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for the Degree of Master of Science

Ketogenic diet deteriorates the symptom of endotoxemia in mice

저탄수화물 고지방 식이가 마우스 내독소혈증에 미치는 영향

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지도교수 윤철희
이 논문을 농학석사학위논문으로 제출함

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Abstract

The present study focuses on the correlation between ketogenic diet composed of low carbohydrate with high fat and endotoxemia causing the acute immune response. There has been an increasing trend to lose body weight by consuming a low carbohydrate with high fat diet, but it is not clear how the immune responses in body change accordingly. Thus, the consequences of an acute immune response when ingesting a ketogenic diet in mouse with endotoxemia were examined in this study.

When endotoxemia was induced in mice after feeding a ketogenic diet for 7 days, functional impairment in lung, kidney, and liver, and over-expression of circulating TNF-α and IL-10 were observed than those of endotoxemic mice fed with control chow diet. Also, ingestion of the ketogenic diet in mice for 7 days caused an increased expression of LPS receptors (TLR4 and CD14), high mobility group box 1 (HMGB1) and plasminogen activator inhibitor-1 (PAI-1) in liver, kidney, lung, and heart at different degree without LPS treatment.

It was intriguing that mortality rate in mice fed with ketogenic diet was significantly higher than mice fed with chow diet suggesting that unbalanced nutrition under certain condition like endotoxemia or sepsis may lead host to be

more vulnerable. Therefore, a ketogenic diet used as a prophylactic or therapeutic purpose for endotoxemia or sepsis may be inappropriate.

Keywords : Ketogenic diet, Endotoxemia, LPS, TNF-α, Organ dysfunction

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List of Abbreviations

AP-1	Activator protein 1
BMDM	Bone-marrow derived macrophage
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
DAMP	Damage associated molecular pattern
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
HMGB1	High mobility group box 1 protein
HRP	Horseradish peroxidase
IFN	Interferon
IL	Interleukin
IRAK	Interleukin 1 receptor associated kinase
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein 1
M-MLV	Moloney murine leukemia virus (Reverse transcriptase)
MyD88	Myeloid differentiation primary response gene 88
NAFLD	Non-alcoholic fatty liver disease
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP	Nod-like receptor pyrin domain containing protein

PAI-1	Plasminogen activator inhibitor 1
PAMP	Pathogen associated molecular pattern
PBST	Phosphate-buffered saline with Tween 20
RAGE	Receptor for advanced glycation end products
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
TF	Tissue factor
TIR	Toll/interleukin 1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
TRIF	TIR domain-containing adaptor inducing IFN-β

I. Review of Literature

The contents herewith will be published elsewhere

as a partial fulfillment of Woo-Jin Son's M. Sc. program

1. Endotoxin

1.1 Characteristics

Endotoxin, also known as lipopolysaccharide (LPS), is molecule consisting of lipid A and a polysaccharide composed of O-antigen and found in the outer membrane of gram-negative bacteria. Among them, lipid A has toxicity.

1.2 TLR4 signaling pathway

Toll-like receptors (TLRs) are a group of receptors sensing pathogen-associated molecular pattern (PAMP), that mostly expressed in innate immune cells such as macrophages and dendritic cells. TLRs can be divided into two sub-family groups depending on whether they are located within intracellular or cell surface. Among diverse TLRs, TLR4 is mostly present on the surface of innate immune cells.

Bacterial LPS is known to be specifically recognized by TLR4 and activates innate immune responses. TLR4 signaling pathway is boosted by producing inflammatory cytokines from innate immune cells [1].

Once activated, TLR4 recruits and interacts with its downstream Toll-interleukin-1 receptor (TIR) domain containing adaptors such as myeloid differentiation primary response gene 88 (MyD88) and TIR domain-containing adaptor inducing IFN-β (TRIF). In other words, TLR4 signaling pathway can be divided into MyD88-dependent and MyD88-independent (TRIF-dependent). MyD88 recruits IL-1 receptor-associated kinases (IRAKs) and TNF receptor-associated factor 6 (TRAF6) to activate some transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein 1 (AP-1) which induce expression of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α. Whilst, TRIF recruits TRAF3 to activate IRF3, NF-κB and AP-1 which induce the secretion of type 1 interferons [2].

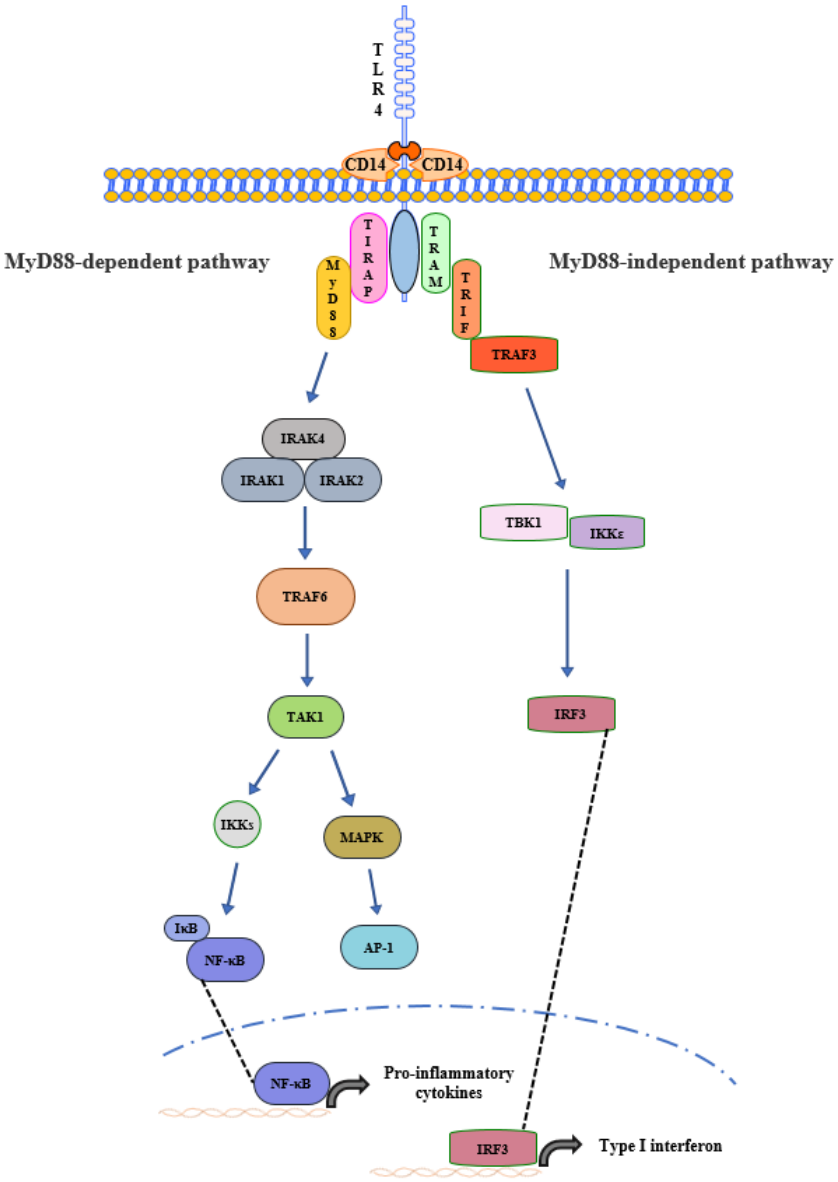


Figure 1. TLR4 signaling pathway.

In response to LPS, signal transduction occurs in MyD88-dependent and -independent (TRIF-dependent) pathways.

1.3 CD14

CD14, along with TLR4, is a co-receptor that recognizes and delivers LPS to TLR4 [3]. So, CD14 increases downstream signaling strength of LPS. CD14 is expressed mainly on macrophages, but moderated expression on neutrophils and dendritic cells. Activated macrophages and monocytes secrete inflammatory molecule, high mobility group box 1 protein (HMGB1) which translocates from the nucleus to the cytoplasm during inflammation like endotoxemia or sepsis through a CD14-dependent mechanism in mice [4]. CD14 is also involved in LPS-induced organ dysfunction. For instance, lung histopathology is alleviated in $CD14^{-/-}$ mice with LPS-induced lung injury compared to normal mice [5].

1.4 Endotoxemia

Borden and Braude firstly proposed endotoxin as a tool causing human gram-negative shock [6, 7]. Although there is concern that data obtained using LPS induced mouse sepsis models may not be applicable to human disease because of differences in sensitivity to LPS between mice and humans, LPS as a single component is a widely used microbial mediator to induce acute systemic pathogenesis in rodent model [8]. Such sepsis-like systemic inflammation induced by LPS is called ‘endotoxemia’.

LPS is one of the PAMPs foreshadowing the threat of gram-negative bacterial invasion into the internal environment. Sudden spread of a large amount of LPS into the body causes the release of inflammatory mediators including cytokines and coagulation promoting factors [9] that are clearly harmful to the host resulting in multi-organ dysfunction. Multi-organ dysfunction, if severe, by excessive immune responses can lead to host death.

1.4.1. Major cytokines in endotoxemia

Endotoxemia causes immune cells in the host to secrete various cytokines such as TNF-α, IL-1β, and HMGB1, representative effector molecules that cause inordinate responses exacerbating symptom of endotoxemia such as inflammatory cell death, organ dysfunction. Excessive production of these molecules causes a hyper-immune response beyond the normal adjustable level, leading to pathological inflammatory disorders. Both TNF-α and IL-1β are best-known cytokines released early time point during the course of endotoxemia [10]. They are observed both in blood from septic patients and in experimental animal models. TNF-α is produced mainly by macrophages, but also by various cells such as dendritic cells, natural killer cells, neutrophils and epithelial cells. High concentration of TNF-α induces cell death like apoptosis [11], pyroptosis [12]

and increases the production of IL-1β and IL-6. TNF-α induces a strong activation of NF-κB signaling pathway through the synergy with IFN-γ in endotoxemic mice [13]. IL-1β is an important mediator of the inflammatory response involved in a variety of cellular activities including cell proliferation, differentiation and apoptosis. IL-1β is also produced by various cells such as macrophages, natural killer cells and neutrophils, as an inactive precursor form called pro IL-1β. Before its releasing, inflammasomes cause the processing of pro IL-1β to a mature and active form [14].

Activation of caspase-1 and Nod-like receptor pyrin domain containing protein 3 (NLRP3) inflammasomes can be seen before HMGB1 release from LPS-primed murine macrophages in endotoxemia [15]. HMGB1, known as a late mediator of endotoxemia, accumulates in serum a few hours after the induction of endotoxemia [16]. HMGB1 has been reported as an important chromatin protein like histone. It enhances the transcription of many genes by interacting with nucleosomes, transcription factors, and histones. However, if inflammation occurs, HMGB1 is secreted by various cells such as macrophages, monocytes and hepatocytes. Its extracellular form binds to receptor for advanced glycation end-products (RAGE) and TLR4 [17]. HMGB1 increases IL-6, TNF-α and mortality in endotoxemic mice in a RAGE-dependent manner [18]. Interacting HMGB1 with TLR4 upregulates NF-κB signaling pathway which increases

production of diverse cytokines [19]. It is also known that HMGB1-LPS complex mediates caspase 11-dependent pyroptosis resulting increase of mortality in endotoxemia models [20].

IL-10 is also important cytokine in endotoxemia. Early produced IL-10 in endotoxemic mice suppresses excessive immune response and increases the survival rate of the host, but continuous expression of IL-10 induces immunoparalysis and has a detrimental effect on the host [21, 22].

1.4.2. Multi-organ dysfunction in endotoxemia

Multi-organ dysfunction, the final stage of severe systemic inflammation, refers to a condition that two or more organs in the body stop functioning properly or become severely dull. Endotoxemia can induce disseminated intravascular coagulation in which forming a microvascular clot, leading to multi-organ dysfunction [23, 24]. The main cause of disseminated intravascular coagulation is PAI-1 produced by hepatocytes, adipocytes, and vascular endothelial cells, which acts to prevent the degradation of fibrin produced during blood coagulation in endotoxemia [23].

Likewise, an uncontrolled excessive inflammatory reaction and tissue damage, is progressed by combination of endotoxemia, sepsis, surgery, metabolism and

drug overdose, leading to local and systemic reactions resulting lung dysfunction, kidney dysfunction, liver dysfunction.

Lung dysfunction by endotoxemia may lead to either rapid breathing (dyspnea) or short breathing (tachypnea) due to alveolar septal thickening and interstitial edema, causing abnormal ventilation [25]. Kidney dysfunction by endotoxemia induces an accumulation of urea or nitrogen-containing substances in the bloodstream [26], causing fatigue, loss of appetite, and nausea. In addition, symptoms of tubular damage include tubular degeneration, vacuolization and dilatation [27, 28]. Liver dysfunction by endotoxemia causes fatal hepatocellular necrosis since liver plays a central role in the synthesis of most, if not all, clotting factors and some inhibitors of clotting and fibrinolysis [29]. Furthermore, severe platelet dysfunction like thrombocytopenia also can be developed. In addition, excessive fat accumulation in liver called non-alcoholic fatty liver disease (NAFLD) deteriorates the symptom of liver dysfunction in endotoxemia [30-32].

2. Ketogenic diet

2.1 Characteristics

The ketogenic diet is defined as a very low in carbohydrates and very high in fat. Because of its very low carbohydrate content, ingesting ketogenic diet will prevent a body from maintaining a glucose at normal level and energy is produced mainly through utilizing fatty acids. Then, ketone bodies are formed through the fatty acid oxidation (β-oxidation) process in liver. When free fatty acids are oxidized in the mitochondria matrix, acetyl-CoA is produced, and the acetyl-CoA forms ketone bodies (acetone, acetoacetate and β-hydroxybutyrate). Ketone bodies are transported and used at various tissues. β-hydroxybutyrate is converted to acetoacetate and becomes acetyl-CoA as an energy source through acetoacetyl-CoA by succinyl-CoA:3-oxoacid-CoA transferase and thiolase in turn [33]. β-hydroxybutyrate and acetoacetate can be converted to each other through a reversible dehydrogenase reaction by β-hydroxybutyrate dehydrogenase, and then acetone is excreted out of the body through urine or ex halation (Figure 2).

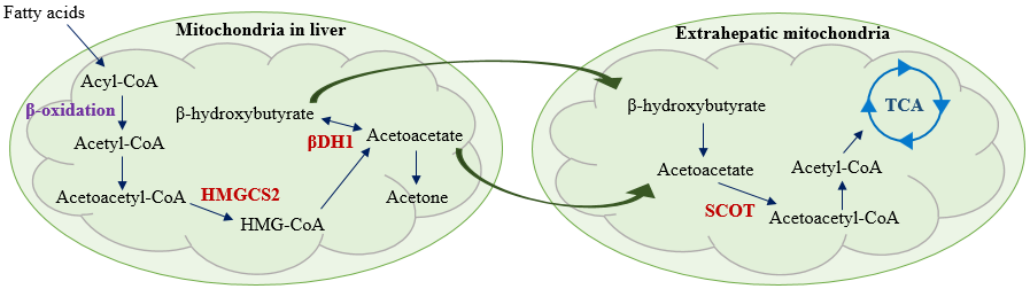


Figure 2. The process of ketone formation.

Ketones (acetoacetate and β-hydroxybutyrate) produced in liver mitochondria move to other tissues and become energy sources. (HMGCS2, 3-hydroxymethylglutaryl-CoA synthase, βDH1, β-hydroxybutyrate dehydrogenase, SCOT, succinyl-CoA:3-oxoacid-CoA transferase)

2.2 Relationship between ketogenic diet and immune system

Several studies have been reported on the correlation between ketogenic diet and immunity. It was noting from numerous studies on the correlation between ketogenic diet and immunity that ketogenic diet induces a pro-inflammatory immune response while others suggesting an anti-inflammatory immune response. Therefore, there is a room for more information on the effects of the ketogenic diet together with its metabolites on the immune system.

2.2.1. Pro-inflammatory effect of ketogenic diet

β-hydroxybutyrate, the final metabolite of ketogenic diet, is known to activate NF-κB signaling pathway in calf hepatocytes [34]. NF-κB signaling pathway increases the expression of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β. It has been suggested that β-hydroxybutyrate induces hepatocyte injury by increasing the expression of TNF-α, IL-6 and IL-1β through NF-κB signaling pathway and proportion of phosphorylated IκB increased through treatment of β-hydroxybutyrate dose dependently (1.2 mM - 2.4 mM) in calf hepatocyte [34].

In mouse study, mice fed with ketogenic diet for 12-week exhibited liver injury, accumulation of monocytes and macrophages in liver. In addition, liver from mice fed with ketogenic diet also exhibited pyknotic hepatocytes, indicative of apoptosis than liver from mice fed with high fat diet or chow diet [35]. According to this study, while small and large droplets macrovesicular steatosis were seen in both livers of mice fed with high fat diet or ketogenic diet, there was no evidence of inflammation, apoptosis in the liver from mice fed with high fat diet, but inflammatory foci and apoptosis were clearly observed in the liver from mice fed with ketogenic diet [35]. These findings suggest that the intake of a ketogenic diet can create an environment that is more prone to inflammation in our body than when a ketogenic diet is consumed.

There have also been cases reported in human cells for the pro-inflammatory effect of ketones. Caspase 1 activation in LPS-stimulated $CD14^+$ human monocytes and granulocytes from human who had fed β-hydroxybutyrate was increased whereas those cells from human who had fed placebo supplement was unchanged [36]. IL-6 involved in increasing tissue factors (TF) that bring about initiation of coagulation [37, 38] was also increased in supernatant from those LPS stimulated $CD14^+$ human monocytes and granulocytes. IL-6 is also produced by macrophages, fibroblasts, endothelial cells, et cetera during the initial stage of inflammation induced by LPS, bacteria, cytokines such as TNF-α and IL-1β and it moves to the liver through the bloodstream followed by the producing a large quantity of acute phase proteins [39]. Increasing complex IL-6/soluble IL-6R by high concentration of IL-6 is implicated in the induction of thrombosis leading to multiple organ dysfunction and disseminated intravascular coagulation in endotoxemia or sepsis [40].

2.2.2. Anti-inflammatory effect of ketogenic diet

It has been reported that β-hydroxybutyrate suppresses the activation of NLRP3 inflammasome in bone marrow-derived macrophages (BMDMs), and NLRP3-mediated IL-1β and IL-18 secretion in $CD14^+$ human monocytes [41]. In NLRP3-

mediated disease in mouse model, both β-hydroxybutyrate and ketogenic diet attenuate caspase-1 activation and IL-1β production. Additionally, β-hydroxybutyrate blocks the secretion of IL-1β in neutrophils through inhibiting NLRP3 inflammasome [42].

The above studies are about how a ketogenic diet attenuates the immune response as well as the protective effect of ingestion of a ketogenic diet in disease models has been reported. Increased numbers of γδ T cells in the lung protect the influenza virus infection in mice fed with ketogenic diet through maintaining the barrier integrity in airway [43]. In addition, there have also been reported cases of benefit by ketone on the memory effect. Mice fed with chow diet and intraperitoneally injected with β-hydroxybutyrate (200 mg/kg) every day exhibited enhanced memory effect of $CD8^+$ T cells through upregulating absolute number of $CD8^+$ T cells and expression of transcription factors related with memory function such as Tcf7, Lef1 and Bcl6 when mice infected with *Lm*-OVA [44].

3. Diet and inflammation

3.1 Nutritional immunology

Appropriate supply of nutrients is essential for maintaining homeostasis and performing respective functions in the host. As public awareness and interest in the relationship between diet and immunity increased, researches in the field of ‘nutritional immunology' has become active. The nutritional status of an individual is important to regulate susceptibility of the immune system against infection and diseases [45, 46]. In line with this, to know the metabolic processes during the processes of infection and diseases would provide new prophylactic or therapeutic approaches. So, one of the main goals of the nutritional immunology is to explore which dietary elements are best for a given condition and how they should be consumed [47].

3.2 Diet and chronic inflammation

A well-known example of the effect of diet is that an excessive fatty tissue stored in the body is one of major cause of inflammation. Obese condition promotes the risk of many chronic diseases. Changes in intestinal microbiota induced by high fat diet are known to cause development of chronic systemic inflammatory condition. Higher relative abundance of the Firmicutes (Mollicutes) including obligate parasites, Mycoplasma and lower relative abundance of the

Bacteroidetes in cecal community show in mice fed with high fat diet compared to mice fed with chow diet [48]. Also overall, diversity of the cecal bacterial community is reduced in mice fed with high fat diet [48]. It has been reported that individuals with high bacterial abundance are associated with less severe adiposity and inflammation than individuals with low bacterial abundance [49].

Moreover, changes in gut microbiota populations cause activation of TLR4 signaling pathway, leading to circulation of LPS throughout the body by increased intestinal permeability [50]. Increased release of LPS or free fatty acids (FFAs) promotes production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α in the gut [51, 52]. Pro-inflammatory cytokines and free fatty acids at high concentration in the systemic circulation also lead to systemic inflammation [53]. Such inflammatory conditions induced by high fat diet induce the differentiation of monocytes to M1 macrophages, inflammatory phenotype which produce pro-inflammatory cytokines [50, 54]. Additionally, high concentration of pro-inflammatory cytokines in the liver and free fatty acids released from gastrointestinal tract lead to hepatic and systemic inflammation [55]. High fat diet induced systemic low grade inflammatory conditions could develop many chronic diseases such as cardiovascular disease [56], intestinal disease [57, 58], kidney disease [59], type 2 diabetes [60].

Likewise, because of these negative effects of the high fat diet on the immune response, studies on other diets that can replace the high fat diet are being conducted, and one of them is the ketogenic diet. Glycemic control is improved by ketogenic diet in patients suffering obesity and type 2 diabetes, but the additional studies are still required to identify metabolic and immune consequences of ketogenic diet [61]. Effects of a ketogenic diet on the microbiome have also been reported. Relative abundances of Bifidobacterium and Lactobacillus in the gut are reduced in mice fed with ketogenic diet compared to mice fed with high fat diet and reduced bifidobacterial growth by ketogenic diet leas to reduction of intestinal Th17 cell accumulation in mice [62].

3.3 Diet and acute inflammation

The relationship between diet and acute immune response has been mainly explored in endotoxemia or sepsis models. Research on diet and acute immune response pays more attention to clinical evidence directly related to host survival such as excessively produced cytokines and rapidly damaged organ, mainly caused by innate immune cells, rather than how the chronically progressive inflammatory baseline environment is established in our body.

Expression level of IL-6 and macrophage chemoattractant protein-1 (MCP-1) in liver was enhanced in high fat diet fed obese mice with sepsis compared to

sham group [63, 64]. And mice fed with a high fat diet had greater damage with higher lipid accumulation to the hepatocytes and hepatocellular ballooning than those of sham group [65]. Endotoxemic mice fed with high fat diet show higher mortality than endotoxemic mice fed with standard fiber-rich chow diet [66].

And the high fat diet induced systemic condition, chronic metabolic inflammation (metaflammation). This metaflammation showed that the expression of IL-6 and TNF-α after endotoxemia was achieved only to a similar degree to those from mice fed with chow diet [66]. This suggests that immunoparalysis is induced after induction of endotoxemia when the mice consumed a high fat diet. The immunoparalysis is also judged by quantifying the ratio of IL-10 and TNF-α in the blood. It is intriguing that this phenomenon is a result that occurs independently of the microbiome because endotoxemia induced in germ-free mice fed with high fat diet shows similar results to those in wild type mice although changes in the gut microbiome increased inflammation in the previous study on chronic diseases affected by a high-fat diet [48].

With a leap of nutritional immunology, more research is needed to determine whether the effects of various diets such as ketogenic diet on acute immune responses are different from those of high fat diet. One research investigated endotoxemic mice fed with ketogenic diet in the post-prandial state (2 h fasting before LPS treatment) and the post-absorptive state (14 h fasting before LPS

treatment) considering the effect of free fatty acids on glucose level in blood [67]. This study reported that endotoxemic mice fed with ketogenic diet exhibited less hypoglycemia postprandially and reduced expression of TNF-α and IL-6 postabsorptively, with reduced hepatic NF-κB expression in both dietary periods compared to mice fed with chow diet. These results suggest that ketogenic diet may alter acute immune response in mice.

Since proper nutritional intake is essential for regulating the immune system of our body, a balanced intake of nutrients is recommended, but the nutrients needed by healthy or unhealthy people may vary depending on their body conditions. Therefore, the direction of nutritional immunology should move toward understanding the overall biological system, such as genes, proteins, and their interactions that are epigenetically changed according to the level of nutrient intake. Therefore, the present study will broaden understanding ketogenic diet on nutritional immunology, especially under endotoxemia condition.

II. Introduction

Endotoxemia or sepsis, leading cause of mortality, is a disease that shows a severe immune imbalance leading to an organ dysfunction [68]. Despite several studies and evidence reported on the clinical relevance of sepsis patients in relation to nutrients [69-71], it is not yet clear how the immune response is regulated. There have only been clinical results showing immunosuppressive or deleterious effects in deceased sepsis patients who were supplemented with soy lipid [70, 72] or glutamine [73, 74]. On the other hand, there is insufficient evidence to confirm the effectiveness of the therapeutic effects of nutrients including arginine [75], vitamin C [76], and omega-3 fatty acids [77, 78]. The relationship between diet and acute immune response, as well as various nutrients, is receiving much attention. The negative effects of a high fat diet on endotoxemic mice have been reported [63-66], now the effects of a ketogenic diet should be attempted. An attempt has been made to evaluate whether the ketogenic diet is suitable for therapeutic purposes for patients with sepsis [79]. Unlike chow diet or high fat diet, the ketogenic diet has been characterized by extremely low carbohydrate content and a fat accounting for most of the calorie intake. Therefore, different patterns in the progression of endotoxemia are expected in mice or human fed with ketogenic diet.

The endotoxemia model has been widely applied for sepsis-related research because endotoxin (lipopolysaccharide, LPS) is a substance that can induce sepsis-like symptom. The downstream signaling pathway is transmitted through the receptors, TLR4 and CD14 resulting increase of various cytokines in a MyD88-dependent and -independent manner during endotoxemia, and then cytokine storming leads to multi-organ dysfunction [80].

Therefore, in the present study, to examine the effect of the ketogenic diet on endotoxemia, survival, organ dysfunction, and factors increasing mortality including cytokines were examined in mice fed with ketogenic diet.

III. Materials and methods

1. Animal

Female, 8-week old, C57BL/6 mice were obtained from Orient Bio (Gapyeong, South Korea) and housed in sterilized cages in a controlled environment with a 12 h light-dark cycle. The mice were divided into groups consumed a chow diet (ZeiGLeR, Rodent NIH-41 diet) or a ketogenic diet (Harlan Laboratories, Teklad, TD. 96355) for 7 days, and then used in the experiment. The sodium 3-hydroxybutyrate (Sigma Aldrich, 54965) which was done to mimic feeding ketogenic diet like conditions. All the experimental procedures were carried out in accordance with the Animal Use and Care protocol approved by the Institutional Animal Care and Use Committee at Seoul National University, Seoul, Korea (Approval No. SNU-200617-3)

2. Endotoxemia model

To induce endotoxemia, mice fed with each chow diet or ketogenic diet with 20 to 25 g in body weight were injected intraperitoneally with 200 μl of LPS (4 mg/kg) on day 7. LPS from *Escherichia coli* O111:B4 (Sigma Aldrich, L2630) was used. The mice were monitored five times daily to observe their behavior

including, but not limited to, appearance, activity, and eye condition. Survival data were obtained from three independent experiments.

3. Depletion of phagocytes

200 μl (7 mg/ml) of clophosome® or control liposome (Formumax, F70101C-N) was intravenously injected to experimental group or control group of mice respectively to deplete phagocytes. Then, LPS was treated 24 h after clodronate injection.

4. Enzyme-linked immunosorbent assay

Blood samples were collected by eye bleeding into heparinized tubes. The sera were then collected by centrifugation for 15 min at 12,000 g and stored at -70 ℃, until further use. Serum samples were examined for the production of IL-1β, IL-6, IL-10, IL12p40, IFN-γ and TNF-α using ELISA kits (R&D systems, Minneapolis, MN, USA). 96-well immunoplate (Nunc, Roskilde, Denmark) was pre-coated with 100 μl/well of capture antibody. After blocking with 1 % bovine serum albumin in phosphate-buffered saline for 1h at room temperature, 100 μl/well of serum sample and recombinant each cytokine (included in the kits)

along with reagent diluent buffer was added and incubated for 2 h at room temperature. After the wash using phosphate-buffered saline with tween 20 for three times, 100 µl/well of biotinylated detection antibody was added and incubated for 2 h at room temperature, followed by addition of the Streptavidin-HRP in reagent diluent buffer. After the incubation for 20 min at room temperature, tetra-methyl benzidine (TMB, Millipore) was added and then the reaction was stopped by adding 50 µl of 2 M H_2SO_4. The absorbance at wavelength 450 nm was measured by a microplate reader (Molecular Devices).

5. RNA isolation and RT-PCR

Total RNA was extracted from perfused murine liver, kidney, lung and heart by using TRIzol (Thermo Fisher Scientific) and isolated by adding chloroform followed by centrifugation at 12,000 g for 15 min at 4℃. Then, isopropanol was added and settled for 10 min at room temperature for RNA precipitation. RNA pellet was obtained by washing with 75 % ethanol and air dried for 10-15 min then resuspended with DEPC water (Sigma Aldrich) and quantified with NanoDrop (Amersham Bioscience, USA) at A260/280. cDNA was synthesized with random primers (500 µg/ml), dNTP (10 mM), 5X first strand buffer, DTT (0.1 M), oligo dT and M-MLV reverse transcriptase (Invitrogen, USA). Quantities of all targets in tested samples were normalized to the corresponding

β-actin levels. And Real-time RT-PCR was done with the SYBR Green PCR Master Mix (Applied Biosystems, USA) using StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Relative quantification of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences used for Real time quantitative PCR are shown in Table 1.

Table 1. Primers for real-time RT-PCR.

Gene	Forward (5' → 3')	Reverse (5' → 3')
HMGB1	GGACTCTCCTTTAACCGC	TTGTGATAGCCTTCGCTGGG
PAI-1	GGACACCCTCAGCATGTTCA	TCTGATGAGTTCAGCATCCAAGA
CD14	GAAGCAGATCTGGGGCAGTT	CGCAGGGCTCCGAATAGAAT
TLR4	TCTGGGGAGGCACATCTTCT	AGGTCCAAGTTGCCGTTTCT
β-actin	TGTCCACCTTCCAGCAGATGT	AGCTCAGTAACAGTCCGCCTA

6. Histology

For histological analysis, mice fed with either chow diet or ketogenic diet for 7 days were sacrificed 30 h after LPS injection. Perfused lung, kidney and liver were fixed each with 4 % paraformaldehyde and embedded into paraffin block for hematoxylin and eosin (H&E) staining. The samples were examined under

the light microscopy (Leica Microsystems, Wetzlar, Germany). All clinical symptoms and histological evaluations were performed in a blinded manner.

7. Statistical analysis

The mean value ± standard deviation was determined for each treatment group in a given experiment. All experiments were performed at least three times.

Using Prism 7 (Graphpad), statistical differences were determined using *t*-test.

IV. Results

1. Ketogenic diet increases the mortality in endotoxemia

Whether ketogenic diet could affect the survival of endotoxemia in mice was first examined. Systemic endotoxemia was induced by intraperitoneal injection of LPS (4 mg/kg). After the injection, the condition of the mice was regularly observed five times a day. The mortality was seen in LPS dose-dependent manner (Figure 1A-D). Furthermore, there was a significantly high mortality in mice fed with ketogenic diet. As shown in Figure 1A, the mice fed with chow diet all survived, while 90 % of the mice fed with ketogenic diet died within about 48 h. Based on the survival data, 4 mg/kg LPS showing clear difference was chosen for further experiment.

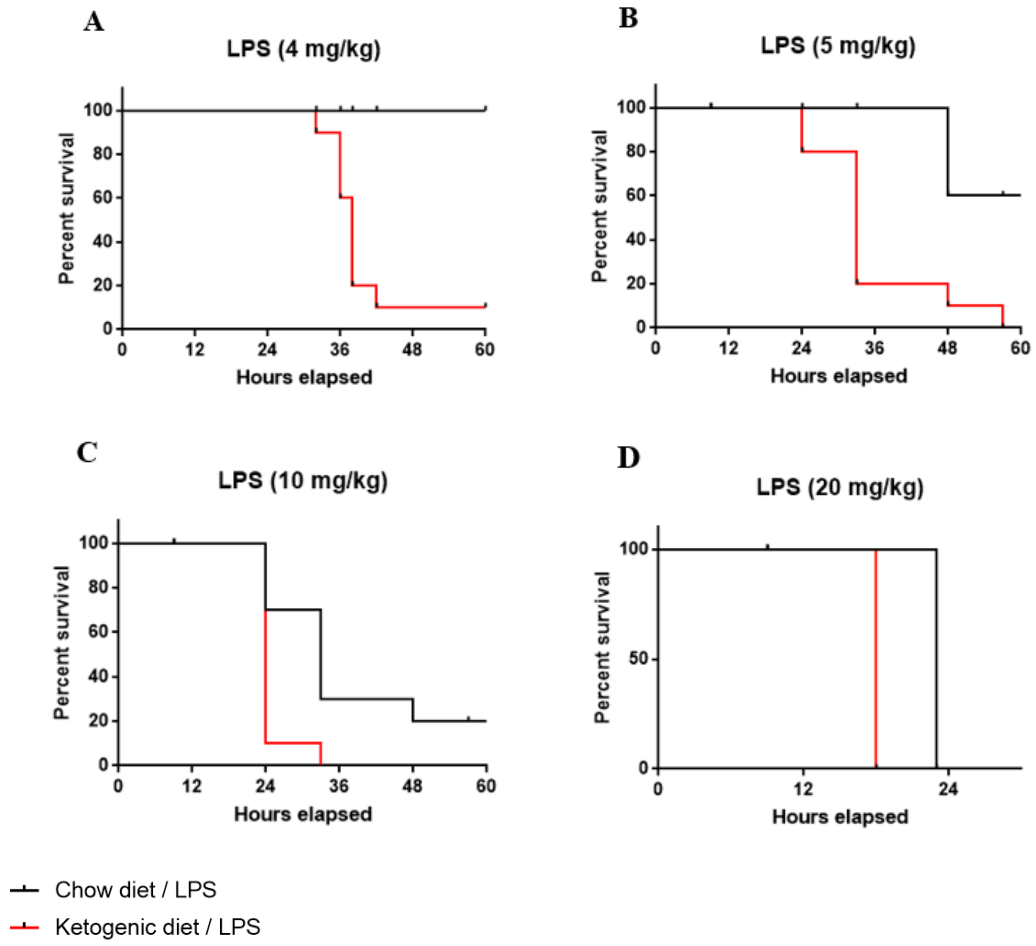


Figure 1. Survival of mice fed with chow or ketogenic diet and then administered with various dose of LPS.

Mice fed with chow or ketogenic diet were injected with 4, 5, 10 or 20 mg/kg of LPS via intraperitoneal route on day 7. The mice were closely examined for their survival (n = 10 per group).

2. Ketogenic diet and LPS impair the function of various organs

Since there were significant differences in survival between mice fed with chow diet and mice fed with ketogenic diet, histology was performed to determine the extent of organ damage to lung, kidney, and liver. In lung, unlike the mice fed with chow diet, mild damage with septal thickening was observed in mice fed with ketogenic diet. In the lung of endotoxemic mice fed with chow diet and endotoxemic mice fed with ketogenic diet, moderate and severe septal thickening was observed, respectively (Figure 2A-2D). Furthermore, enlargement of Bowman's space and tubular dilatation in kidney were observed in endotoxemic mice fed with ketogenic diet unlike other groups (Figure 2E-2H). In liver, hepatocellular ballooning and microvesicular steatosis (33-66%), which are the symptoms of nonalcoholic fatty liver disease (NAFLD), were found in mice fed with ketogenic diet unlike chow diet fed groups. Moreover, hepatocellular ballooning and microvesicular steatosis (>66%) occurred much more seriously in endotoxemic mice fed with ketogenic diet (Figure 2I-2L). NAFLD is known to worsen liver disease in endotoxemia [30, 31]. These results suggest that endotoxemia causes more serious multi-organ dysfunction when the mice fed with ketogenic diet than the mice fed with chow diet.

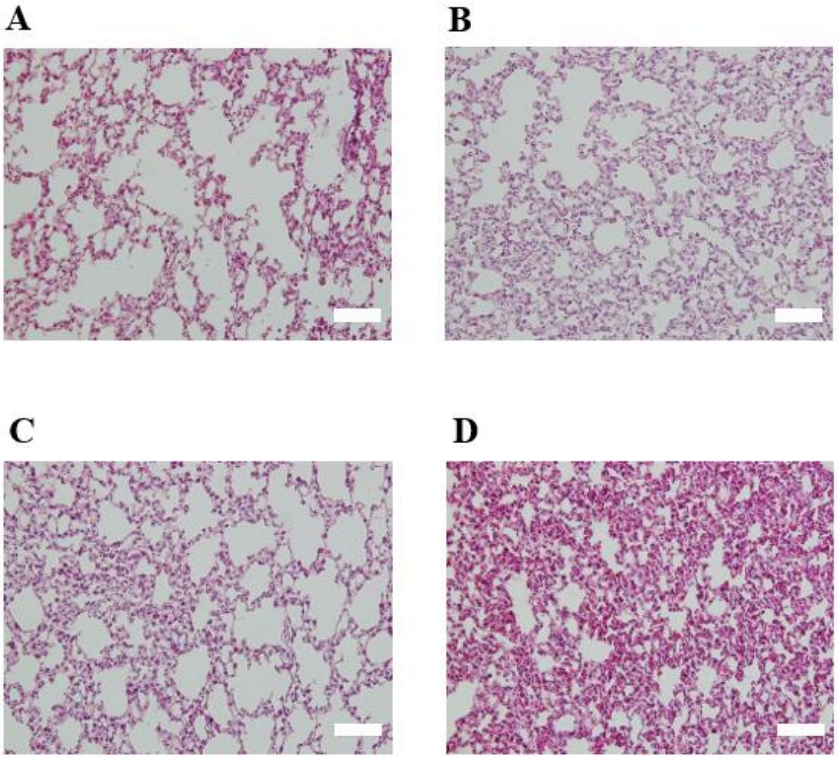
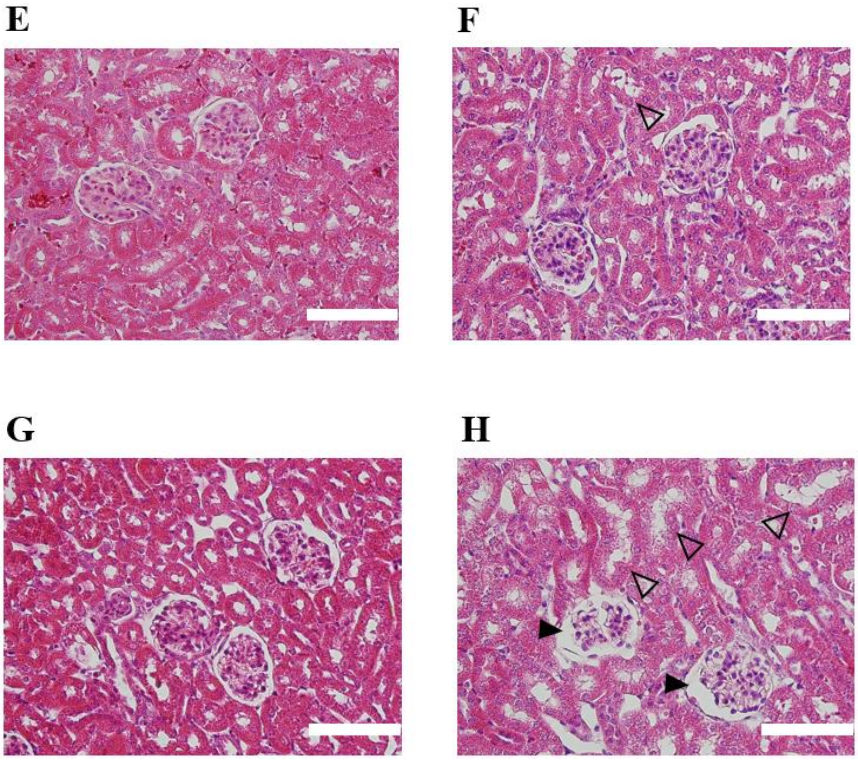


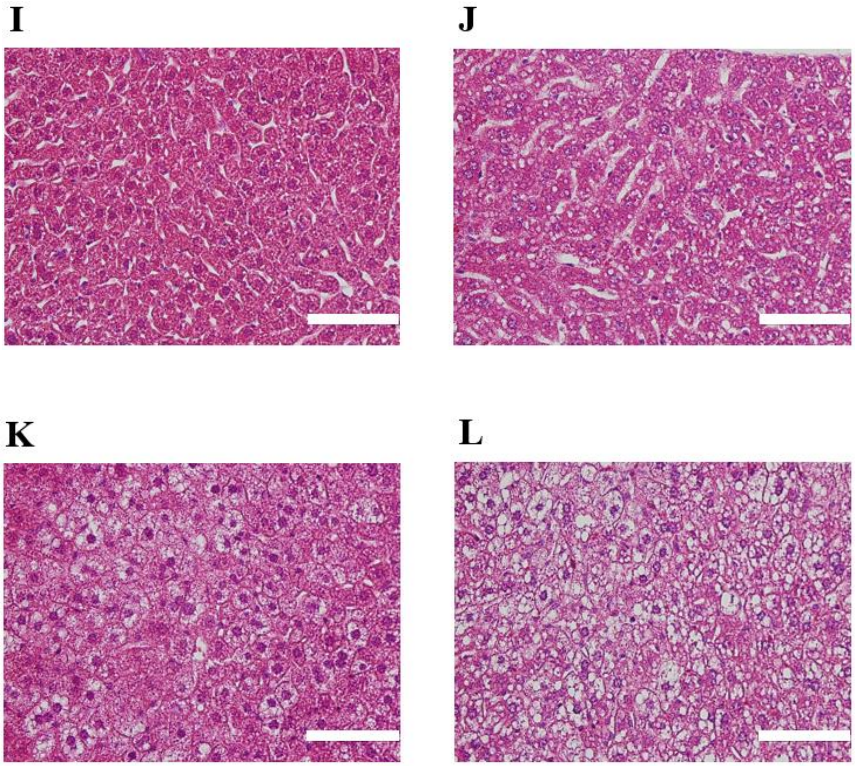
Figure 2. Organ dysfunction in endotoxemic mice fed with chow or ketogenic diet.

Mice fed with chow or ketogenic diet were injected with 4 mg/kg of LPS via intraperitoneal route on day 7. Endotoxemia-induced mice were sacrificed after 30 h, just before the death based on survival data. Perfused lung, kidney and liver were fixed each with 4 % paraformaldehyde and embedded into paraffin block for hematoxylin and eosin (H&E) staining.

Lung of (A) mice fed with chow diet, (B) endotoxemic mice fed with chow diet, and (C) mice fed with ketogenic diet, and (D) endotoxemic mice fed with ketogenic diet. The scale bar indicates 100 μm. The representative pictures are shown from three independent experiments.



Kidney of (E) mice fed with chow diet, (F) endotoxemic mice fed with chow diet, (G) mice fed with ketogenic diet, and (H) endotoxemic mice fed with ketogenic diet. Enlargement of Bowman's space (closed arrowhead) and tubular dilatation (opened arrowhead) are shown. The scale bar indicates 100 μm. The representative pictures are shown from three independent experiments.



Liver of (I) mice fed with chow diet, (J) endotoxemic mice fed with chow diet, (K) mice fed with ketogenic diet, and (L) endotoxemic mice fed with ketogenic diet. The scale bar indicates 100 μm. The representative pictures are shown from three independent experiments.

3. Ketogenic diet affects cytokine expression induced by LPS

Organ dysfunction caused by acute immune responses such as endotoxemia is known to result in excessive cytokine expression [80]. Therefore, the expression of pro-inflammatory cytokines in mice fed with two different diets was examined. To note that blood samples were taken at different time-points and the concentrations of TNF-α, IL-1β, IL-6, IL-12p40 and IFN-γ were compared between the two groups. TNF-α from endotoxemic mice fed with ketogenic diet was expressed about 3-fold higher compared to endotoxemic mice fed with chow diet whereas the expression of other pro-inflammatory cytokines showed no statistically significant differences (Figure 3A). In addition to TNF-α, there was a difference in the expression of IL-10, a representative anti-inflammatory cytokine (Figure 3B). It was decreased in mice fed with chow diet while increased in mice fed with ketogenic diet at 8 h after the induction of endotoxemia. After 12 h, IL-10 and all measured cytokines were gradually decreased and eventually disappear (data not shown). Taken together, these results suggest that excessive expression of TNF-α and IL-10 might have a link to lethal effect in endotoxemic mice fed with ketogenic diet.

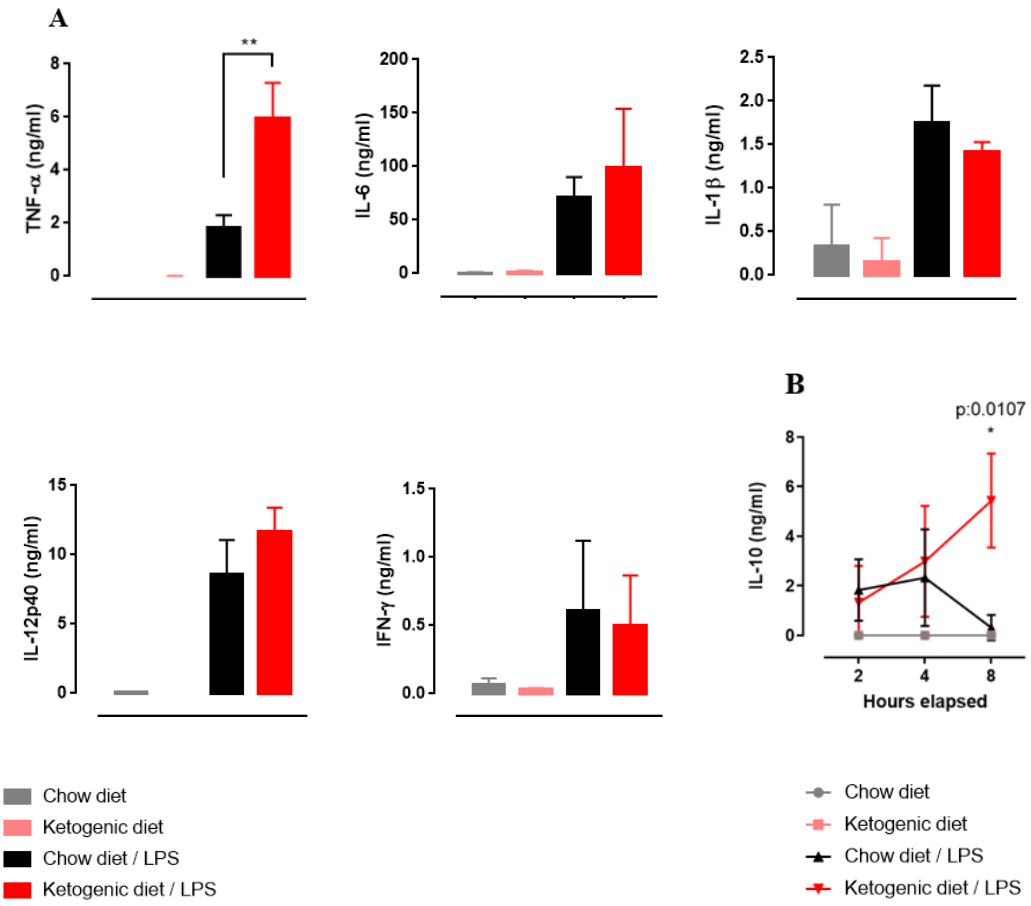


Figure 3. Cytokine expression in endotoxemic mouse fed with chow or ketogenic diet.

Mice fed with chow or ketogenic diet were injected with 4 mg/kg of LPS via intraperitoneal route on day 7. (A) Each cytokine was measured at the time of peak concentration in the blood after induction of endotoxemia (TNF-α: 2h, IL-1β, IL-12p40, IL-6: 4h, IFN-γ: 8h). (B) The IL-10 expression over time. Data were obtained from three independent experiments (n =3 per group).

4. TNF-α produced by macrophages influences survival of endotoxemic mice fed with ketogenic diet

Next, the source of TNF-α was examined. Clodronate is an experimental tool for depletion of phagocytes including macrophages, which are the main source of TNF-α. TNF-α production was 0.8-fold lower in endotoxemic mice fed with chow diet and 0.5-fold lower in endotoxemic mice fed with ketogenic diet by treatment of clodronate (Figure 4A). So, survival rate was examined. Interestingly, the reduction in TNF-α expression increased the survival rate in endotoxemic mice fed with ketogenic diet (Figure 4B). Taken together, these results suggest that the increased TNF-α by macrophages from endotoxemic mice fed with ketogenic diet compared to the mice fed with chow diet makes the mortality rate worse.

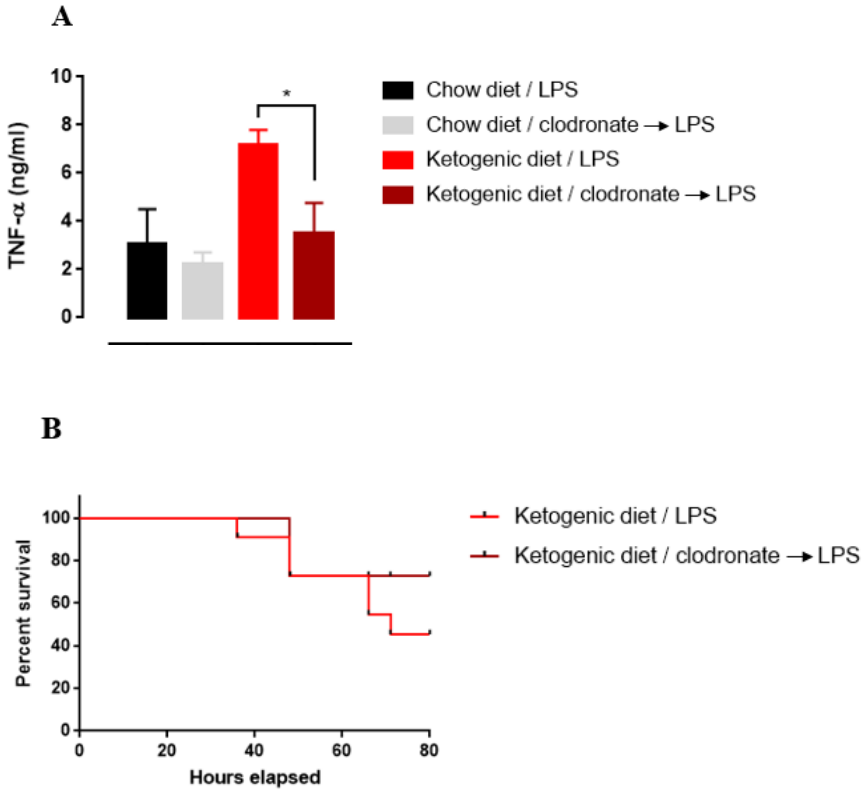


Figure 4. TNF-α expression and survival in endotoxemic mice fed with ketogenic diet were altered by depletion of macrophages.

(A, B) Mice fed with chow or ketogenic diet were treated with 200 μl of clodronate (7 mg/ml) via intravenous route on day 6. 24 h later, the mice were injected with 4 mg/kg LPS via intraperitoneal route. TNF-α expression (n = 3 per group) and survival rate (n = 11 per group) were analyzed.

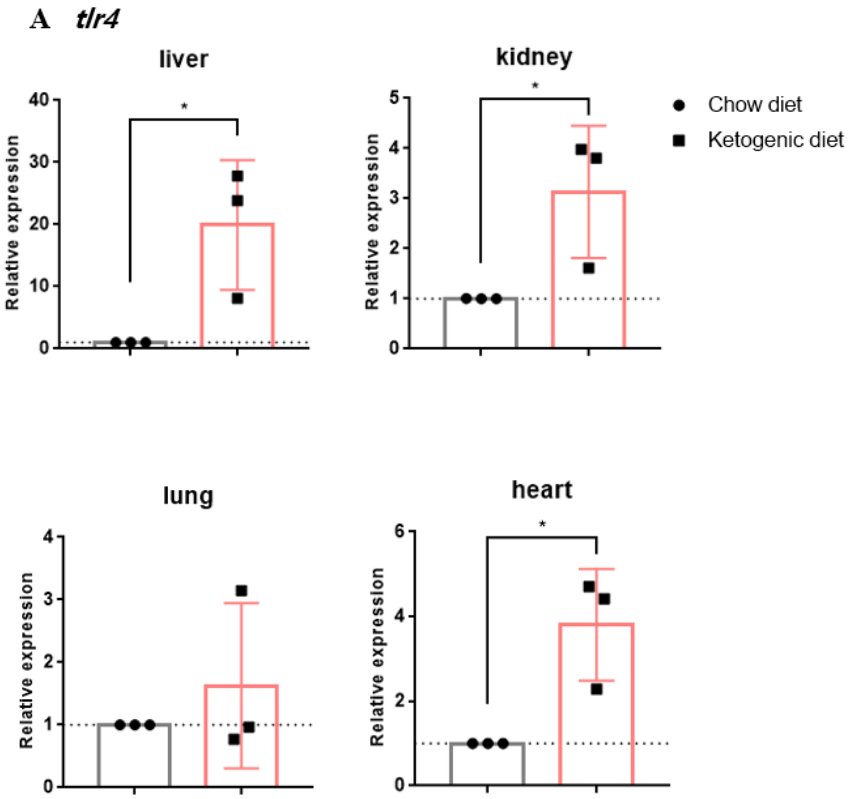
5. Responsiveness to LPS can be increased by Ketogenic diet

Next, whether metabolite of ketogenic diet, β-hydroxybutyrate is the direct factor of death and increased TNF-α and IL-10 in endotoxemic mice fed with ketogenic diet. Mice fed with chow diet were intraperitoneally injected sodium β-hydroxybutyrate (10 mmol/kg) every day to mimic the condition that feeding ketogenic diet. Dose of β-hydroxybutyrate was determined by referring to the results from previous studies [81, 82]. Consequently, none of the β-hydroxybutyrate injected mice fed with chow diet (n =3) died when endotoxemia was induced whereas all endotoxemic mice fed with ketogenic diet (n =3) were died. Therefore, it was determined that β-hydroxybutyrate did not have a direct effect on survival of mice fed with ketogenic diet.

Although it is not yet found what is changed by feeding a ketogenic diet, based on the results of the survival, organ dysfunction, and excessive TNF-α and IL-10 expression, whether the ketogenic diet had an effect on the responsiveness to LPS was investigated. Because, when endotoxemia occurs, LPS spreads throughout the body and causes various cytokine expression and organ dysfunction [23, 25, 27].

Therefore, the expression levels of TLR4 and CD14 in total cells of various organs including liver, kidney, lung and heart were confirmed. TLR4 expression in liver, kidney and heart, and CD14 expression in kidney of the mice fed with

ketogenic diet were significantly increased. The expression of TLR4 was 19.9-fold, 3.1-fold, 3.8-fold higher in liver, kidney and heart, respectively in mice fed with ketogenic diet than mice fed with chow diet (Figure 5A). The expression of CD14 was 4.6-fold higher in kidney in mice fed with ketogenic diet than mice fed with chow diet (Figure 5B). Collectively, these results suggest that overall responsiveness to LPS was higher in mice fed with ketogenic diet than those fed with chow diet.


A tlr4
liver
kidney
lung
heart
Relative expression
Chow diet
Ketogenic diet
*

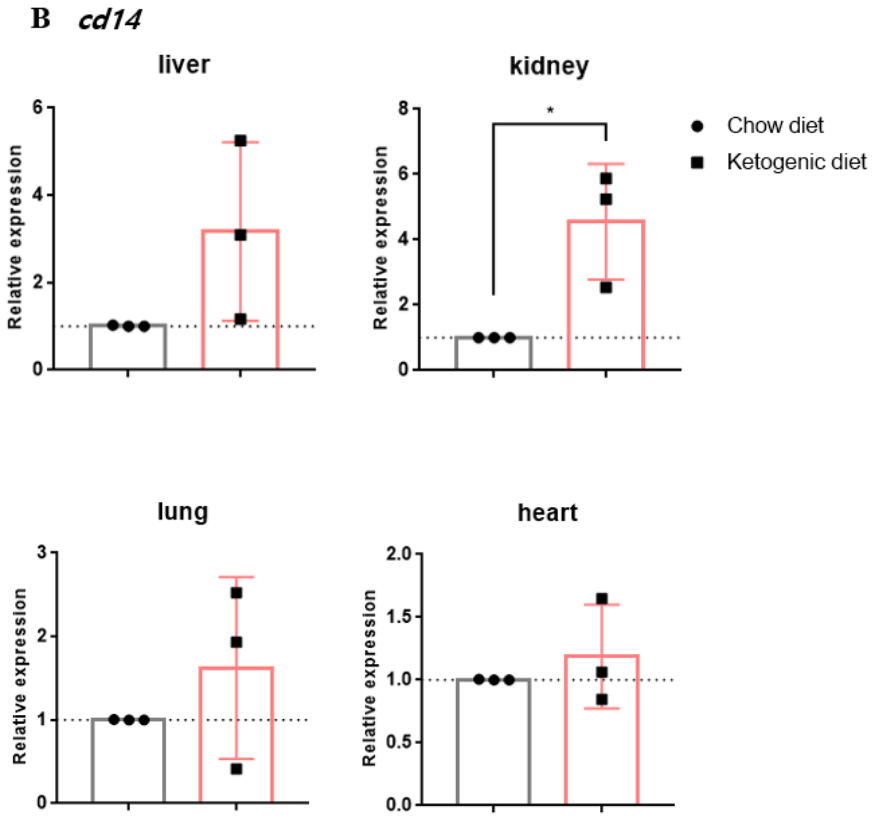


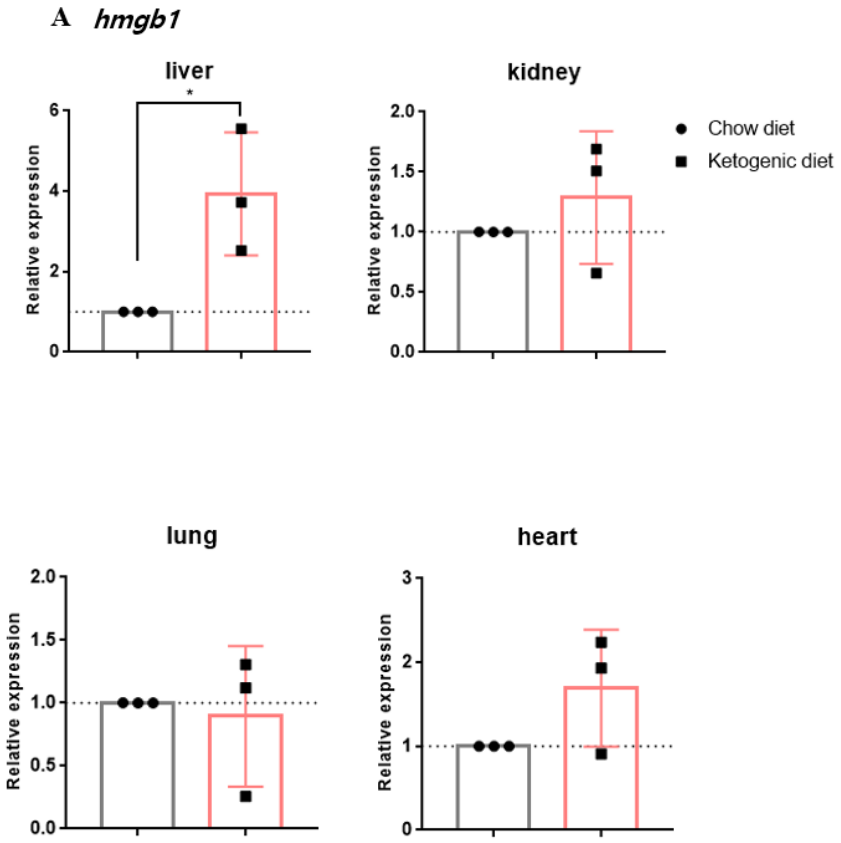
Figure 5. Gene expression of TLR4 and CD14 in liver, kidney, lung and heart of mice fed with chow or ketogenic diet.

Mice fed with chow or ketogenic diet for 7 days were sacrificed. The organs were perfused before harvest for measuring mRNA expression by RT-PCR. Gene expression of (A) TLR4 and (B) CD14 in liver, kidney, lung and heart. Data were obtained from three independent experiments (n =3 per group).

6. Ketogenic diet may increase the mortality in endotoxemia through expression of HMGB1 and PAI-1

Next, the expression of molecules associated with endotoxemia were investigated. HMGB1 is well known to increase mortality of endotoxemia [16]. The main receptor of HMGB1 is RAGE [83], but TLR4 also acts as its receptor [17]. HMGB1 increases IL-6, TNF-α and mortality in endotoxemic mice [18]. A molecule called PAI-1 also significantly affects endotoxemia by causing an inhibition of fibrinolysis [23].

Expression of HMGB1 and PAI-1 was examined in in liver, kidney, lung and heart from mice fed with ketogenic diet. The expression of HMGB1 was 3.9-fold higher in liver in mice fed with ketogenic diet than mice fed with chow diet (Figure 6A). The expression of PAI-1 was 5-fold, and 3.8-fold higher in liver and kidney, respectively in mice fed with ketogenic diet than mice fed with chow diet (Figure 6B). Taken together, these results suggest that HMGB1 and PAI-1, which are highly expressed in liver and kidney of mice fed with ketogenic diet, could have a link with the increased mortality.


A hmgb1
liver
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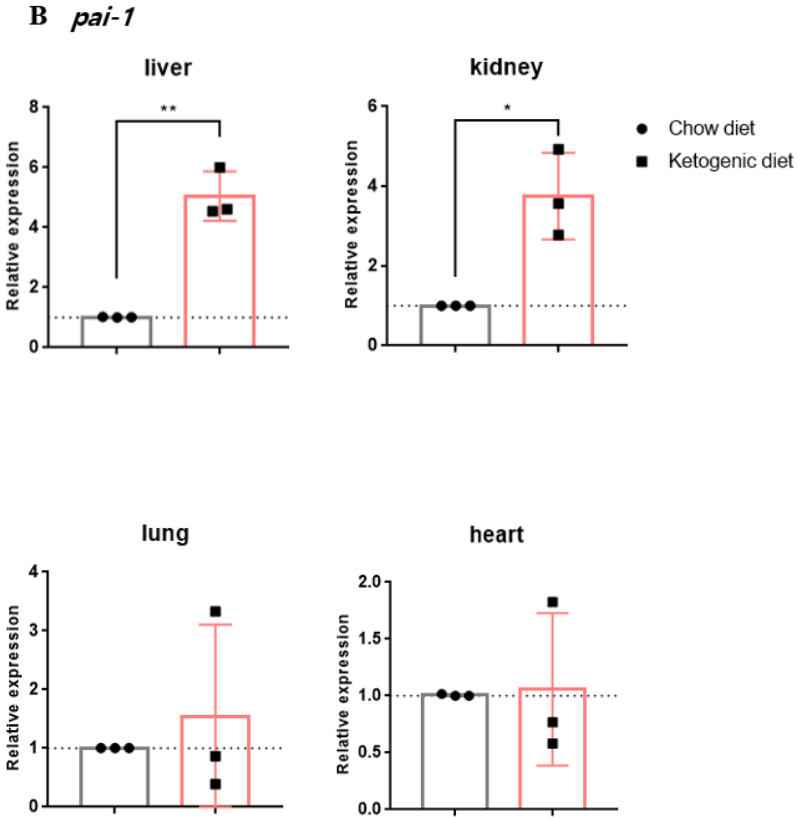

Figure 6. Gene expression of HMGB1 and PAI-1 in liver, kidney, lung and heart of mice fed with chow or ketogenic diet.

Mice fed with chow or ketogenic diet for 7 days were sacrificed. The organs were perfused before harvested for measuring mRNA expression by RT-PCR. Gene expression of (A) HMGB1 and (B) PAI-1 in liver, kidney, lung and heart. The data were obtained from three independent experiments (n =3 per group).

V. Discussion

The ketogenic diet has been characterized by extremely low carbohydrate content with a fat accounting for most of the calorie intake. Mice fed with ketogenic diet shows anti-inflammatory response leading a protective effect against influenza virus [43] or an increase in the memory effect of $CD8^+$ T cells [44]. Other study reported that ketogenic diet induces hepatic inflammation by causing macrophage accumulation in the liver [35]. Therefore, it is likely that the effect of a ketogenic diet on the immune response may vary depending on the environmental factors together with a status of the host.

The present study explored the effect of a ketogenic diet on endotoxemia, which induces an acute immune response. Endotoxemia causes a release of inflammatory mediators including cytokines and coagulation promoting factors [9] that are clearly harmful to the host resulting in multi-organ dysfunction leading to death. In this study, the histology in lung, kidney and liver of endotoxemic mice supports the detrimental effects of a ketogenic diet. In addition, the expression of TNF-α and IL-10 was higher in endotoxemic mice fed with ketogenic diet than those fed with chow diet. TNF-α is generally known as the

early expressed cytokine when inflammation is induced, and mortality is markedly reduced by TNF-α neutralization [13] or blocking TNF-α receptor [84] in the endotoxemia model. IL-10 is also important cytokine in endotoxemia where early produced IL-10 suppresses excessive immune response and increases the survival rate of the endotoxemic mice, whereas continuous expression of IL-10 induces immunoparalysis causing a detrimental effect on the host [21, 22]. Furthermore, reduced expression of TNF-α after the depletion of macrophages enhances the survival of endotoxemic mice fed with ketogenic diet. This finding indicates increased TNF-α in mice fed with ketogenic diet deteriorates the survival of endotoxemic mice. Of course, although this finding is interesting, why the ketogenic diet increased expression of TNF-α and IL-10, excluding other cytokines, needs to be further elucidated. It was noting that the expression of TNF-α in clodronate treated endotoxemic mice fed with ketogenic diet was similar to clodronate untreated endotoxemic mice fed with chow diet, however the survival rates were 73 % and 100 %, respectively. So, it is certain that there are other than TNF-α affecting mortality remains to be explored.

Gene expression of TLR4, CD14, HMGB1 and PAI-1 was higher at different degree in lung, kidney and liver from mice fed with ketogenic diet compared to those fed with chow diet. It is known that activated macrophages and monocytes secrete HMGB1 during endotoxemia or sepsis through CD14-dependent

mechanism in mice [4] and interacting HMGB1 with TLR4 upregulates NF-κB signaling pathway which increases production of diverse cytokines [19]. HMGB1 complexes with LPS to induce caspase 11-dependent pyroptosis in endotoxemic mice [20]. In this study, the gene expression of HMGB1 was higher in the liver from mouse fed with ketogenic diet than those fed with chow diet, so HMGB-1-induced caspase 11-dependent pyroptosis is likely to be a future direction of the study. PAI-1, known to be expressed in various organs of the mouse injected with LPS [85], is fatal by inducing fibrinolysis inhibition in endotoxemic mice [24, 86].

These findings provide important basis that intake of ketogenic diet may cause detrimental immune responses at least in endotoxemia or sepsis in mice. Consequently, this study comprehensively lay the groundwork to enhance our understanding on the effect of ketogenic diet and therapeutic approaches for endotoxemia or sepsis.

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VII. Summary in Korean

본 연구는 저탄수화물 고지방 식단과 급성 면역 반응을 일으키는 내독소혈증과의 상관 관계에 관하여 진행되었다. 최근, 저탄수화물 고지방 식단을 섭취함으로써 체중을 줄이는 추세가 증가하고 있지만 이에 따른 신체의 면역 반응이 어떻게 변화하는지는 명확하게 밝혀지지 않았다. 따라서 본 연구에서는 단기적으로 저탄수화물 고지방 식단을 섭취한 마우스에 내독소혈증을 유도하여 이 식단이 우리 몸에 미치는 영향을 여러가지 면역학적 지표들을 통해 관찰하였다.

7 일 동안 저탄수화물 고지방 식단을 섭취한 마우스의 간, 신장, 폐, 심장에서 일반 식단을 섭취한 대조군 마우스에서 보다 내독소(endotoxin)의 수용체인 TLR4 및 CD14 그리고 HMGB1, PAI-1 의 유전자 수준 발현이 증가하였다. 또한, 저탄수화물 고지방 식단 섭취 후 내독소혈증이 유발되었을 때 대조군 보다 혈중에서 사이토카인 TNF-α 및 IL-10 이 과도하게 생산되었을 뿐만 아니라 간, 신장, 폐 기능 장애가 관찰되었다. 결과적으로, 내독소혈증에 의한 사망률이

대조군에 비해 저탄수화물 고지방 식단을 섭취한 마우스에서 매우 높았다.

따라서 본 연구 결과는 영양소를 균형 있게 섭취하지 못 하는 저탄수화물 고지방 식단은 적어도 내독소혈증이나 패혈증 같은 특정 조건에서 우리 몸을 더 취약하게 만들 수 있으며 내독소혈증이나 패혈증에 대한 예방 및 치료 목적으로의 저탄수화물 고지방 식단은 부적합하다고 창의한다.