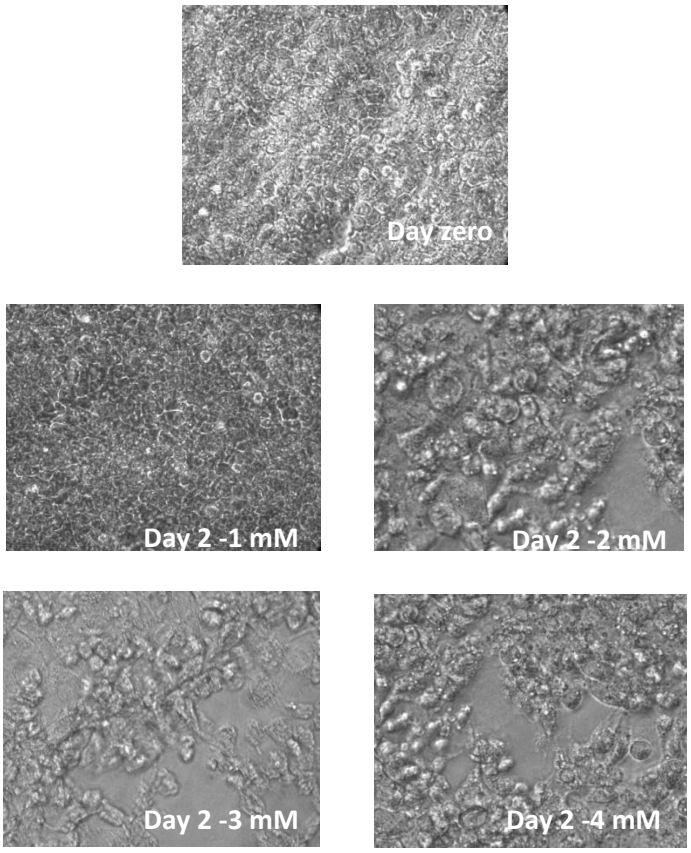


Figure 16: The cell viability for 1, 2, 3, and 4 mM H_2O_2 after two days. The gaps represent cell death in the culture.

Fig-16 compares the cell viability on day zero and day 2 for all four different concentrations of H_2O_2. The gaps between the cells represent the dead cells. There were significant differences between the number of cells on day 2 and day zero for the cells treated with concentrations higher than 1 mM H_2O_2. After two days treatment, the number of live cells for 1, 2, 3, and 4 mM H_2O_2 was about 4%, 23%, 46%, and 58 % respectively less than the day zero (Fig-17).

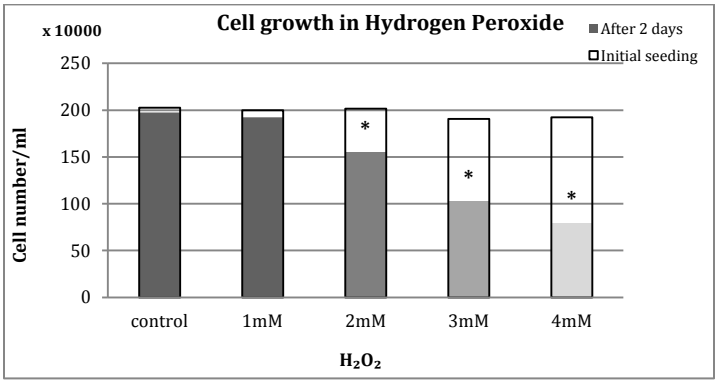


Figure 17: The cell viability after two days cultured in different concentration of H_2O_2.* shows significant decrease in number of cells on day two compared to day zero. Data are the average of three samples at each time point.

Albumin synthesis in both media was almost at the same range (Fig-18), and was suppressed by H_2O_2 ($p << 0.05$), which shows both media from Invitrogen (G-DMEM) and Irvine Scientifics (I-DMEM) are identical and G-DMEM can be replaced by I-DMEM.

As a result, H_2O_2 decreased the cell viability at concentrations higher than 1 mM. It suppressed albumin production for the first three days of the treatment, and then it was increased after day four to reach the control values (Fig-18). The effect of H_2O_2 was reversible after the removal of H_2O_2. The decrease in albumin production at day three exposed to H_2O_2 was 26% compared to the control, and then increased to 9% by day 6.

H_2O_2 showed similar effect as cytokines IL-1ß and IL-6 on albumin synthesis. All three inflammatory mediators suppressed albumin secretion.

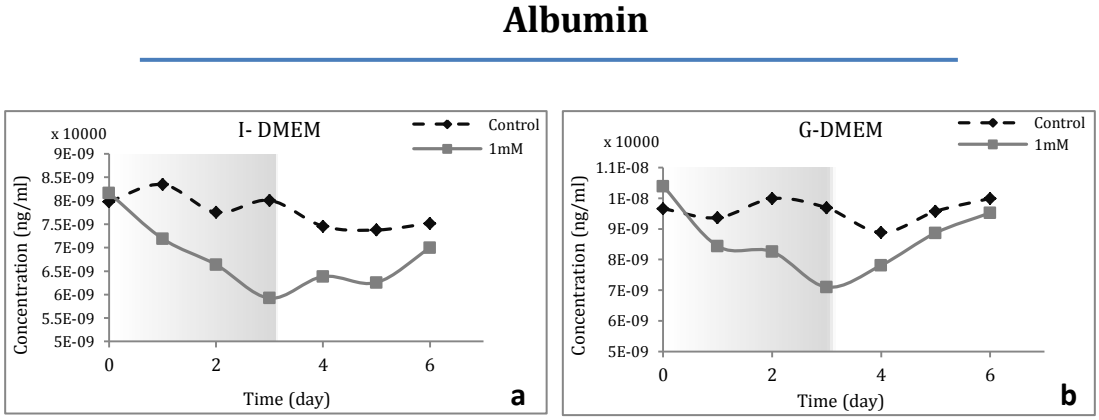


Figure 18: The effect of H_2O_2 (1 mM) on HepG2 cells on albumin synthesis in media from: a) Irvine Scientifics, b) Invitrogen. The shaded area represents the time during which cells were exposed to H_2O_2. Data are the average of three samples at each time point.

Based on the results from this experiment and the previous experiment in Chapter 4, the following experiment was done to investigate the effect of BCAAs as nutritional supplement on HepG2 cells exposed to inflammatory mediators including IL-1ß, IL-6, and H_2O_2 for a 3-day time period and then the cells were returned to mediators-free medium. The purpose is to investigate how albumin secretion would changed in recovery stage (mediator-free medium).

CHAPTER 7

BRANCHED CHAIN AMINO ACIDS SUPPLEMENTATION MITIGATES THE EFFECT OF INFLAMMATORY MEDIATORS ON ALBUMIN PRODUCTION IN HEPG2 CELL LINE

7.1. INTRODUCTION

Systemic inflammatory response (SIR) is initiated by variety of clinical situation including trauma and infection, which mobilizes a series of mediators including cytokines and ROS. ROS promote generation of cytokines including TNFα, IL-1ß, and IL-6, by modulating the nuclear factors responsible for production of these cytokines (Griendling K.K, 2000), and so produce additional tissue damages by effecting on biological molecules (Farber JL, 1990).

Trauma injuries cause a relatively larger increase in amino acid efflux to provide amino acid needs for tissue repair, production of cytokines as well as production of acute phase proteins. Therefore, sufficient quantity and quality of amino acids in the diet is necessary to maximize protein synthesis for optimal immune functions. Besides BCAAs, cysteine, like the other amino acids, is used for protein synthesis. Complementary clinical studies confirmed that supplementation of cysteine improves skeletal muscle function, decreases the plasma levels of the inflammatory cytokines, improves immune function, and increases plasma albumin levels (Cordeiro M.B, 2005). Albumin contains a free thiol group at Cys34 and potentially oxidizes to a mixed disulfide; therefore, it is quantitatively an important redox buffer in blood (Cordeiro M.B, 2005). It was observed in the previous experiment that 1 mM of H_2O_2 suppressed albumin production in HepG2 cells, therefore the purpose of this experiment is to investigate the effect of excess

BCAAs and cysteine on the production of albumin in cells treated with inflammatory mediators in a dose-dependent manner.

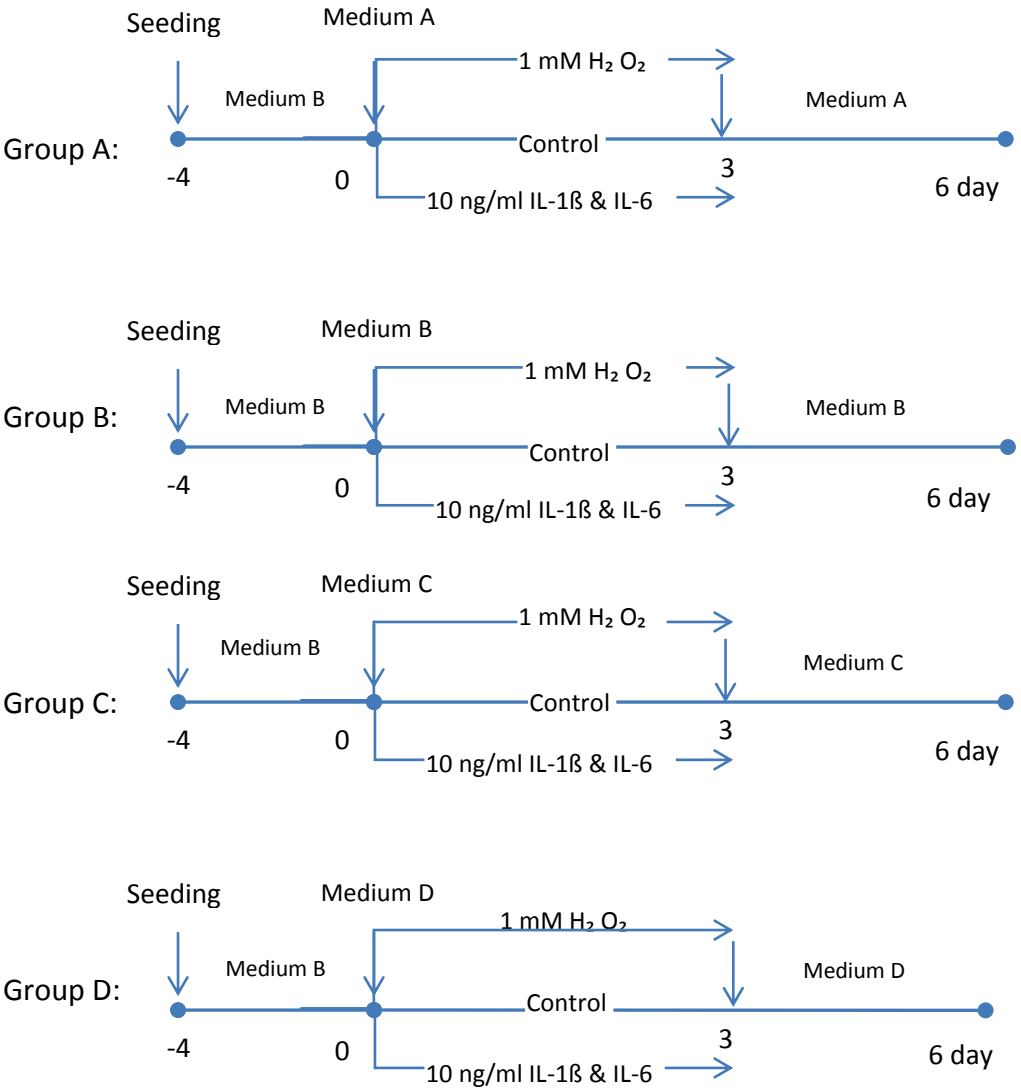
7.2. Materials and Methods

- HepG2/C3A cells from ATCC
- DMEM with 4.5 g/L glucose, no glutamine, no sodium pyruvate, and no BCAAs from Irvine Scientific
- Recombinant Human Il-6 (Cat# 206-IL) from R&D System
- Recombinant Human IL-1ß/IL-1F2 (Cat # 201-LB) from R&D System
- Hydrogen peroxide 30% (W/W) from Sigma
- PBS from Invitrogen
- L-glutamine from Sigma
- Sodium pyruvate from Sigma
- L-leucine, 98.5-101.01% from Sigma
- L-isoleucine, 98.5-101.01% from Sigma
- L-valine, 98.5-101.01% from Sigma
- Cysteine from Sigma

The medium was supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2% penicillin-streptomycin (Invitrogen), 0.227 mM sodium pyruvate, and 2 mM glutamine.

Cell Culture

HepG2/C3A cells were cultured in a medium supplemented with 0.8 mM BCAAs in T-75 plates. At passage 15, cells were harvested and cultured in 24-well plates at 200,000 cells/cm². The media was replaced every 24 h with fresh media for four days until the cells reach confluence. On day 4, cells were divided into four groups (Fig-19). Group ***A*** was cultured in medium without BCAAs; Group ***B*** was cultured in medium with 0.8 mM BCAAs; Group ***C*** was cultured in medium with 2 mM BCAAs; Group ***D*** was cultured in medium with 2 mM BCAAs and 1 mM cysteine.



A: without BCAAs + 0.4 mM cysteine
B: 0.8 mM BCAAs + 0.4 mM cysteine
C: 2 mM BCAAs + 0.4 mM cysteine
D: 2 mM BCAAs + 1 mM cysteine

Figure 19: Experimental design. Cells were cultured in four different medium as A, B, C, D and treated with either 1 mM H_2O_2 or combination of 10 ng/ml IL-1ß & IL-6 for three days.

The concentration of cysteine in groups A, B, and C was 0.4 mM. All groups were treated with either 1 mM H_2O_2, or combination of 10 ng/ml of IL-1ß &IL-6. The exposure to

inflammatory mediators continued for three days and on day four the cells were treated with inflammatory mediators-free media for three more days for recovery state. Every day supernatants were collected and stored in -80°C for further analysis. Cells were also counted every 24 h for all treatments using a hemocytometer.

7.3. Data Analysis

All four groups of cells were treated with either 1 mM H_2O_2, or a combination of 10 ng/ml of IL-1ß &IL-6 for 3 days following 3 days recovery. The collected suppernatants were analyzed for albumin concentration using ELISA (from Bethyl Lab). The cell viability was measured using a hemocytometer with the trypan blue staining method. The concentration of albumin was calculated as the average of three samples per cell every day. The level of production of albumin was statistically compared for each condition using the data analysis software “KaleidaGraph“.

7.3. Results and Discussion

HepG2/C3A cells were treated with inflammatory mediators for 3 days to investigate the effect of excess BCAAs and cysteine on albumin secretion. The cell viability and albumin synthesis rate in cells treated with four different media exposed to either H_2O_2 or combination of cytokines (IL-1ß & IL-6) were statistically analyzed.

7.3.1. Cell viability

The number of cells was counted every 24 h for the period of 6 days for all treatments.

Cells grown in medium A (Without BCAAs + 0.4 mM cysteine):

There was a steady decrease in the number of cells treated with H_2O_2 in medium A by day 3 and gradually increased during the recovery stage (Fig-20, Table-4).

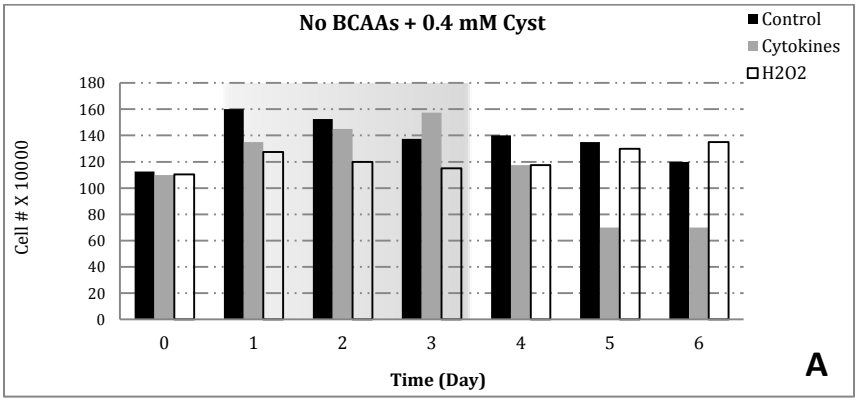


Figure 20: Changes in number of cells grown in medium A (No BCAAs + 0.4 mM cysteine) treated with either of inflammatory mediators compared to control in time. The shaded area represents the time during which cells were exposed to inflammatory mediators.

Media	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
A-Control	42.22%	-4.69%	-9.84%	1.82%	-3.57%	-11.11%
A-Cytokines	22.73%	7.41%	8.62%	-25.40%	-40.43%	0.00%
A-H_2O_2	15.38%	-5.88%	-4.17%	2.17%	10.64%	3.85%

Table 4 : Statistical analysis of the effect of cytokines and H_2O_2 on the number of live cells grown in medium A per day. % represents decrease (-) or increase (+) compared to previous day.

There was a constant increase in the number of cells during the treatment with cytokines; however, there was a sudden decrease after the first day of the recovery stage, which was not recovered by day 6. In the control condition, except for an increase at day 1, there were no significant changes in the number of cells per day (10%).

Cells grown in medium B (0.8 mM BCAAs + 0.4 mM cysteine):

There was a slight decrease in cell viability during the first three days of treatment with H_2O_2 (3%), but it was increased at first day of recovery (19%), and stayed almost constant for the next two days (Fig-21, Table-5).

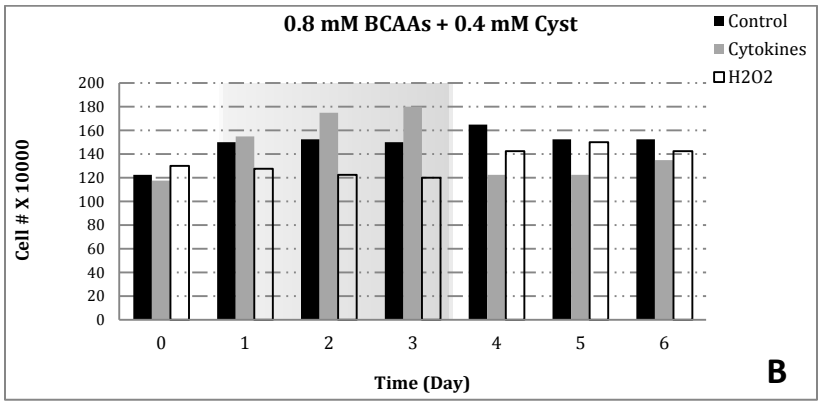


Figure 21: Changes in number of cells grown in medium B (0.8 mM BCAAs + 0.4 mM cysteine) treated with either of inflammatory mediators compared to control in time. The shaded area represents the time during which cells were exposed to inflammatory mediators.

Media	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
B-Control	22.45%	1.67%	-1.64%	10.00%	-7.58%	0.00%
B-Cytokines	31.91%	12.90%	2.86%	-31.94%	0.00%	10.20%
B-H_2O_2	-1.92%	-3.92%	-2.04%	18.75%	5.26%	-5.00%

Table 5: Statistical analysis of the effect of cytokines and H_2O_2 on the number of live cells grown in medium A per day. % represents decrease (-) or increase (+) compared to previous day.

For the cells treated with cytokines, there was an increase at day 1 (31%) which was gradually decreased by day 3, and there was a sudden decrease (31%) after the first day of recovery, but it was increased by day 6 (10%). The number of cells in the control condition was almost stable.

Cells grown in medium C (2 mM BCAAs + 0.4 mM cysteine):

In this condition, there was an increase in the number of cells treated with either of the inflammatory mediators and stayed almost constant after the recovery stage (Fig-22, Table-6). The increase in the number of cells was even more than the control condition after day 4. This might suggest that excess BCAAs can mediate the effect of inflammatory mediators and enhances cell viability during the recovery.

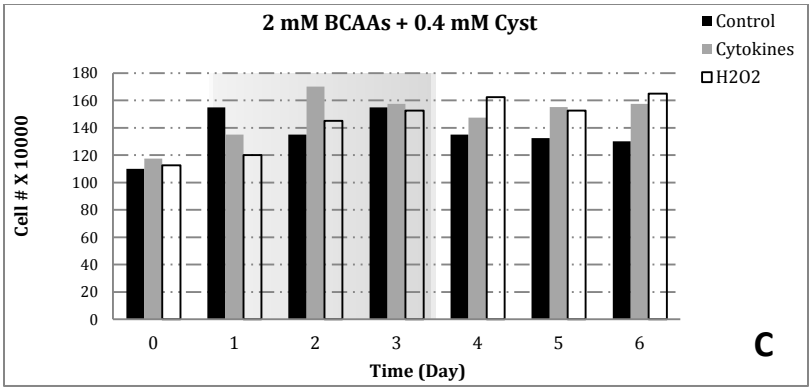


Figure 22: Changes in number of cells grown in medium C (2 mM BCAAs + 0.4 mM cysteine) treated with either of inflammatory mediators compared to control in time. The shaded area represents the time during which cells were exposed to inflammatory mediators.

Media	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
C-Control	40.91%	-12.90%	14.81%	-12.90%	-1.85%	-1.89%
C-Cytokines	14.89%	25.93%	-7.35%	-6.35%	5.22%	1.48%
C-H_2O_2	6.67%	20.83%	5.17%	6.56%	-6.15%	8.20%

Table 6: Statistical analysis of the effect of cytokines and H_2O_2 on the number of live cells grown in medium C per day. % represents decrease (-) or increase (+) compared to previous day.

Cells grown in medium D (2 mM BCAAs + 1 mM cysteine):

In this condition, also there was an increase in number of cells treated with either inflammatory mediator per day, which stayed almost constant during the recovery stage (Fig-23, Table-7). The increase in number of cells treated with cytokines during the

recovery stage was even more than the cells treated with H_2O_2. Comparing medium D with medium C, one might suggest that the excess cysteine might enhance cell viability in this condition.

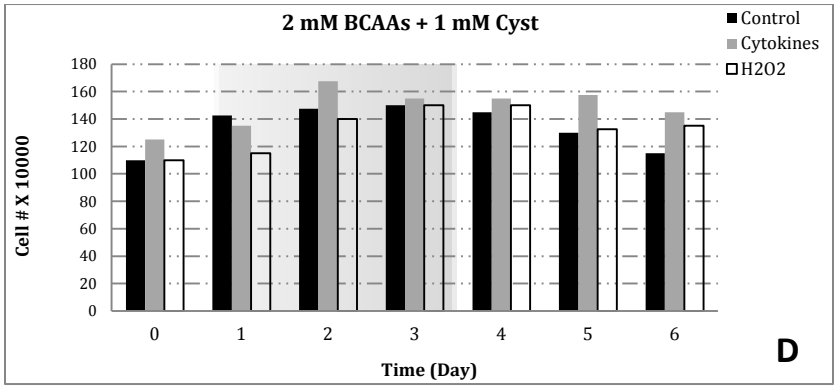


Figure 23: Changes in number of cells grown in medium D (2 mM BCAAs + 0.4 mM cysteine) treated with either of inflammatory mediators compared to control in time. The shaded area represents the time during which cells were exposed to inflammatory mediators.

Media	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
D-Control	29.55%	3.51%	1.69%	-3.33%	-10.34%	-11.54%
D-Cytokines	8.00%	24.07%	-7.46%	0.00%	1.61%	-7.94%
D-H_2O_2	4.55%	21.74%	7.14%	0.00%	-11.67%	1.89%

Table 7: Statistical analysis of the effect of cytokines and H_2O_2 on the number of live cells grown in medium D per day. % represents decrease (-) or increase (+) compared to previous day.

For both media (C and D) with the exception of day 5 in medium D, cell viability almost stayed stable for the rest of the period of 6 days. There was a sudden decrease (12%) in the number of cells treated with H_2O_2 at day 5 for medium D, (that could be due to the human error) but it started to recover at day 6 (2% increased). There was a sudden increase in the number of cells treated with cytokines at day 2 for media C (26%) and D (24%). With the exception of this sudden increase and decrease, cell viability for both

media (C and D) treated with either of the mediators for the time period of 6 days was almost stable (no changes more than 10%).

As a result, comparing the effect of oxidative stress (H_2O_2) on cell viability for all four different media showed an increase in number of cells grown in media C and D compared to media A and B during the treatment (day 1 through day 3) (Fig-24, Table-8). The number of cells for medium C stayed almost constant during the recovery.

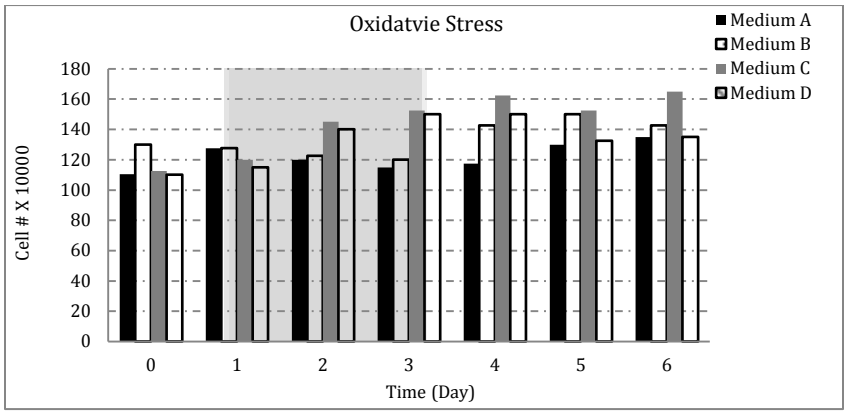


Figure 24: Changes in number of cells grown in media A, B, C, and D treated with H_2O_2. The shaded area represents the time during which cells were exposed to H_2O_2.

Media	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
A-H_2O_2	15.38%	-5.88%	-4.17%	2.17%	10.64%	3.85%
B-H_2O_2	-1.92%	-3.92%	-2.04%	18.75%	5.26%	-5.00%
C-H_2O_2	6.67%	20.83%	5.17%	6.56%	-6.15%	8.20%
D-H_2O_2	4.55%	21.74%	7.14%	0.00%	-11.67%	1.89%

Table 8: Statistical analysis of the effect of H_2O_2 on the number of live cells grown in all four media per day. % represents decrease (-) or increase (+) compared to previous day.

Comparing the effect of cytokines (IL-1ß & IL-6) on cell viability for all four different media showed an increase in number of cells grown in media B, C and D compared to medium A

during the treatment (day 1 through day 3) (Fig-25, Table-9). The overall increase in cell numbers in medium C was more than media B and D through the treatment.

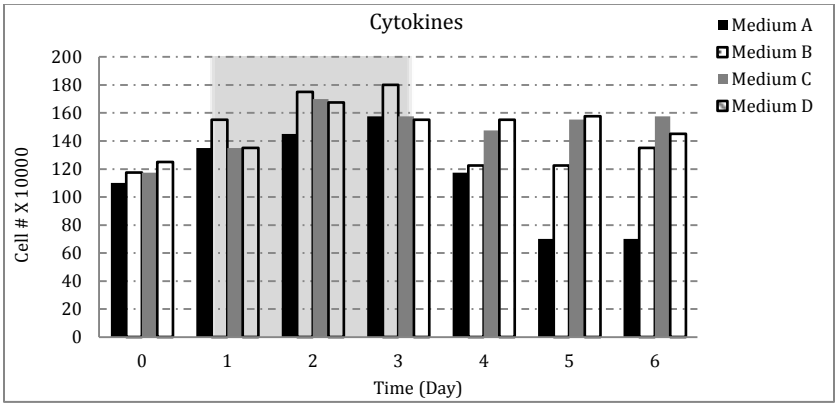


Figure 25: Change in number of cells grown in media A, B, C, and D treated with cytokines (IL-1ß & IL-6). The shaded area represents the time during which cells were exposed to cytokines.

Media	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
A-Cytokines	22.73%	7.41%	8.62%	-25.40%	-40.43%	0.00%
B-Cytokines	31.91%	12.90%	2.86%	-31.94%	0.00%	10.20%
C-Cytokines	14.89%	25.93%	-7.35%	-6.35%	5.22%	1.48%
D-Cytokines	8.00%	24.07%	-7.46%	0.00%	1.61%	-7.94%

Table 9: Statistical analysis of the effect of cytokines (IL-1ß & IL-6) on the number of live cells grown in all four media per day. % represents decrease (-) or increase (+) compared to previous day.

The number of cells grown in media C and D stayed almost stable during the recovery and there was no sudden decrease or increase compared to medium A and B. This would suggest the effect of excess BCAAs in cell viability during the recovery stage.

7.3.2. Albumin secretion

In the previous experiment, it was shown that albumin synthesis is reduced when cells are exposed to inflammatory mediators including cytokines and H_2O_2.

Albumin

Medium A: without BCAAs + 0.4 mM cysteine
***Medium* B**: 0.8 mM BCAAs + 0.4 mM cysteine
***Medium* C**: 2 mM BCAAs + 0.4 mM cysteine
***Medium* D**: 2 mM BCAAs + 1 mM cysteine

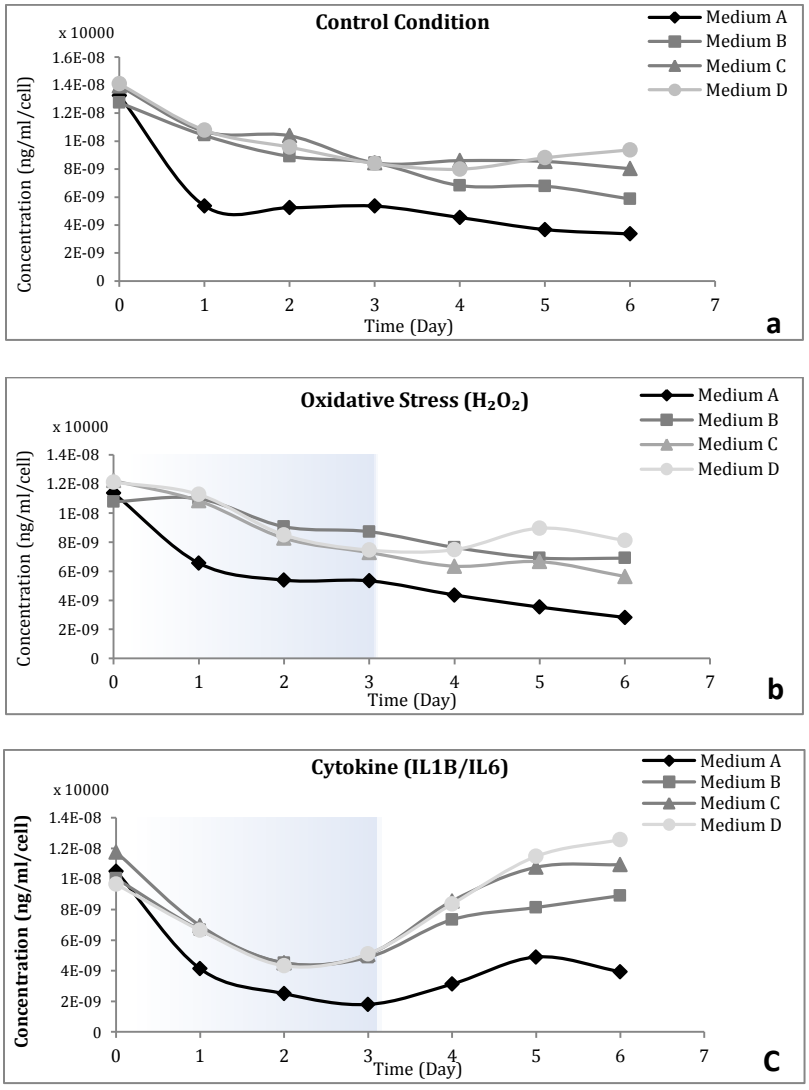


Figure 26:The effect of excess BCAAs and cysteine on albumin production on HepG2 cells treated with inflammatory mediators (H_2O_2 and IL-1ß & IL-6) for three days, followed by three days recovery (treatment with inflammatory mediator-free media). The shaded area represents the time during which cells were exposed to mediators.

In the case of inflammatory mediator-free media (control) (Fig-26-a), there were statistically significant differences in albumin production for cells grown in medium A with either of three media B ($p = 0.0007$), C ($p < 0.0001$), and D ($p < 0.0001$) for the period of 6

days (Table-10). After day three, an increase in albumin production was observed in cells grown in media C and D compared to medium A (medium C: p=0.013, medium D: p=0.0052); also this increase was even more in medium D by day 6 (p=0.0043).

Mediator-Free Media

Media	p value (day 1- 3)	p value (day 3 -6)
B & A	0.0056	0.0019
C & A	0.0024	< 0.0001
D & A	0.0034	< 0.0001

Table 10: Statistical analysis. Comparing the effect of excess BCAAs and Cysteine on albumin production, in mediator-free media.

During the treatment with H_2O_2 (day 1-3), there were no significant changes in albumin production among all four media (Fig-26-b, Table-12), which might suggest that albumin secretion is suppressed due to the effect of oxidative stress even in presence of excess BCAAs and cysteine.

Oxidative Stress

Media	p value (day 1- 3)	p value (day 4 -6)	p value (day 5-6)
B & A	0.0589	0.0005	0.006
C & A	0.1444	0.004	0.0149
D & A	0.1056	< 0.0001	0.0015
D & C	0.9957	0.01	0.0272

Table 11: Statistical analysis. Comparing the effect of excess BCAAs and Cysteine on albumin production due to the oxidative stress response.

However, there were statistically significant changes during the recovery period between medium A and the other three media B ($p = 0.0005$), C ($p = 0.004$), and D ($p < 0.0001$) (Table-11). Production of albumin was increased in media B, C, and D during the recovery compared to medium A ($p \ll 0.05$). Moreover, the difference in albumin secretion for the

recovery period between medium C and D was also significant (p = 0.01). The cells grown in medium C secreted more albumin.

During the treatment with cytokines, there were not significant changes in albumin production among all four media for the first 3 days (Fig-26-c, Table-12); however, there were statistically significant changes for the recovery period between medium A and media C (p = 0.01) and D (p = 0.002), but not with medium B (p = 0.06). It was noticed that there were no statistically significant changes in albumin secretion between medium C and D during the recovery also (p>0.05).

Cytokines

Media	p value (day 1- 3)	p value (day 4 -6)	p value (day 5-6)
B & A	0.1191	0.062	0.042
C & A	0.0953	0.0122	0.0114
D & A	0.118	0.0025	0.0028
D & C	0.9985	0.5996	0.1404

Table 12: Statistical analysis. Comparing the effect of excess BCAAs and Cysteine on albumin production due to the proinflammatory response.

As a result, in the absence of mediators, albumin production was suppressed in BCAAs-free media (media A). Albumin production was enhanced slightly after day 4 in media with excess amount of BCAAs and cysteine (media C and D). The effect of inflammatory mediators on HepG2 cells was reversible in presence of excess BCAAs along with cysteine. This effect was even more in presence of cytokines compared to oxidative stress. Although, cytokines suppressed albumin production at a higher rate compared to H_2O_2, but albumin production increased to level close to day zero during the recovery stage after treatment with cytokines, compared to H_2O_2.

CHAPTER 8

BRANCHED CHAIN AMINO ACIDS SUPPLEMENTATION MODULATES THE GSH:GSSG RATIO IN HEPG2 CELLS

8.1. INTRODUCTION

The supplementation of antioxidants can regulate the harmful effects of ROS. The intracellular regulators are detoxifant enzymes, vitamins C and E or thiol-containing molecules such as glutathione (GSH) (Arrigo A.P, 1999) . Glutathione is the major intraclelular regulator acting through a reversible oxidation of the active thiol group (Arrigo A.P, 1999). In an *in vitro* model study, Lee (Lee K, 2003) showed H_2O_2 oxidizes redox-sensitive molecules and decreases oxygen uptake. This study also showed that the enzyme glutathione peroxidase catalyzes the reduction of H_2O_2 by converting the reduced glutathione (GSH) to the oxidized GSSG (Lee K, 2003).

Glutathione is composed of three amino acids: cysteine, glycine and glutamic acids. The thiol side chain (-SH) in cysteine participates in redox reactions. The thiol group serves as a proton donor and participates in the oxidation of glutathione (GSH) to glutathione disulfide (GSSG). Conversion of cysteine to GSH is strongly influenced by the rate of utilization and transport of GSH within and between the cells (Grimble R.F, 2006). The sulfhydryl-containing side chain of cysteine forms the cornerstone of many redox reactions in the cell (Dominy J.E, 2007). A clinical study showed the lower GSH concentration caused by impaired synthesis is due to the lower concentrations of cysteine (Reid M, 2000), which is the rate-limiting precursor of GSH. Also it is well known that the amino group from

BCAAs can be incorporated into the α-keto-glutarate (α-KG) to produce glutamate through glutamate dehydrogenase (GDH) (Humberto N, 2012).

Therefore this experiment is done to investigate the effect of excess BCAAs as well as cysteine in GSH:GSSG ratio in HepG2 cells treated with inflammatory mediators.

8.2. Materials and Methods

- HepG2/C3A cells from ATCC
- DMEM with 4.5 g/L glucose, no glutamine, no sodium pyruvate, and no BCAAs from Irvine Scientific
- Recombinant Human Il-6 (Cat# 206-IL) from R&D System
- Recombinant Human IL-1ß/IL-1F2 (Cat # 201-LB) from R&D System
- Hydrogen peroxide 30% (W/W) from Sigma
- PBS from Invitrogen
- L-glutamine from Sigma
- Sodium pyruvate from Sigma
- L-leucine, 98.5-101.01% from Sigma
- L-isoleucine, 98.5-101.01% from Sigma
- L-valine, 98.5-101.01% from Sigma
- Cysteine from Sigma

The medium was supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 2% penicillin-streptomycin (Invitrogen), 0.227 mM sodium pyruvate, and 2 mM glutamine.

Cell Culture

HepG2/C3A cells were cultured in medium supplemented with 0.8 mM BCAAs in a T-75 plate. At passage 11, cells were harvested and cultured in 96-well plates at 200,000 cells/cm². After 24 h, the cells were divided in four groups, same as previous experiment in chapter 7: Group **A** was cultured in medium without BCAAs; Group **B** was cultured in medium with 0.8 mM BCAAs; Group **C** was cultured in medium with 2 mM BCAAs; Group **D**

was cultured in medium with 2 mM BCAAs and 1 mM cysteine. The concentration of cysteine in groups A, B, and C was 0.4 mM.

8.3. Data Analysis

All four groups' cells were treated with either 1 mM H_2O_2, or combination of 10 ng/ml of IL-1ß &IL-6 for 24 h. After 24 h the media was removed, cells were washed with PBS, and tested for quantification of total glutathione (GSH + GSSG) and GSH:GSSG ratio using the GSH/GSSG-Glo assay kit from Promega. The concentration was calculated as the average of three cultures per condition and data was analyzed using the data analysis software "KaleidaGraph".

8.3. Results and Discussion

HepG2/C3A cells were treated with inflammatory mediators for 24 h to investigate the effect of excess BCAAs and cysteine on total glutathione and GSH:GSSG ratio. When cells were treated with inflammatory mediator-free media, the GSH:GSSG ratio in group C was significantly higher than the other groups (Fig-27). In oxidative stress situation, the differences were significant between group A (without BCAAs) and groups C and D (Fig-28). This ratio was about 34% more in media C and D compared to medium A (Table-13). In the case of proinflammatory cytokines, this ratio was also low in the absence of BCAAs in group A. The GSH:GSSG ratio was higher at higher concentration of BCAAs (2 mM) in groups C and D compared to group B (Fig-29).

Group A: without BCAAs + 0.4 mM cysteine
***Group* B**: 0.8 mM BCAAs + 0.4 mM cysteine
***Group* C**: 2 mM BCAAs + 0.4 mM cysteine
***Group* D**: 2 mM BCAAs + 1 mM cysteine

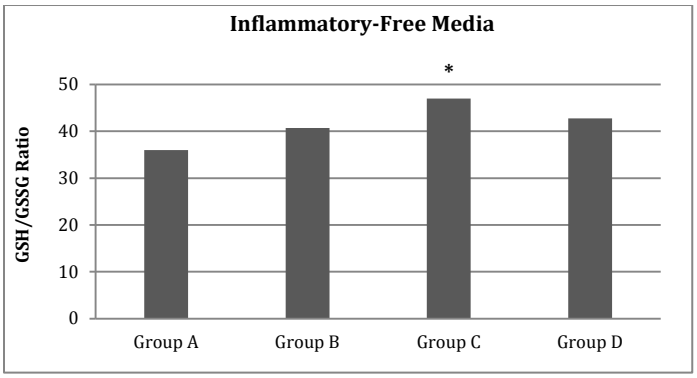


Figure 27: Comparing GSH:GSSG ratio in mediator-free media for all groups.

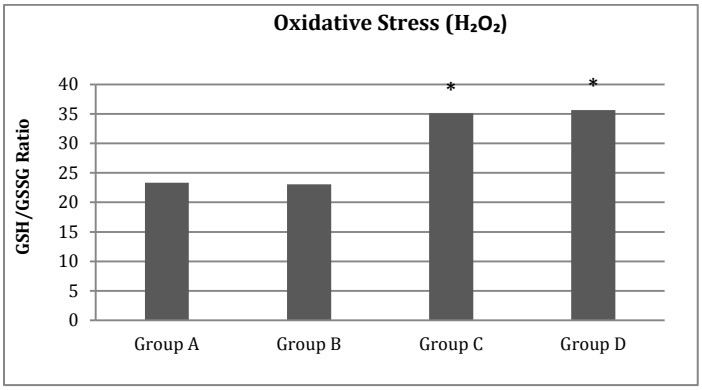


Figure 28: Comparing GSH:GSSG ratio in all groups treated with H_2O_2.

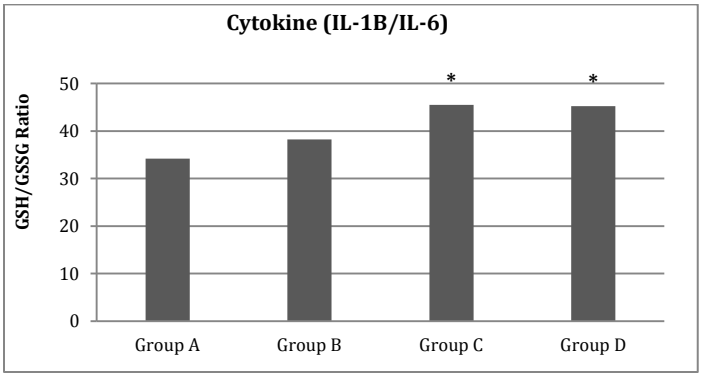


Figure 29: Comparing GSH:GSSG ratio in all groups treated with cytokines.

This might suggest that the excess BCAAs and cysteine enhanced the GSH:GSSG ratio in presence of inflammatory mediators. In overall, all mediators suppressed GSH:GSSG ratio; however, oxidative stress had significant effect on GSH:GSSG ratio compared to proinflammatory cytokines IL-1ß & IL-6 (Table-13).

Condition	Media B & C	Media B& D
Mediator-free	-13.30%	-4.77%
Cytokines	-16.01%	-15.55%
Oxidative Stress	-34.21%	-35.23%

Table 13: Comparing the GSH:GSSG ratio in group B with groups C and D for three different conditions: a) inflammation-free, b)oxidative Stress, c)cytokines.

The total glutathione (GSH + GSSG) for all conditions are shown in Fig-30. The total GSH in medium A was less than the other three media. In the control condition (inflammatory mediator-free) the total (GSH+GSSG) in medium A was about 23% less than the other media. The total (GSH+GSSG) under the effect of cytokines in medium A was 20-27% less than the other media, and in oxidative stress situation the differences was increased to about 40%. Although the p value for all of these conditions was more than 0.05. The total (GSH+GSSG) in medium B compared to media C and D, with excess amount of BCAAs and cysteine, was about 3-6% less.

This might suggest the excess BCAAs and cysteine enhances the total (GSH+GSSG) but not quite significant. However, there was no significant differences in the total (GSH+GSSG) between media B, C, and D (Fig-30).

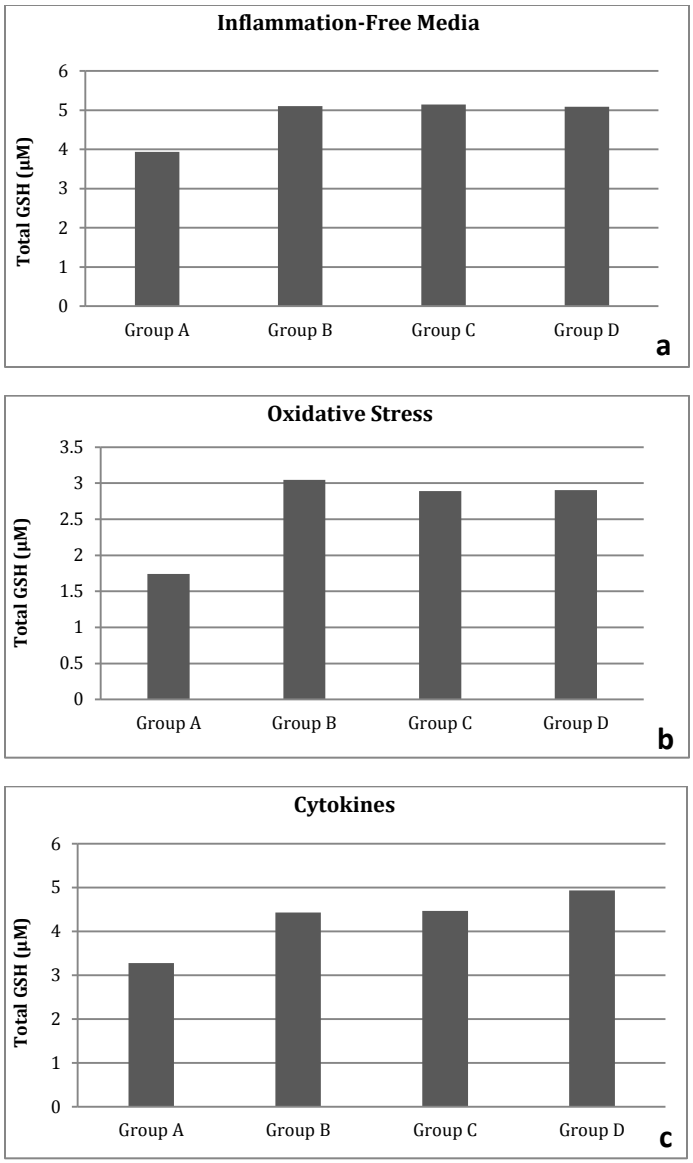


Figure 30: Total GSH in all four media A, B, C, and D for three different conditions: a) inflammation-free, b)oxidative stress, c)cytokines

Media	Mediator-free	Cytokines	Oxidative Stress
B & A	-23.00%	-22.83%	-42.84%
C & A	-23.52%	-20.02%	-39.73%
D & A	-22.67%	-27.50%	-40.05%

Table 14: Statistical analysis. Comparing total GSH in medium B with the other three media under the effect of cytokines, oxidative stress and control

As a result, in the absence of mediators, GSH:GSSG ratio was higher at higher concentration of BCAAs. This ratio decreased in the presence of mediators, but cytokines had less of the effect compared to H_2O_2. It has been also reported that GSH depletion in malnutrition is due to increases in oxidative stress leading to an increase in consumption of GSH (Golden M.H, 1987). The decrease in GSH:GSSG ratio induced by mediator was alleviated at higher concentrations of BCAAs and cysteine, although; total glutathione (GSH+GSSG) was lower in BCAA-free medium, otherwise not affected by BCAAs or cysteine levels.

BCAAs can modulate the inflammatory response through glutamine synthesis. At low concentration of cysteine, the cysteine for GSH synthesis is provided by the intracellular reduction of cystine via cystine/glutamate antiporter (Bannai S, 1986), while at a higher concentraiton of cysteine, this antiporter acts in reverse to take up glutamate instead of cystine (Rimaniol A.C, 2001). Therefore, further experiments need to be done to study the effects of different concentrations of BCAAs and cysteine on GSH:GSSG ratio as well as total GSH to overcome the effects of inflammatory mediators.

Final Discussion

In conclusion, utilization of amino acids in trauma is important for the defense against diseases; however, it reduces body stores of proteins and free amino acids to provide enough amino acids for tissue repair, production of acute phase proteins, and immune activity. Sufficient quality and quantity of nutritional supplementation is required to overcome nitrogen depletion and to maximize protein synthesis for optimal wound

healing and immune function. Several clinical studies have shown among nutritional supplements that BCAAs can enhance liver function in inflammatory states. The purpose of this research was to investigate the effect of proinflammatory mediators (more specifically the cytokines IL-1ß & IL-6), reactive oxygen species H_2O_2, and BCAAs on a cell culture model of liver consisting of HepG2 hepatoma cells. It was found that these mediators reversibly suppress albumin and urea production, and also decrease the amount of the intracellular antioxidant reduced glutathione (GSH). BCAA supplementation mitigates the effect of these inflammatory mediators on albumin production and the GSH:GSSG ratio, and significantly increases albumin production during recovery post removal of inflammatory agents.

Several clinical and *in vitro* studies showed that trauma injuries suppress albumin but increased urea production. Prior studies in rat liver cells (Kang Y.H, 2002) showed that cytokines decrease both albumin and urea production, and our results are consistent with this study. The effect of a high level of BCAAs on albumin production is consistent with another study (Kuwahata M, 2008) on HepG2 cells in the absence of mediators. Prior studies also suggest that BCAAs increase the phosphorylation of mTOR and as a result increase protein synthesis, and mTOR also involved in scavenging of ROS (Avruch J, 2009, Kimball S.R, 2001). This observation is consistent with the result that high level of BCAAs at 2 mM enhanced GSH:GSSG ratio and albumin production.

Appendix-1

1.1 MEDIA FORMULA (from company's website http://www.invitrogen.com)

Media Formulations for Williams' Medium E – Invitrogen (12551)

Components	Molecular Weight	Concentration (mg/L)	mM
Amino Acids			
Glycine	75	50	0.667
L-Alanine	89	90	1.01
L-Arginine	174	50	0.287
L-Asparagine-H2O	150	20	0.133
L-Aspartic acid	133	30	0.226
L-Cysteine	121	40	0.331
L-Cystine 2HCl	313	26.07	0.0833
L-Histidine	155	15	0.0968
L-Isoleucine	131	50	0.382
L-Leucine	131	75	0.573
L-Lysine hydrochloride	183	87.46	0.478
L-Methionine	149	15	0.101
L-Phenylalanine	165	25	0.152
L-Proline	115	30	0.261
L-Serine	105	10	0.0952
L-Threonine	119	40	0.336
L-Tryptophan	204	10	0.049
L-Tyrosine disodium salt dihydrate	261	50.65	0.194
L-Valine	117	50	0.427
Vitamins			
Ascorbic Acid	176	2	0.0114
Biotin	244	0.5	0.00205
Choline chloride	140	1.5	0.0107
D-Calcium pantothenate	477	1	0.0021
Ergocalciferol	397	0.1	0.00025
Folic Acid	441	1	0.00227
Menadione sodium bisulfate	276	0.01	3.6E-05
Niacinamide	122	1	0.0082
Pyridoxal hydrochloride	204	1	0.0049
Riboflavin	376	0.1	0.00027
Thiamine hydrochloride	337	1	0.00297
Vitamin A (acetate)	328	0.1	0.00031
Vitamin B12	1355	0.2	0.00015
alpha Tocopherol acetate Na	702	0.01	1.4E-05
i-Inositol	180	2	0.0111
Inorganic Salts			
Calcium Chloride (CaCl2) (anhyd.)	111	200	1.8
Cupric sulfate (CuSO4-5H2O)	250	0.0001	4E-07
Ferric sulfate (FeSO4-7H2O)	278	0.0001	4E-07
Magnesium Sulfate (MgSO4) (anhyd.)	120	97.67	0.814
Manganese Sulfate (MnSO4-H20)	169	0.0001	6E-07
Potassium Chloride (KCl)	75	400	5.33
Sodium Bicarbonate (NaHCO3)	84	2200	26.19
Sodium Chloride (NaCl)	58	6800	117.24
Sodium Phosphate monobasic (NaH2PO4) anhydrous	138	140	1.01
Zinc sulfate (ZnSO4-7H2O)	288	0.0002	7E-07
Other Components			
D-Glucose (Dextrose)	180	2000	11.11
Glutathione (reduced)	307	0.05	0.00016
Methyl linoleate	295	0.03	0.0001
Phenol Red	376.4	10	0.0266
Sodium Pyruvate	110	25	0.227

Media Formulations for DMEM (11054) - Invitrogen

Low glucose, pyruvate, no glutamine, no phenol red

Components	Molecular Weight	Concentration (mg/L)	mM
Amino Acids			
Glycine	75	30	0.4
L-Arginine hydrochloride	211	84	0.398
L-Cystine 2HCl	313	63	0.201
L-Histidine hydrochloride-H2O	210	42	0.2
L-Isoleucine	131	105	0.802
L-Leucine	131	105	0.802
L-Lysine hydrochloride	183	146	0.798
L-Methionine	149	30	0.201
L-Phenylalanine	165	66	0.4
L-Serine	105	42	0.4
L-Threonine	119	95	0.798
L-Tryptophan	204	16	0.0784
L-Tyrosine disodium salt dihydrate	261	104	0.398
L-Valine	117	94	0.803
Vitamins			
Choline chloride	140	4	0.0286
D-Calcium pantothenate	477	4	0.0084
Folic Acid	441	4	0.0091
Niacinamide	122	4	0.0328
Pyridoxine hydrochloride	204	4	0.0196
Riboflavin	376	0.4	0.0011
Thiamine hydrochloride	337	4	0.0119
i-Inositol	180	7.2	0.04
Inorganic Salts			
Calcium Chloride (CaCl2) (anhyd.)	111	200	1.8
Ferric Nitrate (Fe(NO3)3"9H2O)	404	0.1	0.0002
Magnesium Sulfate (MgSO4) (anhyd.)	120	97.67	0.814
Potassium Chloride (KCl)	75	400	5.33
Sodium Bicarbonate (NaHCO3)	84	3700	44.05
Sodium Chloride (NaCl)	58	6400	110.34
Sodium Phosphate monobasic (NaH2PO4-H2O)	138	125	0.906
Other Components			
D-Glucose (Dextrose)	180	1000	5.56
Sodium Pyruvate	110	110	1

Media Formulations for DMEM (10313) - Invitrogen

High glucose, pyruvate, no glutamine

Components	Molecular Weight	Concentration (mg/L)	mM
Amino Acids			
Glycine	75	30	0.4
L-Arginine hydrochloride	211	84	0.398
L-Cystine 2HCl	313	63	0.201
L-Histidine hydrochloride-H2O	210	42	0.2
L-Isoleucine	131	105	0.802
L-Leucine	131	105	0.802
L-Lysine hydrochloride	183	146	0.798
L-Methionine	149	30	0.201
L-Phenylalanine	165	66	0.4
L-Serine	105	42	0.4
L-Threonine	119	95	0.798
L-Tryptophan	204	16	0.0784
L-Tyrosine disodium salt dihydrate	261	104	0.398
L-Valine	117	94	0.803
Vitamins			
Choline chloride	140	4	0.0286
D-Calcium pantothenate	477	4	0.0084
Folic Acid	441	4	0.0091
Niacinamide	122	4	0.0328
Pyridoxine hydrochloride	206	4	0.0194
Riboflavin	376	0.4	0.0011
Thiamine hydrochloride	337	4	0.0119
i-Inositol	180	7.2	0.04
Inorganic Salts			
Calcium Chloride (CaCl2) (anhyd.)	111	200	1.8
Ferric Nitrate (Fe(NO3)3"9H2O)	404	0.1	0.0002
Magnesium Sulfate (MgSO4) (anhyd.)	120	97.67	0.814
Potassium Chloride (KCl)	75	400	5.33
Sodium Bicarbonate (NaHCO3)	84	3700	44.05
Sodium Chloride (NaCl)	58	6400	110.34
Sodium Phosphate monobasic (NaH2PO4-H2O)	138	125	0.906
Other Components			
D-Glucose (Dextrose)	180	4500	25
Phenol Red	376.4	15	0.0399
Sodium Pyruvate	110	110	1

Media Formulations for DMEM (9024) – Irvine Scientific
(http://www.irvinesci.com)

DME DULBECCO'S MODIFICATION OF EAGLE'S BASAL MEDIUM - LIQUID	High Glucose without L-Glutamine
Catalog Nos.	**9024**
Component	***mg/L***
Sodium Chloride	6400
Potassium Chloride	400
Glucose	4500
L-Arginine HCl	84
L-Cystine 2HCl	63
L-Glutamine	—
Glycine	30
L-Histidine HCl • H_2O	42
L-Isoleucine	--
L-Leucine	--
L-Lysine HCl	146
L-Methionine	30
L-Phenylalanine	66
L-Serine	42
L-Threonine	95
L-Tryptophan	16
L-Tyrosine 2Na • $2H_2O$	104
L-Valine	--
Folic Acid	4
Inositol	7
Nicotinic Acid Amide	4
Riboflavin	0.4
Thiamine HCl	4
Ferric Nitrate	0.1
Phenol Red, Na salt	15
Sodium Phosphate, monobasic NaH_2PO_4 • H_2O	125
Pantothenic Acid, Ca salt	4
Pyridoxine HCl	4
Calcium Chloride, anhyd.	200
Magnesium Sulfate, anhyd. $MgSO_4$	98
Choline Chloride	4
Sodium Bicarbonate	3700
HEPES, Acid Form	—
HEPES, 1 Na Salt	—
Pyruvic Acid, Na salt	—

APPENDIX-2

2.1 Changing medium

This procedure can be listed as:

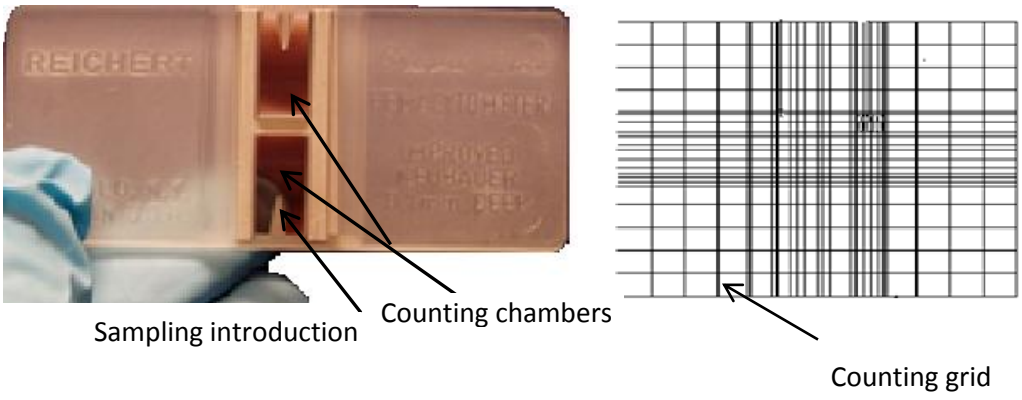
1- Aspirate the medium. If required to collect supernatants, first shake the plate slowly and then collect the medium using micropipette, and then aspirate the leftover medium.

2- Add 1X PBS to the well (the volume depends on the size of the well) and then aspirate.

3- Add new fresh medium

2.2 Cell counting using Hemocytometer

This device is used to determine the number of cells per unit.



First, polish surface and the coverslip carefully. Take 180 µL of trypan blue and pure in a well in 96-well plate. Pipette the cell suspension and add 20 µL of it to trypan blue. Then pipette few times to mix the cell suspension with trypan blue. Take 10 µL of the mix and inject into one of the V-shaped wells in hemocytometer. Place the charged counting

chamber on the microscope to count the cells. The surface area of each square in counting chamber is 1 mm^2, with the depth of 0.1 mm. The entire counting grid has the volume of 1 mm^3. Always follow a specific counting pattern to count the cells. For instance, count the total number of cells in the four large corner squares. Add the number of cells in four squares to find the total number of cells, and then divide by 4 to find the average. So you have X number of cells per mm^3. Since at the beginning you diluted the cells 10 fold, you should multiply the number of cells by 10 and also multiply by 1000 to find the number of cells per mL.

APPENDIX-3

All these protocols are designed for use in 96-well plates and the absorbance should be measured in a micro-plate reader.

3.1. Urea

Urea Reagent: Fisher Cat # NC9976458
Enzyme Reagent: Cat # 2051
Color Reagent: Cat # 2052
Standard: Cat # 1022

Add 50 ml of DI Water to Enzyme Reagent.

⬇

Add 100 µl Enzyme Reagent to each well.

⬇

Add 10 µl of standard or sample.

⬇

Centrifuge for 1min at 1000RPM.

⬇

Incubate at 37°C water bath for 5 minutes.

⬇

Add 100 µl of Color Reagent.

⬇

Centrifuge for 1min at 1000RPM.

⬇

Incubate at 37°C water bath for 5 minutes.

⬇

Measure absorbance at 585 nm.

STD	µg/ml	Urea STd	Medium (µl)
Initial	300	100 µl	0
1	150	50 µl from initial	50
2	75	50 µl from std 1	50
3	37.5	50 µl from std 2	50
4	0	0	50

3.2. Triglyceride/Glycerol

Triglyceride : Sigma
TG Reagent: Cat # T2449-10
Free Glycerol Reagent: Cat # F6428-40
Standard: Cat # G7793-5 (2.5mg/ml)

(Reconstitute the reagents with DI water)
(Materials are stable at 2-8°C for 60 days)
(Dilute samples in water as 1:10)

Add 200 µl of Free Reagent to each well.

⬇

Add 5 mcl of standard or sample.

⬇

Centrifuge for 2 min at 1000RPM.

⬇

Incubate at room temperature for 10min.

⬇

Measure absorbance at 595 nm (Concentration of glycerol)

⬇

Add 50 µl of TG Reagent.

⬇

Incubate at room temperature for 15min.

⬇

Measure absorbance at 595 nm (Concentration of total triglyceride)

STD	mg/ml	TG Standard	DI Water (µl)
Initial	2.5	100 µl	0
1	1.25	50 µl from initial	50
2	0.625	50 µl from std 1	50
3	0.3125	50 µl from std 2	50
4	0	0	50

3.3. Albumin

Albumin Reagents: Bethyl Lab; Cat# E80-129 and Enzyme Substrate, TMB: Cat #E102

Affinity Antibody: A80-129A
HRP Detection Antibody: A80-129P
Standard: RS10-110-4

(Coating Buffer: 1L DI water+ 3.7g Sodium Bicarbonate+ 0.64g Sodium Carbonate; PH=9.6)
(Washing Solution: 1L DI water+ 6.057g Tris+ 8.18g NaCl; PH=8; then add 0.5ml Tween)
(Blocking Solution: 1L DI water+ 6.057g Tris+ 8.18g NaCl; PH=8; then add 10g BSA)
(Sample Diluent Solution: 1L DI water+ 6.057g Tris+ 8.18g NaCl; PH=8; then add 0.5ml Tween + 10g BSA)
(4.8g NaOH + 40ml DI water = 3mol NaOH)
(10ml HCl + 30ml DI water = 3mol HCl)

Note: Run each standard or sample in duplicate.

Add 100 mcl of diluted coating antibody to each well.
(*11ml coating buffer + 110* µl *Affinity antibody for each 96 well plate*)
⬇
Incubate at room temperature (20-25°C) for 1 hour.
⬇
Wash plate FIVE times.
⬇
Add 200 mcl of Blocking Solution to each well.
⬇
Incubate at room temperature for 60 minutes.
⬇
Wash plate FIVE times.
⬇
Add 100 mcl of standard or sample to well.
(1:100 = *300* µl *diluted + 3* µl *sample*)
⬇
Incubate at room temperature for 1 hour.
⬇
Wash plate FIVE times.
⬇
Add 100 mcl of diluted HRP detection antibody to each well.
(*2mcl HRP + 150ml diluted*)
⬇
Incubate at room temperature for 1 hour.
⬇
Wash plate FIVE times.
⬇
Add 100 mcl of TMB Substrate Solution to each well.
⬇
Develop the plate in the dark at room temperature for 10-13 minutes.
⬇
Stop reaction by adding 100 µl of Stop Solution to each well.
(*30ml DI water + 10 ml HCL, good for 5 plates*)
⬇
Measure absorbance on a plate reader at 450 nm.

STD	ng/ml	RS10-110-4 (22 mg/ml Albumin)	Sample Diluent
Initial	10,000	5 µl	11 ml
1	400	100 µl from initial	2.4 ml
2	200	500 µl from std 1	500 µl
3	100	500 µl from std 2	500 µl
4	50	500 µl from std 3	500 µl
5	25	500 µl from std 4	500 µl
6	12.5	500 µl from std 5	500 µl
7	6.25	500 µl from std 6	500 µl
8	0	Blank	500 µl

3.5. Lactate

Lactate Reagent: Trinity Biotech Cat# 735-10
(Standard Solution: 0.018 g Lactic Acid + 20 ml DI Water)
(Dilute samples in water as 1:10)

Add 10 ml of DI Water to lactate Reagent.
⇓
Add 250 µl Reagent to each well.
⇓
Add 2.5 µl of standard or sample.
⇓
Centrifuge for 1min at 1000 RPM.
⇓
Incubate at room temperature for 10 minutes.
⇓
Measure absorbance on a plate reader at 595 nm.

STD	mM	Lactic Acid	DI Water
Initial	10	0.018 g	20 ml
1	5	50 µl from initial	50 µl
2	2.5	50 µl from std 1	50 µl
3	1.25	50 µl from std 2	50 µl
4	0	0	50 µl

3.6. Glucose

Glucose Reagent : Sigma Cat # G3293-20
(Standard Solution: 25 mg D-Glucose + 10ml DI Water)
(Reconstitute the reagent with DI water)
(Dilute samples and media in water as 1:10)

Add 20 ml of DI water to Glucose Reagent and mix well.

⬇

Add 200 µl of Reagent to each well.

⬇

Add 10 µl of standard or sample.

⬇

Centrifuge for 1 min at 1000 RPM.

⬇

The most common radicals in biological systems are oxygen.

⬇

Measure absorbance at 340 nm.

STD	mg/ml	D-Glucose	DI Water
Initial	2.5	25 mg	10 ml
1	1.25	50 µl from initial	50 µl
2	0.625	50 µl from std 1	50 µl
3	0.3125	50 µl from std 2	50 µl
4	0	0	50 µl

Convert mg/ml to mM:

2.5 mg/ml * 1g/1000mg *1/180.2 mol/g * 1000 mmol/mol *1000 ml/L = 13.8724 mmol/L

3.7. Glutamine/Glutamate

Glutamine/Glutamate : Sigma Cat# GLN-1
Acetate buffer: Cat # A-4433
ADP: Cat # A-4558
L-glutamine: Cat # G-6275
L-glutamic acid: Cat # G-6150
Glutamic dehydrogenase: Cat # G-5900
Glutaminase: Cat # G-8880
Hydrazine hydrate: Cat # 207942
NAD: Cat # N-9268
Tris-EDTA: Cat # T3161

(Reconstitute G-6275 , G-6150, N-9268, A-4558 with DI water)

(Dilute A-4433 in water as 1:10 and use 5ml of this to dissolve G-8880)

(Add 1ml of 207942 to 19ml of Tris or add 3ml to 57ml)
(Aliquot reconstituted materials and stor at -20°C for 60 days)

(Dilute samples in water as 1:10)

Reaction A:

Consider two plates as GLN and GLU.
⬇
Add 40 µl of Acetate buffer to both plates.
⬇
Add 20 µl of glutaminase to GLN plate.
⬇
Add 140 µl of STD-GLN and 160 mcl of STD-GLU
and
50 µl of sample to both plates.
⬇
Centrifuge at 1000 RPM for 1 min.
⬇
Incubate at 37°C for 60 min.
⬇

Reaction B:

Consider two more new plates as GLN and GLU, then add
150 µl of Tris-EDTA-hydrazine, 15 µl of NAD, 2 µl of AADP
to both plates.
⬇
Add 75 µl of reaction A
⬇
Centrifuge at 1000 RPM for 1 min.
⬇
Measure absorbance at 340 nm (A1).
⬇
Add 3 mcl of glutamate dehydrogenase.
⬇
Centrifuge at 1000 RPM for 1 min.
⬇
Incubate at 37°C for 40 min.
⬇
Measure absorbance at 340 nm (A2).

Calculation:

GLN plate is actually gives concentration of glutamine + glutamate, and GLU plate gives the concentration of glutamate. A2 – A1 for both STD and samples gives the concentration of glutamine.

STD	mM	Glu- Cat#6150	DI Water (µl)
Initial	1	500 µl	0
1	0.5	500 µl from initial	500
2	0.25	500 µl from std 1	500
3	0.125	500 µl from std 2	500
4	0.0625	500 µl from std 3	500
5	0	0	500

STD	mM	Gln- Cat#6275	DI Water (µl)
Initial	1	500 µl	500
1	0.5	500 µl from initial	500
2	0.25	500 µl from std 1	500
3	0.125	500 µl from std 2	500
4	0.0625	500 µl from std 3	500
5	0	0	500

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