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Hepatoprotective effects of fermented wheat germ extract on primary cultured rat hepatocytes

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1. List of abbreviations

FWGE	Fermented Wheat Germ Extract
PPP	Pentose Phosphate Pathway
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
RR	Ribonucleotide reductase
ATP	Adenosine triphosphate
PARP	Poly (ADP-ribose) Polymerase
MHC-1	Major histocompatibility complex 1
NK cells	Natural Killer cells
ICAM-1	Intercellular adhesion molecule 1
TNF-α	Tumour necrosis factor-alpha
SLE	Systemic lupus erythematosus
Th1	Type 1 T helper cell
Th2	Type 2 T helper cell
RA	Rheumatoid arthritis
AA	Adjuvant arthritis
PCR	Real-time polymerase chain reaction
COX	Cyclooxygenase
PG	Prostaglandin
IB	Infectious bronchitis
ND	Newcastle disease
IBD	Infectious bursal disease
ROS	Reactive oxidant species
SOD	Superoxide dismutase
CAT	Catalase
GSH-Px	Glutathione peroxidase
GST	Glutathione S transferase
MDA	Malondialdehyde
FHL	Feline hepatic lipidosis
FFA	Free fatty acids
TG	Triglycerides
SAMe	S-adenosylmethionine
LPS	Lipopolysaccharides
CCH	Canine chronic hepatitis
GSH	Glutathione
EGTA	Ethylene glycol tetraacetic acid
HBSS	Hank's Balanced Salt Solution
BSA	Bovine serum albumin
FBS	Foetal bovine serum
UDCA	Ursodeoxycholic acid
CCK-8	Cell Counting Kit-8
HRP	Horseradish Peroxidase
DMSO	Dimethylsulfoxide
TBA	Thiobarbituric acid
GR	Glutathione reductase

2. Introduction

Fermented wheat germ extract (FWGE) is characterized by various health improving properties and is successfully used both in human and veterinary medicine. It contains safe, naturally occurring compounds derived from wheat. The basic substance is therefore cheap and easy to obtain and is without any known side effects.

FWGE serves as a medical nutriment for human cancer patients under the trade name Avemar. The ingredients 2-methoxy benzoquinone and 2,6-dimethoxybenzquinone seem to play a significant role in exerting its beneficial effects (Mueller & Voigt, 2011). Avemar reduced the energy supply of cancer cells and decreased their rate of nucleic acid production (Boros et al., 2001). It also strengthened the endogenous anti-tumour defence by modulating the immune response observed for instance in leukaemia cell lines (Fajka-Boja et al., 2002). By means of immunomodulation FWGE could also improve the disease outcome of experimental systemic lupus erythematosus in mice (Ehrenfeld et al., 2001). And it enhanced the humoral immune response after vaccination of broilers (Nagy et al., 2011). Furthermore, an anti-inflammatory effect by the inhibition of cyclooxygenase (COX) could be detected by Telekes et al. (2007) in a rat adjuvant arthritis (AA) model. FWGE's ability to modulate and stimulate the immune system, as well as inhibit inflammation, demonstrates its versatility and usefulness in oncological, inflammatory, and immunosuppressive conditions.

In my study I aimed to focus on the effectiveness of FWGE against another important factor in the pathogenesis of diseases, the oxidative stress. Oxidative stress is defined as an imbalance between the concentration of oxidants and the cellular antioxidant defence system. If this ratio shifts in favour of the oxidants, it can lead to impairment of cell function and irreversible cell damage. The term oxidant refers to highly reactive molecules, named reactive oxygen species (ROS), including oxygen-containing free radicals (Winterbourn et al., 2008). The cellular antioxidant defence system consists of enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) evolved to limit the levels of reactive oxidants (Ames et el., 1993). Oxidative stress plays a relevant role in the development of liver diseases (Li et al., 2015). This includes common liver diseases in companion animals such as feline hepatic lipidosis (FHL) (Begriche et al., 2013) and neutrophilic cholangitis (Mittal, 2014) in cats as well as chronic hepatitis in dogs (Spee et al., 2006). Therefore, the use of antioxidants seems to be useful for the control of such diseases (Vandeweerd et al., 2013). However, further studies and scientific data are needed in this field. Hidvégi et al. (1999) and Barakat et al. (2011) already demonstrated a significant radical-binding effect of FWGE, suggesting its antioxidant activity.

The aim of my study was to consolidate this approach and to examine the hepatoprotective potential *in vitro* on primary cultured rat hepatocytes.

3. Literature review

3.1 The effects of FWGE

3.1.1 Anti-cancer effect

Fermented wheat germ extract (FWGE) sold under the trade name Avemar is a licensed medical nutriment for human cancer patients (Heimbach et al., 2007). It has a multi-substance composition and, besides others, contains 2-methoxy benzoquinone and 2,6-dimethoxybenzoquinone that likely play a significant role in exerting several of its biological properties (Mueller and Voigt, 2011). Their mechanisms of action can be divided into antimetabolic and antiproliferative effects (Hidvégi et al., 1998). Furthermore, FWGE may also modulate the anti-tumour immune response (Fajka-Boja et al., 2002) and led to an ROS-induced cytotoxic effect in different cancer cell lines (Otto et al., 2016).

In contrast to normal cell metabolism, cancer cells display a hypermetabolic state with especially up-regulated utilization of glucose and increased production of lactate (Boros et al., 2002). Normal cells depend to a great extent on oxidative pathways, whereas tumour cells extensively use non-oxidative reactions (Boros et al., 2005). Glucose contributes to the non-oxidative phase of the pentose phosphate pathway (PPP) to produce ribose, which is essential for the increased nucleic acid production in the rapidly dividing tumour cells. FWGE reduced glucose uptake in cancer cells and compromised with enzymes of the anaerobic glycolysis and PPP such as transketolase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase and hexokinase which are required for producing the precursors for RNA and DNA synthesis (Boros et al., 2001). Ribonucleotide reductase (RR) represents an important key factor of DNA synthesis due to its ability to catalyse the conversion of ribonucleotides to deoxyribonucleoside diphosphates (Takeda and Weber, 1981). It was demonstrated in different cancer cell lines that Avemar reduced the activity of RR. The inhibition of these key pathways of DNA synthesis contributes to the antiproliferative effect of FWGE (Illmer et al., 2005; Seiko et al., 2007). Additionally, the impaired glucose consumption of the tumour led to decreased energy production in form of adenosine triphosphate (ATP) resulting in a decrease in tumour growth and an improved metabolic harmony with the host (Telekes et al., 2007). That enhanced the general condition of Avemar-treated patients, facilitated weight gain and improved tolerance for surgeries, chemotherapy or radiation therapy (Boros et al., 2005). The induction of apoptosis in tumour cells by activating the caspase-3-catalyzed cleavage of poly (ADP-ribose) polymerase (PARP) enzyme displays a further anticancer property of FWGE. PARP is a nuclear DNA repair enzyme and has extremely high activity in cancer cells. Its cleavage leads to

instability of the genome, resulting in DNA fragmentation and thus to apoptosis (Virag and Szabo, 2002).

Tumour cells utilize the overexpression of Major histocompatibility complex class 1 (MHC-1) molecules to avoid destruction by the immune system, especially by natural killer (NK) cells (Fajka-Boja et al., 2002). NK cells represent a part of the immune system which is important for the anti-tumour defence. They recognize and are blocked by MHC-1 surface proteins. Consequently, the expression of MHC-1 molecules enables tumour cells to mimic themselves as normal cells and avoid their detection and destruction by NK cells. This approach is a common characteristic of metastatic tumour cells (Kundu and Fulton, 1997). FWGE decreased the MHC-1 expression of cancer cells and sensitized them against NK cells, thus reducing their metastatic activity (Fajka-Boja et al., 2002). Lowered expression of intercellular adhesion molecule 1 (ICAM-1) of solid tumour vessels describes a further strategy of avoiding the immune system. Low ICAM-1 level leads to impaired leukocyte migration through the vessel membrane, resulting in an inhibition of tumour leukocyte infiltration (Griffoen et al., 1996). FWGE up-regulated the synthesis of ICAM-1 on tumour endothelial cells and potentiated the effect of the anti-cancer cytokine, tumour necrosis factor-alpha (TNF-α) (Telekes et al., 2005). Therapeutic potency of FWGE has been detected against head and neck, breast, ovary, colorectal and oral cancer as well as melanoma and leukemia and is used as a complementary and alternative medicine (Patel, 2014).

3.1.2 Immunomodulation

In addition to the immunomodulatory effect used in tumour therapy, further aspects of stimulating the immune system by FWGE were investigated (Boros et al., 2005).

Hidvégi et al. (1999) examined the blastic transformation of peripheral blood lymphocytes of thymectomized mice during the application of FWGE. Spleen cells of the treated animals were removed, cultured and Concanavalin A was added to the cell suspension. The investigation showed a significant increase in H-thymidine incorporation into the splenic cells, highest in the FWGE-treated mice. Consequently, FWGE pre-treatment of mice increased the blastic transformation inducing effect of Concanavalin A on peripheral T lymphocytes (Hidvégi et al., 1999). In the same study the rejection time of skin grafts in mice was explored. The FWGE-treated mice showed significantly shorter skin graft rejection time, which leads to the assumption that FWGE has the ability to restore the immune function. Interestingly, the application of 2,6-dimethoxybenzoquinone alone, instead of the whole extract, did not reduce the graft rejection times (Hidvégi et al., 1999). Nagy et al. (2011) investigated the immune

reactions of broilers under the influence of FWGE. 170 broilers were vaccinated against infectious bronchitis (IB), Newcastle disease (ND) and infectious bursal disease (IBD). The feed of half of these animals was supplemented with FWGE, sold under the trade name Immunovet. Blood samples were taken to assess the humoral antibody level and revealed that FWGE increased the antibody titres significantly (Nagy et al., 2011). The same conclusion was drawn by Ellakany et al. (2017) regarding ND vaccination along with Immunovet administration in broilers.

Furthermore, the effect of FWGE on certain autoimmune diseases was also documented (Patel, 2014). Ehrenfeld et al. (2001) investigated the potential of oral treatment with Avemar on the features of experimental systemic lupus erythematosus (SLE) in naïve mice. The idiotypically induced model showed an upregulation of the Th1 (Type 1 T helper cell) cytokines interleukin-2 and interferon gamma during the initial phase of the disease, while the expression of the Th2 (Type 2 T helper cell) cytokines interleukin-4 and interleukin-10 increased as the autoimmune status progressed (Segal et al., 1997). Analysis of the Th1/Th2 cytokine response showed that the treatment with FWGE prior to disease induction delayed the Th2 response. Furthermore, FWGE-treated mice showed a significantly lower humoral response (with approximately 50%) as shown by the production of autoantibodies. This was associated with the normal values of erythrocyte sedimentation rate (ESR), white blood count (WBC) and moderate proteinuria. Whereas the mice that did not receive FWGE developed high titres of autoantibodies, increased ESR, leukopenia and significant proteinuria. Therefore, it appeared that administration of Avemar starting prior to disease induction promoted amelioration of the disease (Ehrenfeld et al., 2001). Further investigations were performed by Telekes et al. (2007) in a rat adjuvant arthritis (AA) model regarding the anti-inflammatory effect of FWGE. Different groups were treated either with one anti-inflammatory drug (diclofenac, dexamethasone) alone or in combination with Avemar. The assessment was carried out by plethysmographies of the paws, histological investigation of synovial tissues, detection of CD4+ and CD8+ T lymphocytes and determination of gene expression of cyclooxygenase (COX)-1 and -2 by real-time polymerase chain reaction (PCR). Histological examinations revealed no or minimal inflammatory infiltration with almost complete disappearance of CD4+ T lymphocytes and minimal degree of fibrosis in the joints of the FWGE-treated rats. Additionally, FWGE possessed dose-dependent down-regulation of COX-1 and -2 genes and an additive effect with diclofenac (Telekes et al., 2007). Therefore, FWGE provides significant anti-inflammatory properties and could be suitable for the treatment of RA in humans or in animals (Telekes et al., 2007). The

down-regulation of autoantibody production in experimental SLE and disappearance of CD4+ T lymphocytes in rat AA model showed that FWGE causes not only a stimulation of the immune system, but rather a modulation of the immune response.

3.1.3 Free radical binding

Reactive oxygen species (ROS) including oxygen-containing free radicals form a broad spectrum of unstable, highly reactive compounds. Mitochondrial respiration is one of the main sources for the physiological production of ROS. However, further intrinsic, or extrinsic factors such as UV radiation and the metabolism of various drugs and xenobiotics can also lead to their accumulation (Winterbourn, 2008). During the chain reactions of lipid peroxidation free radicals disrupt the cell membrane by "stealing" an electron from its phospholipid bilayer (Barakat et al. 2011). Further, elevated intracellular ROS levels can also contribute to DNA and protein damage, impairing cell function. Therefore, the increased ROS exposure causes oxidative stress with irreversible cell damage up to cell death. Consequently, they play an important role in the pathogenesis of many conditions, including cancer and cardiovascular, inflammatory, and degenerative diseases. ROS can be subdivided in one electron (radical) oxidants such as superoxide anion radical (O_2^-), hydroxyl radical (OH^-) etc. and two-electron (nonradical) oxidants like hydrogen peroxide (H_2O_2) (Winterbourn, 2008).

The animal and human organism has several protective mechanisms to reduce their accumulation and the following consequences. These include enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione S transferase (GST) (Ames et al., 1993). They serve to maintain the so-called redox homeostasis as shown in **Figure 1** (Li et al., 2015). In addition to these endogenous defence systems, the consumption of dietary antioxidants like ascorbate (Vitamin C), tocopherol (Vitamin E) and carotenoids can also lead to a reduction of such harmful molecules (Ames et al., 1993).

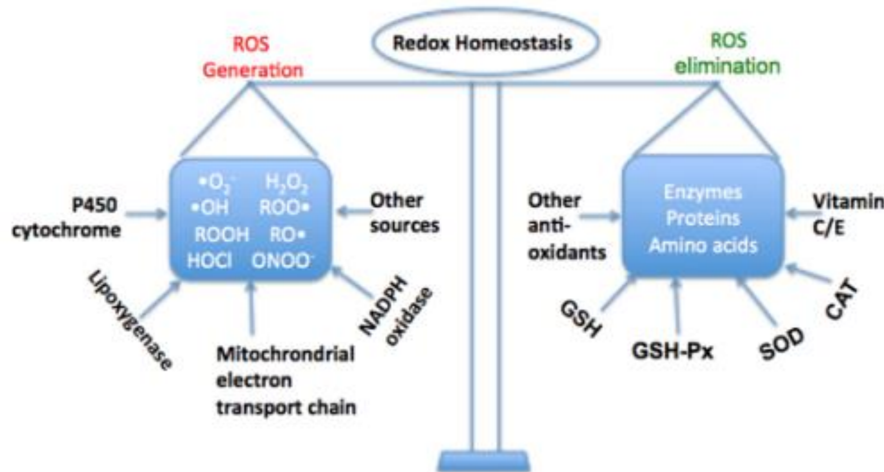


Figure 1: The redox homeostasis: The balance between ROS generation and elimination. (Li et al., 2015)

Hidvégi et al. concluded already in 1999 that FWGE has a significant radical-binding effect by measuring its superoxide scavenging efficiency using electron spin resonance spin trapping method. The result was compared to the scavenging capacity of SOD and led to the assumption that FWGE acts as less powerful but still suitable antioxidant (Hidvégi et al., 1999). Among others, this hypothesis was confirmed by a study of Barakat et al. (2011). In this research the potential influence of FWGE (Avemar) on liver and kidney function alteration and oxidative stress induced by chlorpyrifos was investigated in rats. The FWGE-treated rats showed a significant increase in erythrocyte SOD, plasma CAT and GST activities, coupled to decreased concentration of serum malondialdehyde (MDA), a product of lipid peroxidation. Furthermore, an amelioration of different liver and kidney function parameters as well as an improvement of the lipid profile could be observed compared to the chlorpyrifos exposed rats without FWGE treatment (Barakat et al., 2011). This allows us to conclude that FWGE may act as a powerful antioxidant.

3.2 Common liver diseases in dogs & cats

3.2.1 Feline hepatic lipidosis

Feline hepatic lipidosis (FHL) is one of the most common forms of liver diseases in cats. It is an acute hepatopathy with massive accumulation of fat in the hepatocytes (hepatocellular steatosis) leading to an acute loss of function (Nelson & Couto, 2014). Affected cats are usually obese, suffering from stress or a disease resulting in anorexia (Zachary, 2016). It is classified

into primary idiopathic and secondary FHL. The pathogenesis of the primary FHL is not fully understood, but a combination of excessive peripheral fat mobilization into the liver, a lack of nutrients necessary for fat metabolism and transport, and loss of appetite is suspected. Secondary FHL usually occurs in connection with diseases associated with anorexia, such as pancreatitis, diabetes mellitus, other liver diseases, inflammatory bowel disease or neoplasia (Nelson & Couto, 2014). During prolonged anorexia or stressful conditions intense peripheral lipolysis causes a dramatic increase of free fatty acids (FFA) concentration in the blood, which are finally taken up by the liver. They can either enter the mitochondria for beta-oxidation with the help of L-carnitine or be converted to triglycerides (TG). TGs accumulate in vacuoles within the hepatocytes and are partly incorporated into very low density lipoproteins and secreted into the blood (Armstrong & Blanchard, 2009). Since cats cannot store and produce certain amino acids and biogenic amines, including arginine, methionine and taurine, they need to obtain them from their diet. This can lead to a shortage, particularly in the case of increased fat metabolism and reduced feed intake. Significantly decreased plasma concentrations of alanine, methionine, taurine, citrulline, arginine and tryptophan (≥50%) could be detected in experimentally induced FHL (Biourge et al., 1994). Methionine plays a major role in the production of carnitine, which is responsible for the FFA transport into the mitochondria and therefore essential for the process of beta-oxidation (Center, 2005). If the carnitine present cannot ensure sufficient beta-oxidation, the amount of unmetabolized fatty acids increases and in turn leads to the induction of ROS generation and impaired energy (ATP) production (Begriche et al., 2013). Furthermore, methionine serves as a precursor of S-adenosylmethionine (SAMe). SAMe acts as a methyl donor and is needed for the synthesis of glutathione, a crucial intracellular antioxidant. In addition to insufficient production, a depletion of antioxidants due to the increased amount of ROS leads to an impairment of hepatic antioxidant defence, thus to oxidative damage (Center, 2005).

Masarone et al. (2018) illustrated that a FFA overload may lead to increased permeability of the inner mitochondrial membrane resulting in the dissipation of the membrane potential and reduced ATP synthesis capacity. This energy imbalance favours the development of ROS due to mitochondrial dysfunction. The increased beta-oxidation following lipid overload leads to an enhanced electron flux in the electron transport chain, generating an “electron leakage”. These electrons can react directly with oxygen, leading to the formation of ROS as shown in **Figure 2**. The generated free radicals trigger lipid peroxidation, resulting in the production of highly

reactive substances such as MDA and deterioration of mitochondrial dysfunction. The reduced proton extrusion leads to further impairment of the ATP production (Masarone et al., 2018).

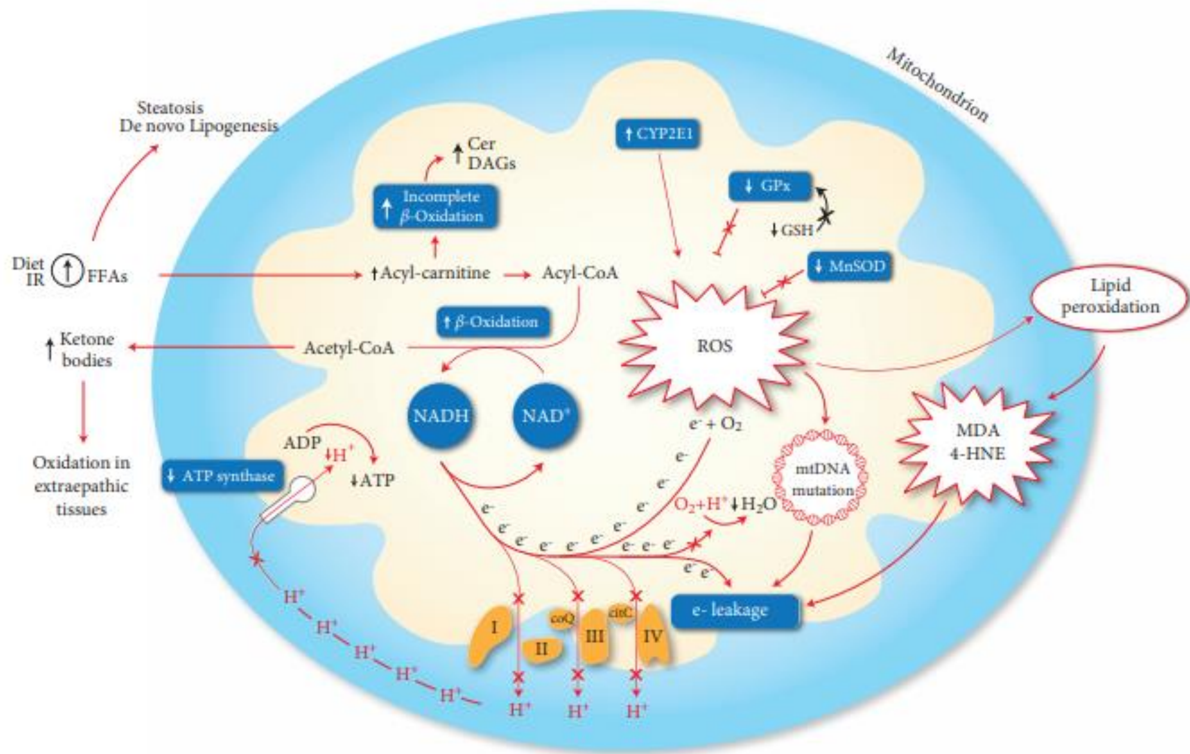


Figure 2: Increased FFA concentration leads to oxidative stress due to mitochondrial dysfunction. (Masarone et al., 2018)

Further abbreviations: 4-HNE = 4-hydroxy-2-nonenal; Cer = ceramides; CYP2E1 = cytochrome P450 2E1; DAGs = diacylglycerols; GPx = glutathione peroxidase; MnSOD = manganese superoxide dismutase

3.2.2 Feline cholangitis-cholangiohepatitis complex

Neutrophilic and lymphocytic cholangitis belong to another group of feline liver disorders, called Feline cholangitis-cholangiohepatitis complex. The neutrophilic or suppurative cholangitis represents the second most common liver disease in cats. The origin is assumed to be a bacterial infection ascending from the small intestine. The most frequently isolated bacteria are the Gram-negative *Escherichia coli*, but *Streptococci*, *Clostridia* and in some cases, *Salmonella* may be involved as well (Nelson & Couto, 2014). The result is an infiltration and accumulation of neutrophils within and surrounding the bile ducts. If the inflammation extends to the liver

parenchyma, the term cholangiohepatitis can be used (Zachary, 2016). A special characteristic of the anatomy of cats is the combined pancreatic and biliary duct, which open together into the proximal duodenum. Due to this proximity, neutrophilic cholangitis usually occurs with an accompanying pancreatitis or intestinal disease (Nelson & Couto, 2014). During inflammatory processes ROS serve as key signaling molecules by attracting cells of the innate immune system and promoting their migration. They are produced by polymorphonuclear neutrophils for the destruction of pathogens, a process called oxidative burst. Therefore, ROS also contribute to the protection of the organism. However, marked inflammatory reactions can lead to tissue damage and a depletion of antioxidants due to the enhanced accumulation of ROS (Mittal, 2014). In addition, studies showed that lipopolysaccharides (LPS) derived from the cell wall of Gram-negative bacteria such as *Escherichia coli*, damaged liver cells and induced ROS production (Nourbakhsh et al., 2019). Beyond that, cholangitis as well as FHL can result in cholestasis (Day, 1995; Armtrong & Blanchard, 2009). A study by Sokol et al. (1995) demonstrated that the accumulation of bile acids, as in the case of cholestasis, led to damage of the hepatocytes including mitochondrial dysfunction and therefore increased oxidative stress.

3.2.3 Canine chronic hepatitis

In contrast to cats, dogs suffer more often from a chronic disease affecting the liver parenchyma instead of the bile duct system. A common example is the canine chronic hepatitis (CCH) (Nelson & Couto, 2014). It is defined by hepatocellular apoptosis and necrosis, infiltration with diverse inflammatory cells, regeneration and fibrosis (Van Den Ingh et el., 2006). The loss of liver tissue and the resulting portal hypertension commonly leads to cholestasis. The cause of this disease stays mostly unidentified (Nelson & Couto, 2014). Possible initiators include canine adenovirus type I, *Leptospira* species, toxins and drugs such as phenobarbital, defects in copper metabolism, breed predisposition and autoimmunity (Watson, 2004). Again, oxidative stress seems to play a decisive role in the pathogenesis. The study of Spee et al. (2006) revealed a reduction of the ROS defence system in chronic inflammatory and cholestatic liver diseases in dogs. The gene expression of the enzymatic antioxidants SOD and CAT showed a significant decline in all diseased groups. Also, the concentration of the reduced form of glutathione (GSH) was greatly diminished in the liver, whereas the level of the oxidized form (GSSG) significantly increased. Consequently, the GSH/GSSG ratios were decreased compared to the control group (Spee et al., 2006). In line with this, Center (2002) mentioned lowered glutathione levels in necroinflammatory liver disorders and extrahepatic bile duct occlusion in dogs as well as in FHL in cats.

3.3 Antioxidants in liver therapy

As the liver is one of the main targets of oxidative stress, the treatment with antioxidants becomes more and more important (Li et al., 2015). The liver is the biggest internal organ and consists to a great extent of parenchymal cells (Zachary, 2016), which contain mitochondria and peroxisomes. These are the major cell organelles involved in ROS generation by metabolizing oxygen (Sánchez-Valle et al., 2012). In case of a redox imbalance, the Kupffer cells in the liver are triggered to produce cytokines like TNF-alpha, which might enhance inflammatory and apoptotic processes. Furthermore, ROS-mediated lipid peroxidation activates the hepatic stellate cells, which leads to increased collagen synthesis contributing to the development of fibrosis. Oxidative stress can alter lipids, proteins and DNA contents and negatively influence the pathways of normal biological functions (Li et al., 2015). Therefore, oxidative stress plays a fundamental role in the pathophysiology of liver diseases as shown in **Figure 3**.

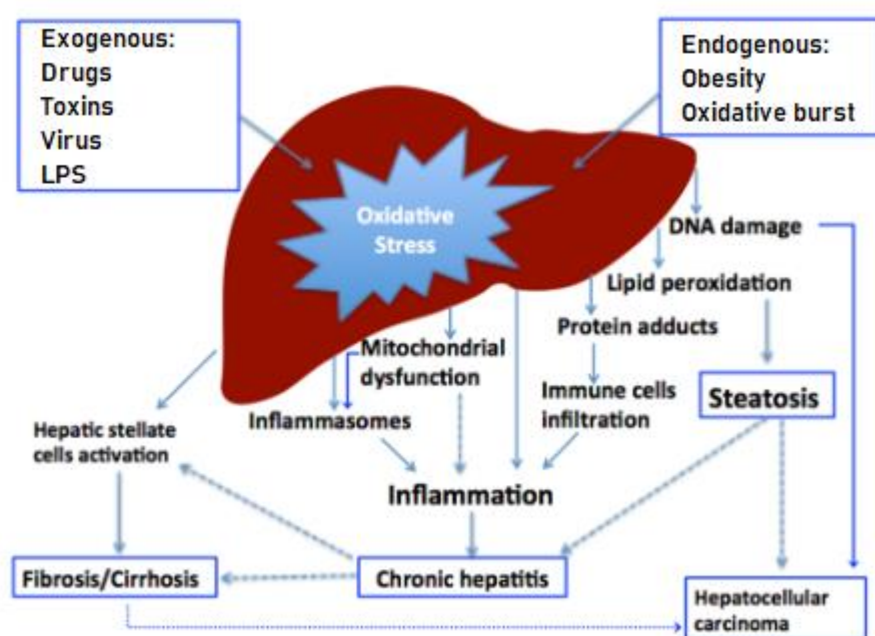


Figure 3: The induction of oxidative stress and the resulting progression of liver diseases. (Adapted from Li et al., 2015)

To support the cellular pro-oxidant/antioxidant redox balance in the liver, several hepatoprotective substances were subjected in medical studies as a complementation of liver disease therapies. For instance, vitamin E (α-tocopherol) can terminate free radical chain reactions as described, among others, by Burtan & Traber (1990). Sokol et al. (1998)

demonstrated in a rat model that the pre-treatment with vitamin E significantly reduced the bile acid induced oxidant injury to hepatic mitochondria. It seems to be beneficial to use vitamin E for the concomitant treatment of hepatobiliary diseases (Watson, 2004; Vandeweerd et al., 2013). However, further scientific data are needed to support this hypothesis (Vandeweerd et al., 2013).

Another naturally occurring antioxidant is silymarin, a flavonoid complex extracted from milk thistle (*Silybum marianum*). Besides silydianine and silychristine, the main active isoflavonoid of this complex is silibinin. Silymarin was found to be an effective radical scavenger (Köksal et al., 2009) and it led to an improved redox state by significantly increasing the glutathione content in the liver of rats (Velenzuela et al., 1988). It proved hepatoprotective effect against paracetamol intoxication in cats (Avizeh et al., 2010) and against mebendazole-provoked hepatotoxicity in dogs (Mosallanejad et al., 2013).

A further way to reduce oxidative stress includes the administration of SAMe and N-acetylcysteine. Both are part of endogenous glutathione synthesis and therefore strengthen the hepatic antioxidant defence system by counteracting its depletion (Webster & Cooper, 2009).

Ursodeoxycholic acid (UDCA) is also widely used in various liver diseases, especially in cholestatic disorders. It is a hydrophilic bile acid and represents only a small fraction of the naturally occurring bile acid pool. The majority consists of hydrophobic bile acids, which in contrast to hydrophilic ones induce a hepatotoxic effect in case of their accumulation (Lazaridis et al., 2001). It was demonstrated by Stiehl et al. (1999) that the hepatoprotective property of UDCA treatment may correlate with competitive displacement of hydrophobic bile acids. Besides that, UDCA provoked the secretion of bile acids, reducing their harmful accumulation (Trauner and Graziadei, 1999). Furthermore, Heuman et al. (1991) investigated cytoprotective properties of UDCA and emphasized its direct interaction with plasma membranes of hepatocytes improving their membrane stability. However, Lukivskaya et al. (2001) studied the antioxidant mechanism of hepatoprotection by UDCA in a rat model. It was found that the UDCA treatment led to decreased production of ROS and lipid peroxidation products (e.g. MDA) and reduced activities of the antioxidant defence system. Therefore, the destruction of the liver due to oxidative stress could be reduced by the antioxidant effects of UDCA (Lukivskaya et al., 2001).

This short list is only a small selection of known antioxidants, moreover further studies and clinical trials are needed to fully understand and evaluate their effectiveness. But if oxidative

stress is considered as a fundamental factor in the pathogenesis of liver diseases, further research on antioxidants seems to be essential.

4. Hypothesis and Aims

Based on literature data, FWGE possesses ability to bind free radicals, resulting in a reduction of oxidative stress. Furthermore, FWGE strengthens the immune system, combats inflammatory reactions and is safe to use. Since oxidative stress plays an important role in the development of several diseases, particularly concerning the liver, this naturally occurring substance may decelerate their progression and improve the general health status.

This study aimed to assess the antioxidant mechanism of hepatoprotection by FWGE. The effect of FWGE on cellular redox homeostasis in cultured rat liver cells was investigated by exposing the primary hepatocyte cell cultures to inflammation-triggering lipopolysaccharides (LPS) with or without FWGE (Immunovet®) supplementation. In addition, the supplementation of UDCA and silymarin was used to provide a comparison with widely used hepatoprotective substances. To monitor its hepatoprotective effects, different parameters regarding oxidative stress, antioxidant defence system and the cellular metabolic activity were measured. These include hydrogen peroxide as a ROS, MDA as a product of lipid peroxidation and the activity of GSH-Px as an enzymatic antioxidant.

The use of rats as an approved model in animal research provides scientific data, which are also transferable to companion animals or even humans.

5. Materials and Methods

All reagents used in this study were acquired from Sigma-Aldrich (Darmstadt, Germany), unless otherwise declared. The performed animal procedures were conducted in strict accordance with the national and international law as well as institutional guidelines. Their implementation was approved by the Local Animal Welfare Committee of the University of Veterinary Medicine, Budapest and by the Government Office of Pest County, Food Chain Safety, Plant Protection and Soil Conservation Directorate, Budapest, Hungary (permission number: PEI/001/1430-4/2015).

5.1 Cell isolation, Culturing and Treatment

The samples were taken from 8-week-old Wistar rats (approx. 200-250 g). Husbandry and feeding were carried out in compliance with the actual Hungarian and European animal welfare laws. Median laparotomy was performed after applying carbon dioxide narcosis. Thereupon the cannulation of the *vena portae* and the thoracic section of the *vena cava caudalis* was conducted. These access points were used to flush and exsanguinate the liver through the portal system as shown in **Figure 4**.

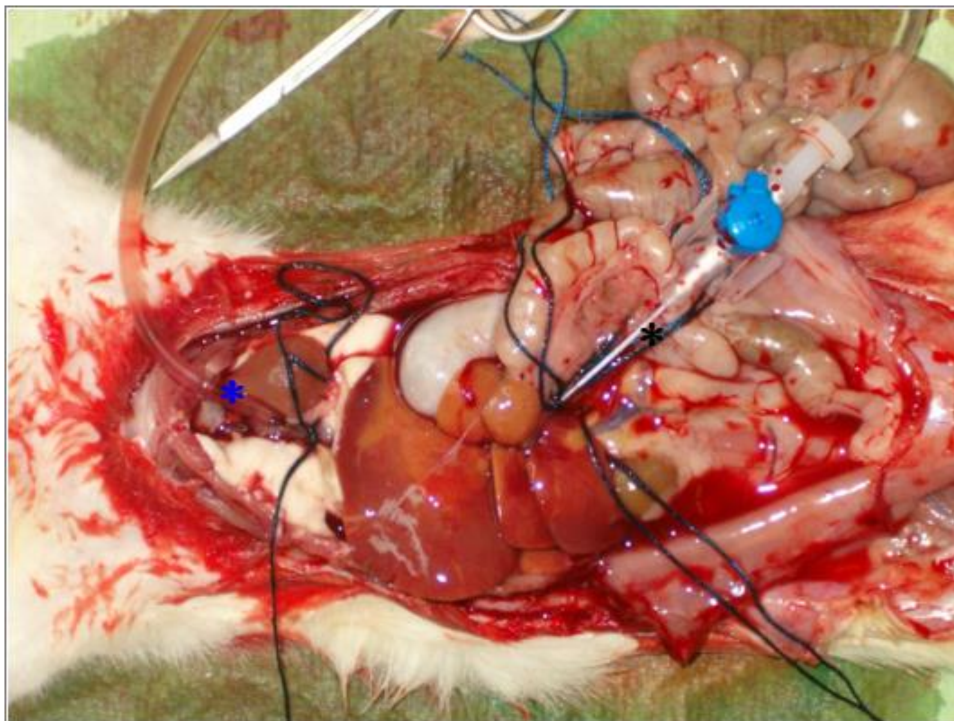


Figure 4: The liver perfusion excess points. The perfusion solutions are introduced into the liver via the *vena portae* (in-flow, ∗) and will be resumed via the thoracal section of the *vena cava caudalis* (out-flow, ∗).

A multi-step perfusion with different buffers was used and introduced via the *vena portae*. To enable the recirculation of the buffers, the effused solutions were resumed via the *vena cava caudalis*. Three different buffer solutions were applied for liver perfusion: at first, 300 mL ethylene glycol tetraacetic acid (EGTA, 0.5 mM) containing Hank's Balanced Salt Solution (HBSS) buffer, then 200 mL EGTA-free HBSS buffer and finally, 130 mL EGTA-free HBSS buffer, supplemented with 50 mg type IV. collagenase (Serva, Duisburg, Germany), 2.5 mM $CaCl_2$ and $MgCl_2$. During this process, all reagents were kept at a temperature of 40°C and were previously oxygenated with Carbogen (95% O_2, 5% CO_2). The perfusion rate was adjusted to 30 ml/min. In order to loosen the hepatocytes within the liver parenchyma, the collagenase containing buffer was recirculated until the complete disintegration. Following the removal of the liver and severing the capsule, the cell suspension could be filtered through sterile gauze sheets. The obtained suspension was mixed for 50 min with 25 mg/mL bovine serum albumin (BSA) and ice-cold HBSS to prevent cluster formation.

Low speed multi-step differential centrifugation (3 times, 100x g, 2 min) was then used to isolate the hepatocytes by forming cell pellets. They were resuspended in Williams' Medium E supplemented with 50 mg/mL gentamicin, 2 mM glutamine, 20 IU/L insulin, 4 µg/L dexamethasone, 0.22% $NaHCO_3$ and in the first 24 h of culturing with 5% foetal bovine serum (FBS). Following this step, the viability of the hepatocytes was examined. Therefore, trypan blue exclusion test was applied and always revealed a result of more than 90% viable cells. The number of hepatocytes in turn was monitored by cell counting in Bürker's chamber and the desired concentration of 10^6 cells/ mL was adjusted with the appropriate dilution. 96- and 6-well Greiner Advanced TC cell culture dishes (Greiner Bio-One Hungary Kft., Mosonmagyaróvár, Hungary) were previously coated with collagen type I (10 µg/cm^2) to aid adherence and growth. The hepatocytes were seeded on them using 200 µg/well seeding volume in the 96-well plates and 2 mL/well in the 6-well plates. The incubation was performed at 37°C and 100% relative air humidity and the cell culture media were replaced after 4 h. Confluent monolayer cell cultures were gained after 24 h incubation as shown in **Figure 5**.

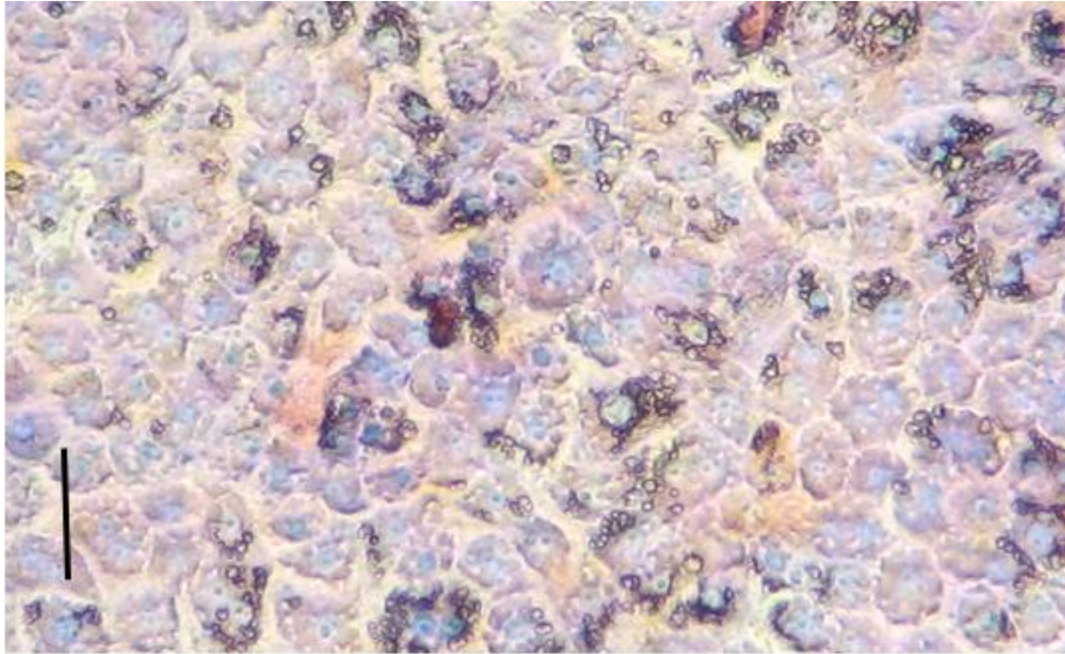


Figure 5: Primary cultured rat hepatocytes after 24 h incubation. Giemsa staining. Bar = 30 µm

The cell cultures were exposed to LPS after 24 h incubation to trigger increased oxidative stress as a result of an inflammatory reaction. Therefore, cell culture media were mixed with 0 (control) or 10 µg/mL *Salmonella enterica* serovar. Typhimurium derived LPS for 2 and 8 h incubation time. The LPS-free as well as the LPS-challenged cultures were divided into five further subgroups and supplemented with 0.1% or 1% FWGE prepared from Immunovet®, silymarin (50 µg/mL), ursodeoxycholic acid (UDCA, 200 µg/mL) or without supplementation. The composition and abbreviation of the experimental groups and subgroups are shown in **Table 1**.

Table 1: Abbreviations of the cell culture groups and subgroups

1. **LPS0** = Cell cultures not exposed to LPS	2. **LPS10** = Cell cultures exposed to 10 µg/mL LPS
Supplemented with:	
1.1 **CRT =** control group without supplementation	2.1 **CRT** = control group without supplementation
1.2 **FWGE 01** = 1 mg/mL FWGE	2.2 **FWGE 01** = 1 mg/mL FWGE
1.3 **FWGE 1** = 10 mg/mL FWGE	2.3 **FWGE 1** = 10 mg/mL FWGE
1.4 **SILY** = 50 µg/mL silymarin	2.4 **SILY** = 50 µg/mL silymarin
1.5 **UDCA** = 200 µg/mL UDCA	2.5 **UDCA** = 200 µg/mL UDCA

As proved hepatoprotective and antioxidant substances, silymarin and UDCA served as benchmarks for assessment of the FWGE effect. In order to produce a suitable FWGE solution, Immunovet® granules were processed into a fine powder with the means of a mortar. Afterwards it was dissolved in sterile phosphate buffered saline solution (PBS). The buffer was used to ensure a constant pH and isotonic conditions. The prepared solution was finally filtered in three steps by using gauze sheets, a cell strainer (70 µm pore size) and a sterile filter (0.22 µm pore size) (Merck Millipore, Burlington, MA, USA). Concentration of the stock solution was 100 mg/mL (10%), freshly diluted to the appropriate working concentration. On both the 96- and 6-well plates, 6 replicates were made for each treatment group (n = 6). After either 2 h or 8 h of incubation, samples from cell culture media of the 6-well plates were collected. Additionally, wells were washed with PBS, and the cells were lysed with Mammalian Protein Extraction Reagent (M-PER™, Thermo Fisher Scientific, Waltham, MA, USA). Finally, the collected samples were stored at -80°C.

5.2 Measurements

5.2.1 Cellular metabolic activity

After the treatment, the metabolic activity of the cells was examined by using the Cell Counting Kit-8 (CCK-8) (Dojindo, Rockville, USA). The contained WST-8 reagent is getting reduced by dehydrogenase activities in cells to give an orange coloured product (formazan), suitable for sensitive colorimetric assays. Thus, the total amount of $NADH+H^+$ produced in the aerobe catabolic reactions could be displayed, leading to the assessment of metabolic activity (**Figure 6**).

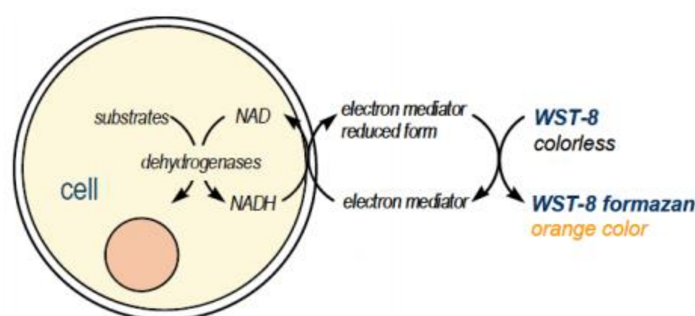


Figure 6: Formazan dye generated by cellular dehydrogenases in the CCK-8 assay (Dojindo Molecular Technologies, Inc. CCK-8: Technical Manual. Rockville, MD, USA)

As described in the manufacturer's instructions, 100 µl fresh Williams' Medium E and 10 µl of the CCK-8 solution was added to each well of the cell cultures in the 96-well plates. Following 2 h incubation time at 37°C, the absorbance was measured at 450 nm with a Multiskan GO 3.2 reader (Thermo Fisher Scientific, Waltham, MA, USA).

5.2.2 H_2O_2 production

In order to evaluate the extracellular ROS concentration, H_2O_2 production was measured using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). In the presence of peroxidase, the Amplex® Red reagent reacts with H_2O_2 to produce the red fluorescent oxidation product, resorufin. Resorufin can finally be measured fluorometrically or spectrophotometrically. Just prior to use, the stock solutions were prepared as described in the manufacturer's instructions. In order to obtain 10 mM Amplex® Red reagent stock solution, one vial of 154 µg Amplex® Red reagent was dissolved in 60 µL Dimethylsulfoxide (DMSO) at room temperature. Afterwards, 4 mL of 5X Reaction Buffer was added to 16 mL of deionized water resulting in 20 mL of 1X Reaction Buffer working solution. Then one vial of Horseradish Peroxidase (HRP) was dissolved in 1.0 mL of 1X Reaction Buffer gaining 10 U/mL HRP stock solution. Additionally, 20 mM H_2O_2 working solution was prepared with 3% H_2O_2 and 1X Reaction Buffer and was used to create a H_2O_2 standard curve including a control with no H_2O_2. Finally, a working solution of 100 µM Amplex® Red reagent and 0.2 U/mL HRP was prepared using 50 µL of 10 mM Amplex® Red reagent stock solution, 100 µL of 10 U/mL HRP stock solution and 4.85 mL of 1X Reaction Buffer. 50 µL of the gained solution was added to 50 µL cell culture medium and incubated for 30 min at room temperature (21°C). The resulting fluorescence ($\lambda ex = 560$ nm; $\lambda em = 590$ nm) of resorufin was detected using a Victor X2 2030 fluorometer (Perkin Elmer, Waltham, MA, USA).

5.2.3 Malondialdehyde concentration

As a result of oxidative damage, lipid peroxidation leads to the production of highly reactive substances such as MDA. The Lipid Peroxidation (MDA) Assay Kit was used to measure this end product in the cell culture media. The principle of this test based on the reaction of MDA with thiobarbituric acid (TBA) forming a colorimetric product, proportional to the MDA present. Ultrapure water was used for the preparation of all reagents. First the TBA stock solution was prepared according the protocol. Therefore, one bottle of TBA was reconstituted with 7.5 mL of glacial acetic acid and then adjusted to the final volume of 25 mL with water. Then the MDA standards for colorimetric detection were designed as followed. 10 µL of the

4.17 M MDA Standard Solution was diluted with 407 µL of water to prepare a 0.1 M MDA Standard Solution. Further 20 µL of the 0.1 M MDA Standard Solution was diluted with 980 µL of water to gain a 2 mM MDA Standard. 0, 2, 4, 6, 8, and 10 µL of the gained solution was added into separate microcentrifuge tubes, generating 0 (blank), 4, 8, 12, 16, and 20 nM standards. Each tube was complemented with water to bring the volume to 200 µL. 300 µL TBA stock solution was mixed with 100 µl cell culture media and was incubated at 95°C for 1 h. Afterwards 10 min cooling on ice to room temperature followed. Absorbance was measured at 532 nm with a Multiskan GO 3.2 reader (Thermo Fisher Scientific, Waltham, MA, USA). The evaluation of the values was carried out using the MDA standard curve and the formula provided by the manufacturer.

5.2.4 Glutathione peroxidase activity

As one of the most important enzymatic antioxidants, glutathione peroxidase (GSH-Px) plays a crucial role in the defence system against oxidative stress. Its activity was measured in the cell lysate by using Glutathione Peroxidase Cellular Activity Assay Kit. The oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalysed by GSH-Px, is coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH+H^+ as shown in **Figure 7**. The principle of this test kit is an indirect method, measuring the decrease in NADPH+H^+ absorbance, which is indicative of GSH-Px activity.

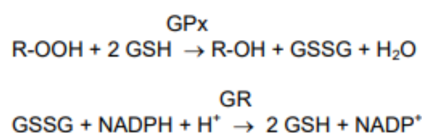


Figure 7: NADPH+H^+ as a part of the GSSG recycling process (Sigma-Aldrich, Glutathione Peroxidase Cellular Activity Assay Kit: Technical Bulletin, St. Louis, MO, USA. 2017)

Further abbreviations: GPx = glutathione peroxidase, R-OOH = organic peroxide

According to the protocol, a sufficient amount of GSH-Px buffer was adjusted to room temperature and 1 vial of NADPH Assay Reagent was reconstituted in 1.25 mL of water, stored at 2-8 °C. Then 21.5 µl of Luperox TBH70X was diluted in water with a total volume of 5 mL to prepare a 30 mM tert-Butyl Hydroperoxide solution. As described in the instruction manual, a glutathione peroxidase solution of 0.25 U/ml was produced for the positive control. Additionally, a blank probe was prepared excluding the enzyme solution and cell culture sample. Finally, 455 µl of GSH-Px Assay Buffer was mixed with 25 µl of NADPH Assay Reagent, 5 µl of tert-Butyl Hyperoxide solution and 15 µl of the cell lysate. The decrease of

absorbance was continuously detected at 340 nm (initial delay: 15 sec; interval: 10 sec; number of readings: 6). Enzyme activity was calculated using the formula provided by the manufacturer.

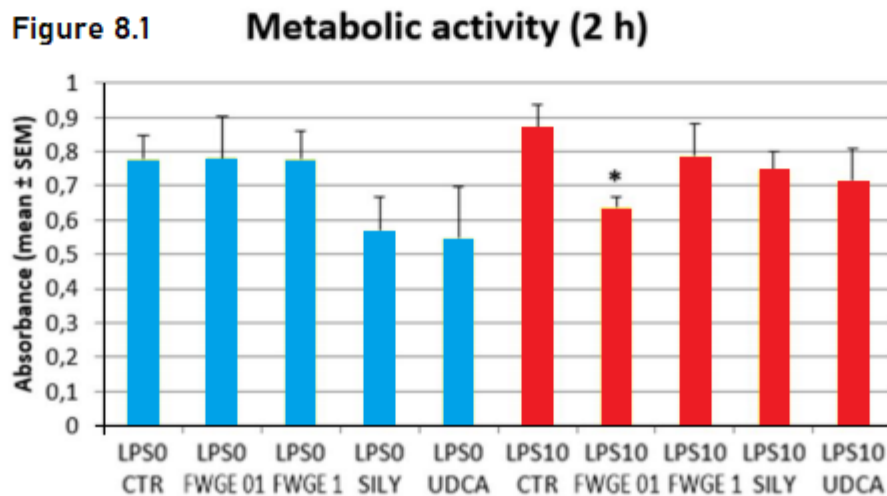
5.3 Statistics

All the data analysis was performed using R 3.5.3. software (GNU General Public License, Free Software Foundation, Boston, MA, USA). On both of 96- and 6-well plates, six wells were included in one treatment group. Differences between groups were assessed using one-way analysis of variance (ANOVA) and post-hoc tests for pairwise comparisons. Results were assessed as the mean ± standard error of the mean (SEM). Differences were assumed significant at $P<0.05$. Results of the FWGE, silymarin and UDCA treated groups were compared to the respective control groups (LPS free or LPS supplemented control groups). The effects of LPS supplementation was considered as main effect compared to the control groups without LPS treatment.

6. Results

Cellular metabolic activity

CCK-8 assay was used to measure the metabolic activity of the cultured cells. The results revealed that most of the applied treatments did not influence this parameter. However, the addition of 0.1% FWGE (P=0.016; **Figure 8.1**) led to a significant decrease after 2 h incubation time in the LPS challenged groups. A further significant reduction in metabolic activity was observed in case of the 8 h long silymarin and UDCA (P=0.029 and P<0.001; **Figure 8.2**) treatments of the LPS free control cells.



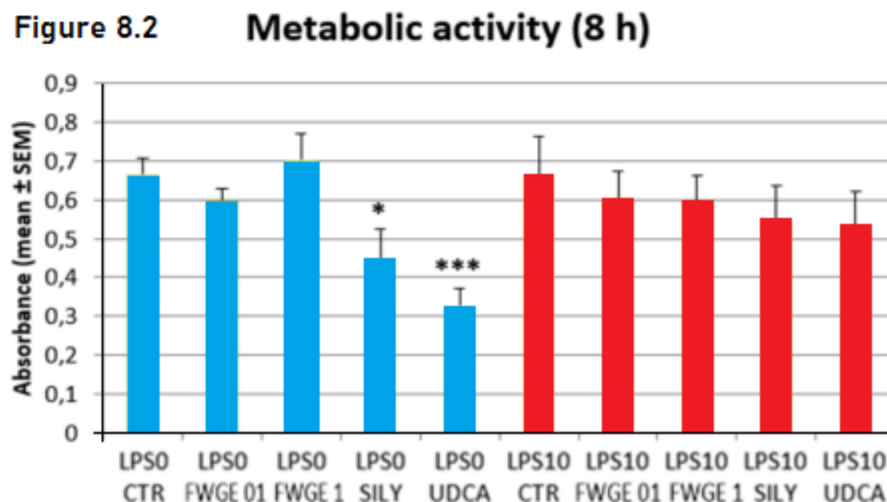
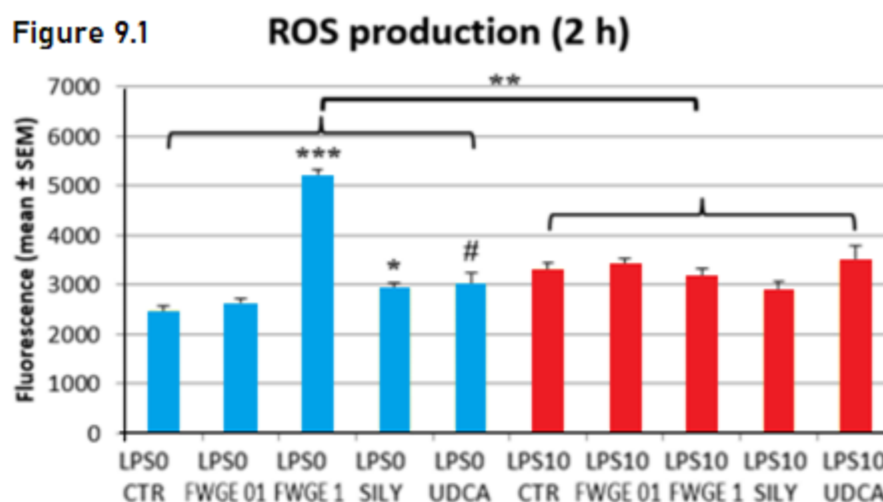


Figure 8: Measurements of cellular metabolic activity after 2 h (**8.1**) and 8 h (**8.2**) incubation using CCK-8 assay. LPS 0= cultures without LPS treatment, LPS 10= LPS treated cultures (10 µg/ml); FWGE 01= 0.1% FWGE, FWGE 1= 1% FWGE, SILY= silymarin (50 µg/ml), UDCA= ursodeoxycholic acid (200 µg/ml). Mean ± SEM, *P<0.05, ***P<0.001.

H_2O_2 production

In order to evaluate the extracellular ROS concentration, H_2O_2 production was measured using the Amplex® Red method. The results showed that the LPS exposure of the cell cultures increased the ROS concentration after 2 h and 8 h incubation (2 h incubation: P=0.001; 8 h incubation: P=0.036; **Figure 9)**. The treatment of LPS exposed cells with FWGE and silymarin showed a significant decrease in this value after 8 h incubation as shown in **Figure 9.2** (0.1% FWGE: P=0.020; 1% FWGE: P=0.027; silymarin: P=0.006).

On the other hand, the supplementation with 1% FWGE led to a significant increase of ROS after 2 h and 8 h incubation in case of the LPS free cell cultures (P<0.001 and P=0.007, **Figure 9**). A significant increase of ROS in non-LPS treated cell cultures was also observed after 2 h incubation with silymarin and UDCA (silymarin: P= 0.014, and UDCA: P= 0.058, **Figure 9.1**).



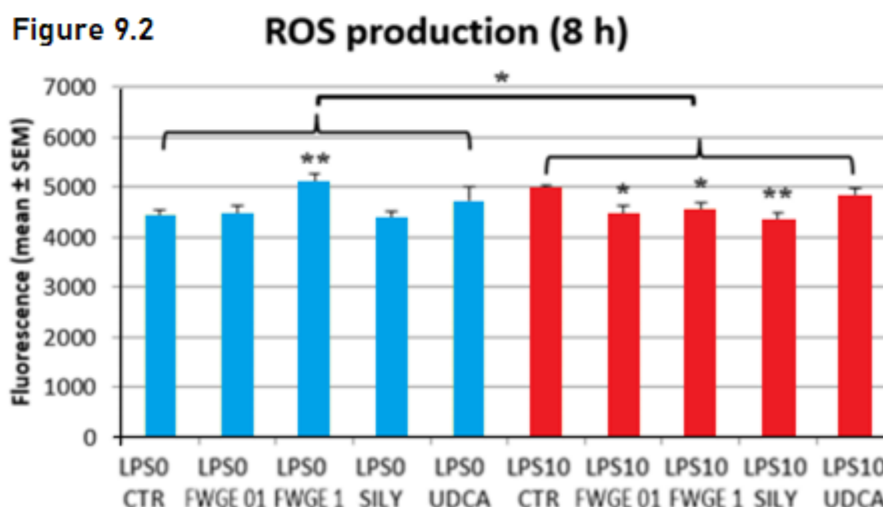
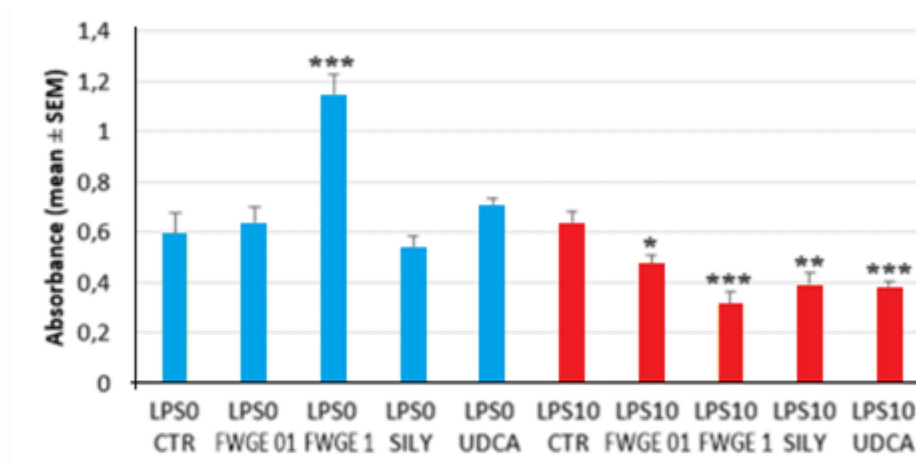


Figure 9: Measurements of extracellular ROS production of cell cultures after 2 h (**9.1**) and 8 h (**9.2**) incubation using Amplex red method. LPS 0 = cultures without LPS treatment, LPS 10 = LPS treated cultures (10 µg/ml); FWGE 01 = 0.1% FWGE, FWGE 1 = 1% FWGE, SILY = silymarin (50 µg/mL), UDCA = ursodeoxycholic acid (200 µg/mL). Mean ± SEM, #P<0.10, *P<0.05, **<0.01, ***P<0.001.

Malondialdehyde concentration

As a result of oxidative damage, lipid peroxidation leads to the production of highly reactive substances such as MDA. The Lipid Peroxidation (MDA) Assay Kit was used to measure this end product in the cell culture media after 2 h and 8 h incubation. The treatment with 1% FWGE showed a significant elevation of MDA concentration after both incubation times in case of the LPS free cell cultures (2 h: P<0.001; 8 h: P=0.003; **Figure 10**). In contrast, the supplementation with 1% FWGE in LPS-treated cell cultures revealed a significantly lowered MDA concentration after 2 h as well as 8 h incubation (2 h: P<0.001; 8 h: P=0.044; **Figure 10**). A significantly decreased MDA concentration was also measured after 2 h incubation with 0.1% FWGE, silymarin and UDCA in the LPS treated cell cultures (0.1% FWGE: P=0.018, silymarin: P=0.004, UDCA: P<0.001; **Figure 10.1**).



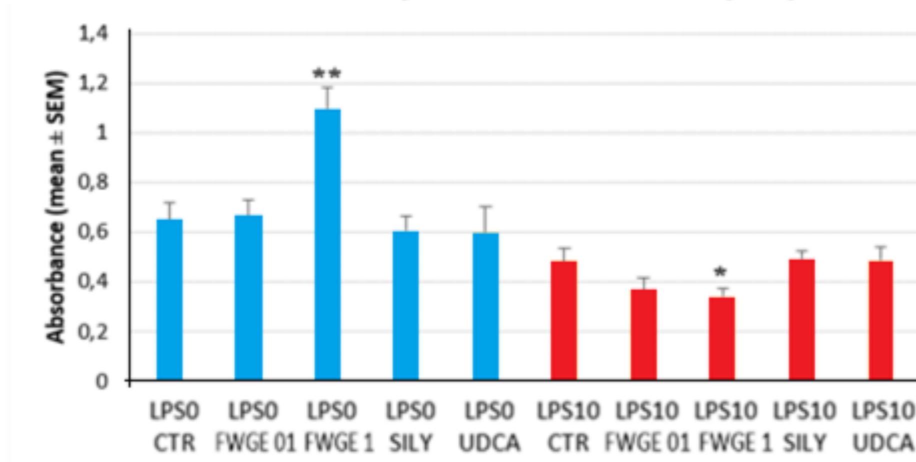


Figure 10: Measurements of MDA concentration in the cell culture media after 2 h (**10.1**) and 8 h (**10.2**) incubation. LPS 0 = cultures without LPS treatment, LPS 10 = LPS treated cultures (10 µg/ml); FWGE 01 = 0.1% FWGE, FWGE 1 = 1% FWGE, SILY = silymarin (50 µg/mL), UDCA = ursodeoxycholic acid (200 µg/mL). Mean ± SEM, *P<0.05, **<0.01, ***P<0.001.

Glutathione Peroxidase activity

As one of the most important enzymatic antioxidants, glutathione peroxidase (GSH-Px) plays a crucial role in the defence system against oxidative stress. Its activity in the cell lysate was measured by using Glutathione Peroxidase Cellular Activity Assay Kit after 8 h incubation. In case of the LPS treated groups a significantly increased GSH-Px activity was measured compared to cells without exposure (P<0.001; **Figure 11**). Only the UDCA supplementation could significantly counteract this increase (P=0.002; **Figure 11**). However, both FWGE

concentrations, silymarin and UDCA led to a decreased GSH-Px activity in the LPS free cell cultures (0.1% FWGE, silymarin and UDCA: P<0.001, 1% FWGE: P=0.004).

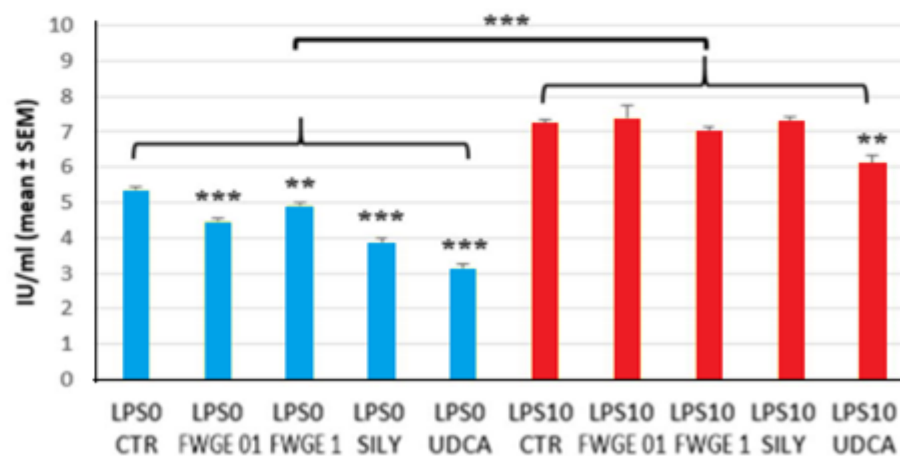


Figure 11: Measurements of glutathione peroxidase activity of the cultured cells after 8 h incubation. LPS 0 = cultures without LPS treatment, LPS 10 = LPS treated cultures (10 µg/ml); FWGE 01 = 0.1% FWGE, FWGE 1 = 1% FWGE, SILY = silymarin (50 µg/mL), UDCA = ursodeoxycholic acid (200 µg/mL). Mean ± SEM, **P<0.01, ***P<0.001.

7. Discussion

This study investigated the hepatoprotective effect of FWGE (gained from Immunovet®) on cultured rat liver cells by measuring extra- and intracellular redox parameters. Since oxidative stress plays an important role in the pathogenesis of liver diseases, it represents a possible target of liver therapies and their investigation (Li et al., 2015). Consequently, several studies focused on the investigation of antioxidants with regards to their hepatoprotective properties (Sokol et al., 1998; Avizeh et al., 2010). For instance, silymarin and UDCA are widely used in liver therapy and were applied as comparative agents in the present study. In the past, FWGE was primarily researched and used in the field of tumour therapy in humans, sold under the trade name Avemar (Boros et al., 2001; Saiko et al., 2007). It was shown that 1% FWGE (10 mg/mL) led to an ROS-induced cytotoxic effect in different cancer cell lines after 24h incubation (Otto et al., 2016). My study, on the other hand, emphasizes the less investigated antioxidant effect of FWGE, in accordance with the results of Hidvégi et al. (1999) and Barakat et al. (2011). The effect on tumorigenic cells does not necessarily mean that FWGE has a cytotoxic effect in non-carcinogenic tissue. In order to clarify possible cytotoxicity in liver cells, 0.1% and 1% (1 and 10 mg/mL) FWGE was applied. The rat model represents a widely used and approved method in the research of liver diseases (Lee et al., 2011). Therefore, the primary hepatocyte cell culture of rat origin was chosen to investigate the effect of FWGE *in vitro*. The applied treatment with LPS to induce an inflammatory reaction in liver cells was already successfully used by Mátis et al. (2017). In my study this procedure was also performed, using 10 μg/mL LPS and the incubation times of 2 h and 8 h according to the literature data. It created a suitable basis to investigate the hepatoprotective property of FWGE, as LPS treatment led to increased ROS production and GSH-Px activity.

The metabolic activity of the cultured cells was measured using the CCK-8 assay. This method displays the total amount of NADH+H^+ produced in cellular catabolic reactions. In case of the treatment with 0.1% FWGE a slight but significant decrease in metabolic activity in the LPS challenged cell cultures was detected after 2h incubation (**Figure 8.1**). The slight reduction cannot be interpreted as cytotoxicity but indicates an influence on the metabolic processes within the cell. However, in the LPS free cell cultures 0.1% as well as 1% FWGE did not lead to a reduction of this value in case of both incubation times (**Figure 8.1** and **8.2**). This supports the assumption that the applied concentrations of FWGE have no cytotoxic effect on liver cells under physiological circumstances.

In order to evaluate the extracellular ROS concentration as an indicator of oxidative stress, H_2O_2 production was measured using the Amplex® Red method. According to the results, 1% FWGE significantly increased the ROS level after 2 h and 8 h incubation in the LPS free cell culture media (**Figure 9.1 and 9.2**). Therefore, the use of FWGE in higher concentration may exhibit a mild pro-oxidant effect in healthy liver cells. In accordance with other studies, different concentrations and environmental circumstances of antioxidants can lead to a pro-oxidant effect. This phenomenon was observed for instance with vitamin C, vitamin E (Poljšak et al., 2008) and even flavonoids (Procházková et al., 2011). However, the treatment with 0.1% and 1% FWGE as well as with silymarin showed a significant reduction of the ROS production after 8 h incubation in the LPS challenged cell culture media (**Figure 9.2**). These results in turn illustrate the potential of FWGE as an antioxidant in the case of inflammatory liver cell damage.

ROS can induce the harmful chain reaction of lipid peroxidation, which results in impairment of the cell membrane and consequently in disruption of the cell function. MDA represents a product of lipid peroxidation and was measured in the present study using Lipid Peroxidation (MDA) Assay Kit. The treatment of the LPS free cell cultures with 1% FWGE resulted in significantly increased MDA concentrations after 2 h and 8 h incubation, whereas 0.1% FWGE did not trigger such elevation (**Figure 10.1 and 10.2**). These results are consistent with the measurements of the extracellular ROS concentration, because both lead to the conclusion that the increased dosage of FWGE may have a pro-oxidant effect on healthy liver cells. However, in case of the LPS treated cell cultures, the addition of 1% FWGE could cause a significant reduction in MDA concentration after both incubation times (**Figure 10.1 and 10.2**), and the treatment with 0.1% FWGE led to significantly decreased MDA production after 2 h incubation (**Figure 10.1**). Consequently, the assumed pro-oxidant effect of the increased FWGE concentration does not apply to the inflammatory liver cells, also reflected by the MDA measurements. Decreased MDA concentration was also found in the study by Barakat et al. (2011) in FWGE-treated mice. Additionally, Hidvégi et al. (1999) recorded the superoxide scavenging efficiency of FWGE. Therefore, FWGE appears to be effective in combating oxidative stress and lipid peroxidation in LPS exposed cells, but the appropriate dosage is particularly important in healthy tissue to avoid a pro-oxidant effect.

The whole glutathione defence system was evaluated using the Glutathione Peroxidase Cellular Activity Assay Kit. This method relies on the indirect measurement of $NADPH+H^+$ in the cell lysate, which factor is necessary for the recycling process of GSSG to GSH. The oxidation of GSH to GSSG in turn, is catalysed by GSH-Px. Since GSH-Px plays a crucial role in the

regulation of oxidative stress in the liver, its assessment was used to obtain a better understanding of the oxidative status of the examined cell cultures. According to the gained results, the treatment with LPS led to an increased activity of GSH-Px (**Figure 11**). This increase indicates the reduction of oxidative stress in correlation with the enhanced use of the glutathione defence system.

As ROS are also involved in the immune response, they represent an integral part of inflammatory reactions (Mittal, 2014), which could be achieved by the LPS treatment. In addition, further studies showed increased GSH-Px activity in inflammatory liver tissue of rats (Iskusnykh et al., 2013). In the present study, only the supplementation with UDCA could significantly decrease the GSH-Px activity in the LPS-exposed cell cultures. This emphasizes the hepatoprotective property of UDCA, which was used as comparative agent in this study. On the other hand, FWGE was able to reduce the lipid peroxidation to greater extent compared to UDCA in the LPS treated cell cultures. For both concentrations of FWGE as well as silymarin and UDCA a mildly decreased activity of GSH-Px was measured in the LPS free cell cultures. Therefore, the oxidative status of these cells was not greatly affected as reflected by the glutathione defence system, even with 1% FWGE, which indicated oxidative stress when based on ROS and MDA production. Thus, the increased concentration of FWGE in normal physiological circumstances may contribute to an elevation in ROS, but not necessarily lead to oxidative distress.

8. Conclusion

Based on the results of this study and the collected literature data, it can be concluded that FWGE had a hepatoprotective effect *in vitro* on a primary liver cell culture of rat origin.

Since oxidative stress plays a fundamental role in the pathogenesis of liver diseases in companion animals (Center, 2002; Spee et al., 2006), FWGE may positively influence the course of the disease due to its antioxidative effect. In the present study 0.1% as well as 1% FWGE proved to significantly decrease the extracellular ROS production in LPS exposed cell cultures. Furthermore, the MDA concentration, as a marker of lipid peroxidation, could be also reduced by FWGE treatment. Therefore, it can be concluded that FWGE is able to alleviate oxidative stress and the subsequent lipid peroxidation, ameliorating inflammatory changes and improving general health condition.

However, the higher concentration of FWGE (10mg/mL) led to an increase in ROS and MDA levels in the healthy cell cultures, which suggests a pro-oxidative effect. Nevertheless, no cytotoxic effect or increase in GSH-Px activity could be detected in these cases. Consequently, FWGE may contribute to an elevation in ROS release under normal physiological circumstances, but not necessarily lead to oxidative distress. Therefore, FWGE should be applied in the appropriate dosage to avoid any possible pro-oxidative effect.

The results of this study form a reasonable basis for considering FWGE (Immunovet®) in liver therapy of companion animals. Further investigations in this field are nevertheless needed and should be complemented by *in vivo* studies.

9. Abstract

9.1 English Abstract

Fermented wheat germ extract (FWGE) is used both in human and veterinary medicine and is characterized by various health improving properties. The ingredients 2-methoxy benzoquinone and 2,6-dimethoxybenzquinone seem to play a significant role in exerting its beneficial effects. Additionally, wheat germ has a high concentration of vitamin E, omega-3 and omega-6 fatty acids. According to the literature data FWGE demonstrated antimetabolic, antiproliferative and reactive oxygen species (ROS)-induced cytotoxic effect in different cancer cell lines. In further studies, the immunomodulatory and free radical binding activity of FWGE was also demonstrated. However, as data regarding the antioxidant activity of FWGE are limited, the aim of our study was to investigate its effects on the hepatic redox homeostasis applying primary hepatocyte cell cultures of rat origin, also serving as a model for companion animals.

Liver cells were gained from 8-week-old Wistar rats and monolayer cell cultures were prepared. The cells were exposed to lipopolysaccharides (LPS, 10 µg/mL) and incubated for 2 and 8 h resulting in an inflammatory reaction and increased oxidative stress. After that, they were supplemented with 0.1% and 1% (1 and 10 mg/L) or without FWGE (Immunovet®). For comparison with approved hepatoprotective substances, ursodeoxycholic acid (UDCA) or silymarin were also added. Finally, different parameters regarding the oxidative status of the cell cultures were measured. These include the cellular metabolic activity, hydrogen peroxide as a ROS, malondialdehyde (MDA) as a product of lipid peroxidation and the activity of glutathione peroxidase (GSH-Px) as an enzymatic antioxidant.

The results of the present study showed that none of the applied FWGE concentrations caused cytotoxicity to the investigated liver cells. Even though 1% FWGE led to an increased ROS and MDA level in the LPS free cell cultures, the GSH-Px activity was reduced at the same time. These findings indicate that a higher concentration of FWGE under normal physiological circumstances can contribute to an elevation in ROS, but not necessarily lead to oxidative distress. Therefore, FWGE should be applied in the appropriate dosage to avoid a pro-oxidative effect. On the other hand, both applied FWGE concentrations significantly decreased the ROS and MDA levels in case of the LPS challenged cell cultures. The latter was even more reduced compared to the corresponding values of UDCA and silymarin treatment. Consequently, the results of this study showed that FWGE is able to reduce inflammation associated oxidative stress and thereby provide a hepatoprotective effect *in vitro*. Hence, properly dosed FWGE may

serve as a promising candidate in the supplementary therapy of patients suffering from inflammatory diseases, decreasing the generation of free radicals, thus avoiding the occurrence of harmful cytotoxic effects.

9.2 Hungarian Abstract

A fermentált búzacsíra-kivonatot (fermented wheat germ extract, FWGE) sokrétű – elsősorban a 2-metoxi-benzokinonnak és a 2,6-dimetoxi-benzokinonnak köszönhető – előnyös hatásai révén a humán és állatgyógyászatban egyaránt széles körben alkalmazzák. Irodalmi adatok alapján az FWGE daganatos sejtvonalakon antimetabolikus, antiproliferatív és a reaktív oxigén vegyületek (ROS) által közvetített citotoxikus aktivitást fejt ki, valamint a kivonat immunmoduláns és szabadgyökkötő hatását is igazolták. Mivel azonban az FWGE antioxidáns aktivitásával kapcsolatban kevés információ áll rendelkezésünkre, kutatómunkánk célja a kivonat redox homeosztázisra gyakorolt hatásainak vizsgálata volt, a háziállatfajok számára is modellként szolgáló patkány eredetű primer májsejttenyészeten.

A májsejteket 8 hetes Wistar patkányok májából többlépcsős perfúzió és differenciáló centrifugálás segítségével izoláltuk, majd azokból egyrétegű sejttenyészeteket hoztunk létre. A sejteket lipopoliszacharidokkal (LPS, 10 µg/ml) kezeltük 2, ill. 8 óra időtartamig, gyulladásos reakció és oxidatív stressz kiváltása céljából. Ezzel egyidejűleg a tenyészetek tápfolyadékát 0,1% és 1% (1 és 10 mg/l) koncentrációjú FWGE-vel (Immunovet®) egészítettük ki. Összehasonlító vizsgálatok céljából ismert hepatoprotektív anyagokkal, urzodezoxikólsavval (UDCA) és szilimarinnal kezelt csoportok is kialakításra kerültek. A sejtek oxidatív státuszának vizsgálatára azok metabolikus aktivitását, a hidrogén-peroxid és a lipidperoxidációs markerként szolgáló malondialdehid (MDA) extracelluláris koncentrációját, valamint a glutation-peroxidáz (GSH-Px) aktivitását határoztuk meg.

Eredményeink alapján egyik alkalmazott FWGE-koncentráció sem bizonyult citotoxikusnak a májsejttenyészeteken. Az 1%-ban adagolt FWGE hatására szignifikánsan emelkedett a hidrogén-peroxid és az MDA koncentrációja az LPS-sel nem kezelt sejtek tápfolyadékában, miközben a GSH-Px aktivitása csökkent. Ebből arra következtethetünk, hogy az élettani körülmények között, egészséges sejteken nagy koncentrációban alkalmazott FWGE a reaktív vegyületek fokozott termelődéséhez vezethet, de ez nem feltétlenül jár együtt oxidatív distressz kialakulásával. Eredményeink tehát felhívják a figyelmet az FWGE megfelelő adagolásának fontosságára, elkerülendő az esetleges prooxidáns hatásokat.

Ezzel szemben az LPS-sel kezelt sejteken mindkét FWGE-koncentráció szignifikánsan csökkentette a ROS- és MDA-szintet, az utóbbi esetében az UDCA-nál és a szilimarinnál is kifejezettebb mértékben. Az FWGE tehát hatékonyan csökkenti a gyulladással járó oxidatív stresszt, és így hepatoprotektív hatást fejt ki *in vitro* körülmények között. A megfelelően adagolt FWGE tehát ígéretes jelölt lehet a gyulladásos kórképekben szenvedő betegek kiegészítő terápiája számára, mérsékelve a szabad gyökök termelődését és az ezzel járó sejtkárosító hatásokat.

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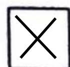

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