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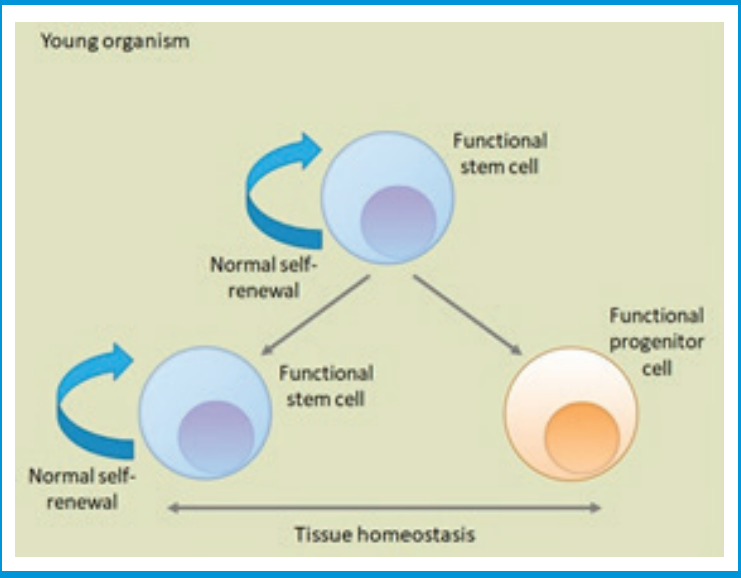

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Slov Vet Res 2019; 56 (1)

Review Article

Jazbec K, Jež M, Justin M, Rožman P. Molecular mechanisms of stem cell aging.....5

Original Research Articles

Marolt Banek I, Roša J, Ježek D, Delaš I. Effect of metabolic cage housing on metabolic changes in the liver of young male laboratory rats.....13

Ujčič-Vrhovnik I, Nataša Kopitar A, Malovrh T, Ježek J, Jakovac-Strajn B. Effects of feeding grains naturally contaminated with *Fusarium* toxins on selected haematological parameters and lymphocyte subsets in primiparous sows 21

Pathirana HNKS, Wimalasena SHMP, De Silva BCJ, Hossain S, Gang-Joon H. Antibacterial activity of clove essential oil and eugenol against fish pathogenic bacteria isolated from cultured olive flounder (*Paralichthys olivaceus*) 31

Slov Vet Res 2019: 56 (1): 5–12
DOI 10.26873/SVR-545-2018
UDC 576.32/.36:577.2

Review Article

MOLECULAR MECHANISMS OF STEM CELL AGING

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Abstract: The aging of multicellular organisms is a complex process, which is a result of various mutually complementary causes. One of these causes is the aging of stem cells. The biological function of stem cells is the replacement of cells that are lost due to illness, injury or normal fluctuations in the maintenance of tissue homeostasis. Molecular mechanisms involved in stem cell aging are similar to those involved in the aging of somatic cells. They include DNA damage and mutations, cell senescence, stem cell exhaustion, telomere shortening, epigenetic changes (alterations of histones and DNA and the consequent dysregulation of gene expression), changes in microRNAs, changes in metabolism, nutrient sensing, decline in mitochondrial integrity and biogenesis, alterations in microenvironment, accumulation of paracrine factors, and loss of cell polarity and proteostasis. Stem cells have developed special mechanisms that compensate for age-related accumulations of errors and they manage to maintain their stemness for a long time, however, they are able to keep cells in a good condition only for a limited period. This article describes the various mechanisms of stem cell aging and their consequences.

Key words: stem cell; aging; nutrient sensing; niche

Introduction

Stem cells (SC) have the ability to go through numerous cell cycles while maintaining an undifferentiated state (self-renewal) and the capacity to differentiate into specialized cell types, which is referred to as potency. In contrast to more differentiated daughter cells, SCs are theoretically regarded as "immortal" and can go through an unlimited number of divisions. In tissues they replace cells lost due to homeostatic turnover (fast renewing tissues) or due to injury and disease. With age, their ability to self-renew declines and their ability to differentiate into various cell types is altered, which can lead to degeneration and dysfunction of the aging tissues and organs. Molecular mechanisms involved in the aging of adult SCs are very similar to the mechanisms involved in the aging of other somatic cells. Aging is caused by various factors that interact and operate simultaneously. Molecular mechanisms that cause cell aging include DNA damage and mutations, cell senescence, exhaustion of the stem cell pool, telomere shortening, epigenetic changes (alterations of histones, DNA and the consequent dysregulation of gene expression), changes in microRNAs, changes in metabolism, nutrient sensing, decline in mitochondrial integrity and biogenesis, alternations in microenvironment and paracrine factors, as well as loss of cell polarity and proteostasis (Figure 1) (1, 2).

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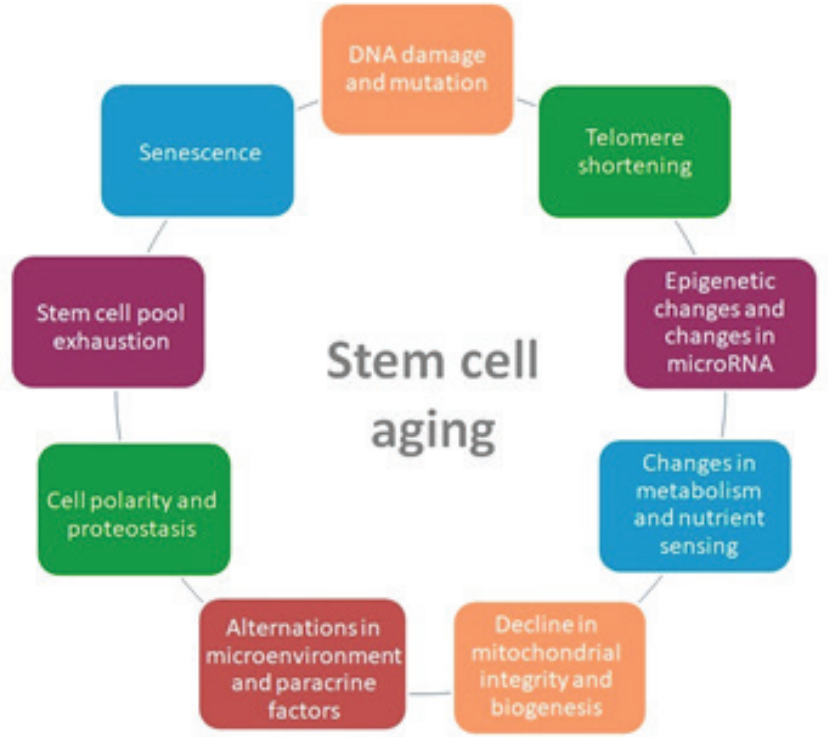

Figure 1: Mechanisms involved in the aging of stem cells

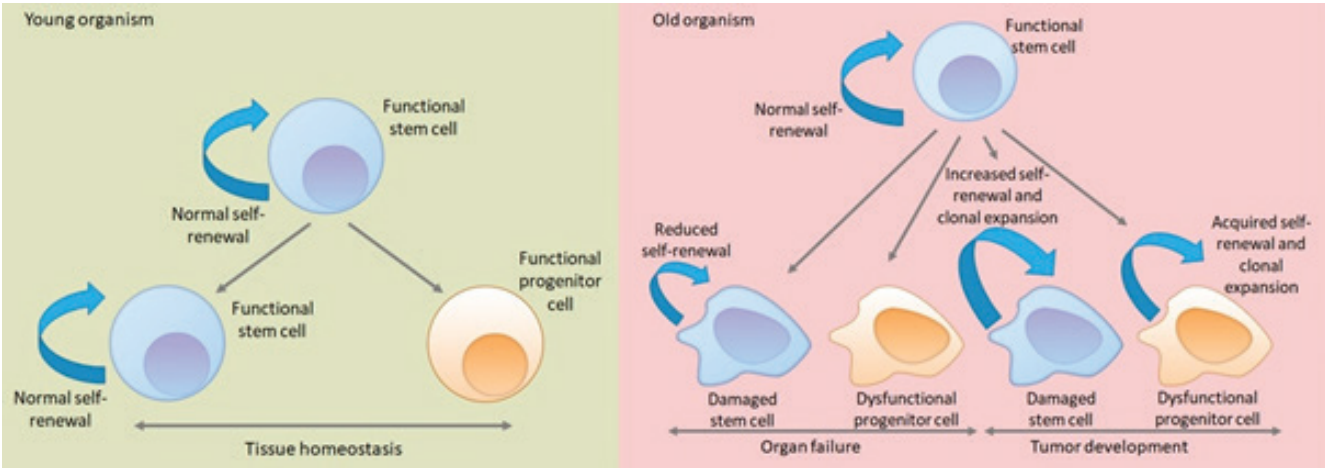

Figure 2: The accumulation of mutations reduces the functionality of stem cells

Stem Cell Aging

1. Exhaustion of stem cell pool

One of the many theories of aging, the so called "*Stem cell theory of aging*", proposes that a decline in the ability of different types of SCs to efficiently regenerate tissues contributes to the aging of organs and the whole organism (3). This inability to regenerate tissues is mostly caused by changes in the production of specialized progenitors, and in some cases, by a decline in the number of SCs.

One of the major discoveries in recent years showed an age-related decline in the number of hematopoietic stem cell (HSC) clones whereas in a normal adult approximately 10 000 clones are available. The study of hematopoiesis in a 115 years old woman demonstrated that the majority of her white blood cells were the offspring of just two HSC clones. Furthermore, telomeres of her white blood cells were significantly shorter

than telomeres of other somatic cells (4). Aging is also associated with a decline in the number of circulating CD34+ hematopoietic stem and progenitor cells. Mandraffino and colleagues (2017) monitored 100 octogenarians and demonstrated that the number of their circulating CD34+ progenitor cells was a better predictor of their lifespan than other risk factors, such as blood pressure, smoking, and cholesterol levels (5). In our study, we also observed a decline in the number of CD271+ cells (mesenchymal stem/stromal cells) with increasing age (unpublished). It is now becoming clear that maintaining a sufficient pool of functional SCs contributes to extended longevity and a longer health span.

2. DNA damage and mutations

In most mammalian cells, spontaneous mutations in DNA occur on a daily basis. Many of them are repaired by DNA repair mechanisms, but some persist. Mutations accumulate throughout life and are responsible for many age-related cellular events. The "*DNA damage theory of aging"* was one of the first theories to explain the process of aging (6). DNA damage can be caused by external factors (ionizing radiation, ultraviolet radiation, or environmental toxins) or by a cell's own metabolic processes (for example, by the accumulation of reactive oxygen species that are generated during the mitochondrial respiration process). Old SCs with accumulated mutations are still functional up until the DNA alterations affect the essential regulatory genes, which cause them to become dysfunctional. Clonal expansion of the damaged stem and progenitor cells can lead to cancer and tumor development. A decrease in their ability to self-renew and the consequent production of dysfunctional progenitor cells leads to organ failure (Figure 2) (7).

3. Telomere shortening

Telomeres are repetitive sequences at the end of the chromosomes that maintain chromosome stability and protect chromosomes from undesirable recombination and fusion with each other. In most somatic cells, telomeres shorten with each cell division, which limits the number of somatic cell divisions. In contrast to differentiated somatic cells, adult stem (and cancer) cells express the enzyme telomerase, which catalyzes the extension of telomere sequences and theoretically enables an unlimited number of cell divisions. However, despite the action of telomerase, it has been demonstrated that telomeres in SCs also shorten with time (8). When telomeres reach a certain shortened length, the cell enters a state of senescence, stops dividing, and can become apoptotic. Though there is a lot of evidence that telomere shortening is inversely related to lifespan, the direct association of telomere length with aging still remains controversial. Researchers have developed mice without the telomerase RNA gene (TERC) and did not find any age-related phenotypes in comparison to the wild type mice (9), which leaves this an open issue.

We have also determined telomere length and expression of telomerase in old and young CD34+ cells intended for autologous transplantation but the telomere length did not correlate with the success of cell therapy (10).

4. Epigenetic changes and changes in microRNA

Epigenetic changes include altered DNA methylation profiles, histone modifications, chromatin remodeling, and changes in microRNA (miRNA). Alterations in epigenetic regulation lead to SC dysfunction and an increased risk of developing hematologic cancer diseases (3). For example, an age-related increase in trimethylation of lysine 4 on the histone H3 protein subunit (H3K4me3) has been found to be associated with a loss of differentiation ability (11).

miRNAs are small noncoding RNAs that regulate the posttranscriptional expression of target genes and are important in the regulation of SC self-renewal and differentiation. They tend to be altered in aged stem cells. It has been demonstrated that some miRNAs inhibit the translation of target mRNAs in SCs and their differentiating daughter cells. This miRNAs function was proven in embryonic and in various adult SCs (12).

5. Decline in mitochondrial integrity and biogenesis

Many studies have demonstrated that mitochondrial dysfunction is one of the major

factors contributing to the aging of cells. Evidence shows that a decline in mitochondrial functions is caused by an accumulation of mutations in mitochondrial DNA (mtDNA), an increase in oxidative damage, a decrease in the number and function of mitochondria, a destroyed calcium homeostasis and a failure in mitochondria dynamics (fusion and division), all of which may contribute to the aging process (13, 14, 1).

Despite many studies, the exact pathophysiological link between damage in mtDNA and aging is still unclear. Mutations in mtDNA are difficult to detect because every mutation is unique and their detection would be possible only if every mitochondria produced the same mutation. Nevertheless, these mutations can be detected in induced pluripotent stem cell (iPS) lines because in the process of iPS derivation, researchers use clones of individual blood or skin cells. Every iPS cell derived from the same cell thus contains the same mutations in mtDNA as the original adult cell (15).

The "*Free radical theory of aging*" describes aging as a result of an increased production of reactive oxygen species (ROS) and subsequent increased oxidative damage in aging cells. However, it is still unclear if an increased production of ROS is a cause or the result of aging. While high levels of ROS can cause oxidative damage to the main cellular components, such as DNA, proteins and lipids, a mild increase in ROS formation during stress can help an organism to survive and does not have a negative effect on longevity. In fact, it can even prolong its lifespan (13, 16). This concept is known as mitochondrial homeostasis or mitohormesis (17, 18).

6. Changes in metabolism and nutrient sensing

SCs, like other cells, generate energy via glycolysis or oxidative phosphorylation. Dormant SCs mostly use glycolysis as the main process for generating energy. This is probably because fewer ROS are generated during this process. Perhaps, due to this fact, most SCs reside in hypoxic environments (19, 20). When there is an increased need for energy (for example, when cells start to proliferate), SCs switch from glycolysis to oxidative phosphorylation, which makes them more susceptible to oxidative damage.

Glucose is the main cell nutrient. Many age-associated regulatory mechanisms maintain concentration of glucose at physiological levels. There is an age-related decrease in hormone levels, including growth hormone and its secondary mediator IGF-1 (insulin like growth factor 1). The presence of glucose induces the insulin/IGF-1 signaling (IIS) pathway. The IIS pathway is one of the most evolutionary preserved pathways, which controls aging. The main targets are the FOXO family of transcription factors and the mammalian target of rapamycin (mTOR) complexes. Mutations and a decrease in levels of expression of those key targets promote longevity. At the same time, mutations in genes for the same targets can lead to uncontrolled cell division and cancer development (21). There are three additional, related and interconnected nutrient-sensing systems that participate in glucose-sensing: the mTOR signaling pathway, which senses high amino acid concentrations; the AMPK (AMP-activated protein kinase) signaling pathway, which senses low energy states by detecting high AMP levels; and the sirtuin pathway, which senses low energy states by detecting high NAD+ levels (22). Researchers managed to increase longevity in yeast, nematodes and fruit flies by lowering mTOR1 expression levels. Similarly, longevity was increased in mice which received the mTOR inhibitor rapamycin (23, 24). By promoting catabolic processes, the AMPK and sirtuin pathways have an opposite effect to the IIS and mTOR pathways. An increased expression of AMPK and sirtuins promote healthy aging (25), whereas the anabolic activity, induced by the IIS and mTOR1 pathways seem to be a major accelerator of aging.

One of the most robust interventions that delays aging in diverse species from yeast to mammals, is caloric restriction. It is defined as a reduction in caloric intake while maintaining essential nutrient requirements. Research in this area has discovered molecules – small-molecule caloric restriction mimetics (rapamycin, metformin) – that provide health benefits and extended lifespan without food intake reduction. It has been shown that these molecules extend the lifespan in mice (24). Caloric restriction also has a positive impact on functions of different populations of SC, for example it increases the number of satellite cells in muscles and improves the function of HSC and germinal SC in fruit flies (26, 27). Caloric

restriction influences SC function through the IIS pathway, mTOR signaling pathways, AMPK, sirtuins, and FOXO transcriptional activity. To summarize, signaling pathways involved in anabolic processes accelerate aging, while limited caloric intake promotes longevity (28).

7. Alterations in microenvironment and the effect of paracrine factors

Similarly, as aging affects SCs, it also affects the microenvironment, the so called SC niche. The bone marrow niche consists of mesenchymal stromal/ stem cells (MSC), osteoblasts, adipocytes, other stromal cells, and extracellular matrix. The ability of the MSCs to proliferate declines with age, whereas the number of adipocytes in the bone marrow increases (29, 30, 31). Molecular mechanisms that promote aging of the SC niche are probably similar to mechanisms involved in the aging of other types of cells (for example, accumulation of ROS, etc.). The extent to which the aged bone marrow niche contributes to the aging of HSCs has not yet been fully explained. One of the main reasons is the inaccessibility of the niche in the bone marrow, its complex architecture and its three-dimensional geometry. It has been demonstrated that the old microenvironment has a negative effect on SC engraftment after transplantation. When young HSC were transplanted into an aged niche, HSCs had less success in engrafting and repopulating the niche, and they also exhibited enhanced myelopoiesis (32). One of the many mechanisms of aging is associated with alterations in adhesions between SCs and niche cells. An aged bone marrow niche also contributes to the loss of HSC polarity, skewing toward myeloid differentiation, expansion of granulocyte-macrophage progenitor cells and dysfunction in lymphocytes B differentiation (33, 34, 35, 36).

Various circulating factor levels also have an influence on the aging of SCs. Many of them were identified because of their rejuvenating effects detected in the blood or plasma of young animals, or animals on caloric restriction diets. Experiments with parabiosis in mice demonstrated that a decline in neural and muscle SC function in old mice can be reversed by circulating factors from young mice (37, 38). It has been observed that most of the therapeutic effects after transplantation probably occur due to soluble factors and are not a result of direct integration of transplanted cells (39).

8. Cell polarity and proteostasis

Proteostasis is a mechanism that regulates the proper folding, functioning, and degradation of cellular proteins through regulated protein translation, chaperone assisted protein folding, and protein degradation pathways. Many researchers have demonstrated that proteostasis alters with age, and there is consequently an accumulation of misfolded or damaged proteins (40). The accumulation of unfolded or misfolded proteins results in the development of some age-related diseases, such as Alzheimer's disease, Parkinson's disease, and cataracts (41). The activity of the two main proteolytic systems, the autophagy-lysosomal system, and the ubiquitin-proteasome system, involved in protein quality control, also declines with age (42, 43).

To prevent the accumulation of the damaged components, SCs have developed a mechanism called asymmetric segregation. After an asymmetric division, the damaged components are distributed into the differentiating cell, whereas the SC remains youthful (6, 44). In this way, SCs have been shown to asymmetrically segregate damaged proteins and mitochondria. In order to divide asymmetrically, the cell has to be polarized. The ability of HSCs to polarize decreases with age, and one of the reasons for this are alterations in the Wnt signaling pathway (45).

SCs also have high autophagy and proteasome activity. Both processes participate in the degradation of damaged proteins. Increased autophagy activity was found in HSC and skin SCs (46), while increased activity of proteasome was found only in embryonic SCs and has not been described in adult SCs (47).

9. Cellular senescence

Cellular senescence is defined as an arrest of the cell cycle. It is triggered by various stress factors like telomere shortening, ROS, damage of nuclear DNA, activation of certain oncogenes or reactivation of tumor suppressor genes. Senescent cells secrete different pro-inflammatory cytokines and chemotactic factors, which are recognized by the immune cells, which then destroy the senescent cell.

When a cell loses its tumor suppressor defense, which includes proteins p53, retinoblastoma proteins, and telomeres, it enters senescence. If

a cell does not enter senescence, its telomerases reactivate, or the cell develops an alternative path of telomere lengthening, it can become malignant (48). In addition to apoptosis, senescence is therefore one of the beneficial mechanisms that contribute to the removal of damaged and potentially oncogenic cells under normal conditions. However, it contributes to the plethora of aging mechanisms if it is not compensated for by a renewal of cells.

Conclusion

We have witnessed major progress in the development of advanced cell therapies in recent decades. SCs that are used by clinicians for various cell-therapies need to be of the best quality, meaning that cell products should not contain senescent or other dysfunctional cells. Understanding the SC aging processes will have a major impact on the quality of advanced cell therapies. It will also affect the selection of donors donating cells for therapies. On the other hand, the quality of cells could be improved by the process of rejuvenation, using the counter-aging mechanisms.

One of the major goals in science today is to improve the quality of life of the aging population. In the future, we will probably be able to rejuvenate tissues and organs. This will improve the healthspan, and consequently increase the lifespan of people. Science will soon establish whether SCs are the "fountain of youth" people have been in search of for over a thousand of years.

Acknowledgements

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MOLEKULARNI MEHANIZMI STARANJA MATIČNIH CELIC

K. Jazbec, M. Jež, M. Justin, P. Rožman

Povzetek: Staranje večceličnih organizmov je kompleksen proces, ki je posledica različnih, med seboj dopolnjujočih se vzrokov. Eden od teh je tudi staranje matičnih celic, katerih biološka funkcija je nadomeščanje celic, ki propadejo zaradi bolezni, poškodb ali normalnega obnavljanja pri ohranjanju homeostaze tkiv. Molekularni mehanizmi, ki so vpleteni v staranje matičnih celic, so podobni kot pri staranju telesnih celic. Vključujejo poškodbe DNK in mutacije, celično senescenco, izčrpavanje zalog matičnih celic, krajšanje telomer, epigenetske spremembe (spremembe histonov in DNKA ter posledično spremenjeno izražanje genov), spremembe v mikroRNK, spremembe v zaznavanju hranil in presnovi, zmanjšano število mitohondrijev in njihovo oslabljeno funkcijo, spremembe v mikrookolju in kopičenje različnih parakrinih dejavnikov ter izgubo celične polarnosti in proteostaze. Matične celice so razvile posebne mehanizme, s katerimi kompenzirajo s staranjem povezano kopičenje napak in ohranjajo svojo matičnost, vendar jih ti mehanizmi v dobri kondiciji lahko ohranjajo le določen čas. V članku opisujemo različne mehanizme staranja matičnih celic in njihove posledice.

Ključne besede: matične celice; staranje; hranilna snov; zaznavanje; niša

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Original Research Article

EFFECT OF METABOLIC CAGE HOUSING ON METABOLIC CHANGES IN THE LIVER OF YOUNG MALE LABORATORY RATS

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Abstract: The aim of this study is to investigate if a metabolic cage housing is appropriate for studies of metabolic changes in rat liver. Metabolic cages are intended for separately collecting animal urine and feces and for measuring of feed and water consumption. They are often used in biomedical research, especially in studies involving metabolism and nutrition, where it is necessary to control the total intake of feed and water. In numerous studies, researchers have found that housing in a condition similar to housing in metabolic cages i.e. grid floor, lack of bedding, lack of movement, and single housing, could cause stress and thus alter the results and influence the research. Two months old male Wistar rats were randomly assigned to two different housing systems for a three week period. One group of rats was housed individually in metabolic cages and rats in the other group were paired and housed in polycarbonate type IV cages with wood shavings bedding material. The body mass and feed consumption were monitored daily. At the end of the study, four rats from each group were anesthetized and the liver and blood samples were taken. The rest of the rats were used for primary hepatocytes cell culture to determinate glucose production. Metabolic cage housing conditions in our study did not cause stress of sufficient impact to alter the test parameters.

Key words: metabolic cage; hepatocytes; insulin resistance; plasma lipids

Introduction

The metabolic cage has been designed for efficient separate collection of urine and feces samples. It is important in biomedical as well as in nutritional experiments where it is imperative to closely monitor total intake of feed and water and to collect urine or feces samples separately. Typical metabolic cage is constructed with a polycarbonate upper chamber with a grid floor and a funnel in the lower chamber. The feeder is placed outside the cage (1). In studies on nutrition and metabolism, there is a strong need to control feeding of the animals and to obtain samples and perform measuring by avoiding stress in animals, which is known to interfere with metabolic status. Stress can lead to fatty liver and other metabolic changes linked with a metabolic syndrome (2). Metabolic cages are also known for not allowing the natural behavior of laboratory animals. Cage is rather small, with no bedding on grid bottom and no hiding places for the animal. Usually, cage holds a single animal. In a normal environment rats and mice are very social animals that live in groups. There are numerous studies on the effect of housing conditions on animal welfare and stress. The lack of bedding seems to be a major problem and grid cages flooring can cause injuries. There is also a strong concern about the

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lack of movement and socialization. In research on single or group housing, female rats demonstrated significant variation in feed intake and triglyceride levels, but not in total cholesterol or body mass gain (3). It was observed that rats housed alone had stress-like responses to common procedures such as cage change, restraint, and subcutaneous or tail vein injection. Rats housed individually had higher heart rate and mean arterial pressure than rats housed in groups, but rats housed in pairs had similar results as rats housed individually (4). Alternating housing conditions in male rats between a standard housing and metabolic cages on grid floor showed reduction in body mass gain and increased excretion of feces, indicating mild stress in animals when housed in metabolic cages. Cortisol levels did not significantly differ between housing conditions (5). In another study, rats were housed on solid bottom or grid floor over a two weeks period and then crossed over to alternate condition for another two weeks. Grid bottom or solid bottom bedded cages had no effect on corticosterone levels, growth, energy expenditure and behavior (6). Considering all possible stressful conditions of a metabolic cage (individual housing, grid floor, no bedding) which can alter metabolic status in rats, control animals were housed in pairs, on a standard solid floor with wood shavings for bedding.

Materials and methods

Animals and housing

Twelve male Wistar rats, two months old, were randomly divided into two groups. The control group, Standard Cage Group (SCG), was kept in standard 425x266x185mm polycarbonate cages, floor area 800 cm^2 (Techniplast, Buguggiate, Italy) with bedding of wood shavings. To reduce social isolation the animals were kept in pairs. The animals in a Metabolic Cage Group (MCG) were housed in metabolic cages with a floor area of 320 cm^2 (Techniplast, Buguggiate, Italy) and kept individually, with no bedding or any enrichment. The room in which the animals were kept was at a constant temperature of 21 °C with twelve hours light and dark cycle.

Principles of animal care (Croatian Animal Welfare Regulation Acts NN135/06; NN 37/13; NN55/13) were followed.

Feed consumption and body mass

Animals were given a standard rodent diet (Mucedola, Milano, Italy), but for the group in a metabolic cage, the pellets were ground. Feed consumption and body mass were monitored daily and the monitoring was held by the same trained handler at the end of the light cycle. The process lasted 3-5 minutes per animal. Animals had access to feed and water *ad libitum*.

Sampling and analysis

After three weeks of monitoring mass gain and feed consumption, four animals from the each group were fasted overnight and were anesthetized by an intraperitoneal injection of sodium thiopental (Rotexmedica GmbH, Trittau, Germany), 10 µg/100 g body mass. The skin of the tail tip was cut for the glucose sampling with the test strip. Three mL of blood from *v. cava inferior* and a liver were taken. The liver was washed in a cold saline, weighed and cut in small sections for histological analysis. The rest of the animals were used to obtain primary liver cell culture. Those animals had not have fasted overnight.

Biochemical analysis

After taking three mL of venous blood in EDTA test tubes, plasma was removed after centrifugation at 3500*g* for 30 min and stored at 4 °C or frozen at -80 °C until evacuation. The serum concentrations of triacylglycerols (TG), total cholesterol (CH), and HDL cholesterol (HDL-CH), were measured enzymatically using commercial kits (Herbos Dijagnostika plc., Sisak, Croatia).

Fasting glucose was measured with a drop of blood from the tip of the tail and was picked on a test strip and measured using Accu Chek glucose meter (Roche Diagnostic, Mannheim, Germany).

Histology

Immediately after cutting the liver into small sections, samples were put in a Carnoy fixative. After appropriate time in fixative, the samples were set in paraffin blocks, cut and stained with hematoxylin and eosin stain.

Primary cell culture

Hepatocytes were isolated by a modified collagen perfusion technique (7) from two animals of each group. Animals were anesthetized with sodium thiopental (10 µg/100 g body mass) via intraperitoneal injection. For the liver perfusion, calcium-free Swim's S-77 medium was used with the addition of collagenase (0.5 g/L). After washing twice with the same media but without added collagenase and insulin, the cells were suspended in M-199 medium containing in addition 2 g bovine albumin, 900 µg L-glutamine and 2.2 g $NaHCO_3$ per liter, to the concentration of one million cells per mL of media. The viability of the cells was greater than 95 % as determined by trypan blue dye exclusion. Three mL of suspension was placed in collagen-coated Petri dishes of 60 mm. The plates were kept in an incubator at 37 °C and 5 % CO_2 gas and 95 % air mixture. After four hours, the media was replaced.

Collagen was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany), collagenase, bovine albumin, L-glutamine and M-199 medium from Sigma-Aldrich (St Luis, MO, USA).

Glucose production in cultured hepatocytes

After 24 hours of incubation, the medium was removed and the hepatocytes were incubated in Hans-Hepes medium without glucose with pyruvate (10 mmol /L). After 0, 60, 120 and 180 minutes of incubation 100 µL samples were collected for determination of glucose. The GOD-PAP method was used using Glucose Liquicolor set. After three hours of incubation, the medium was removed from dishes and the cultures were frozen in liquid nitrogen. Hepatocytes were digested in 0.2 N NaOH to obtain samples needed for the determination of protein by Lowry's method (8).

Insulin and pyruvate were purchased from Sigma-Aldrich (St Luis, MO, USA), Glucose Liquicolor from Human GmbH (Wiesbaden, Germany)

Statistics

For all statistical analyses, two-group comparisons were carried out using Student's t-test. Results were shown as mean SEM and values of $p \leq 0.05$ were considered statistically significant.

Results

Mass gain and feed consumption

Animals had an almost linear body mass gain in both groups. The initial body mass was 253 ± 6.1 g and the final body mass was 310 ± 16.1 for SCG. For MCG the initial mass was 260 ± 7.7 g, and the final was 316 ± 8.2 g (Figure 1). There were no statistical differences in the final body mass gain and feed consumption between groups (Figure 2). Liver mass and liver mass/body mass ratio was the same in SCG and MCG group (Figure 3).

Histology

There were no visible differences in livers between groups. Macroscopically there was no evidence of liver fat accumulation. Liver histology slides have shown no fat accumulation in hepatocytes or other histopathological signs in either of the groups (Figure 4).

Blood lipids and fasting glucose

Results for blood lipids and glucose are shown in Table 1. There were no statistically significant differences between groups. Metabolic cage did not influence on lipids or fasting glucose in blood.

Glucose production

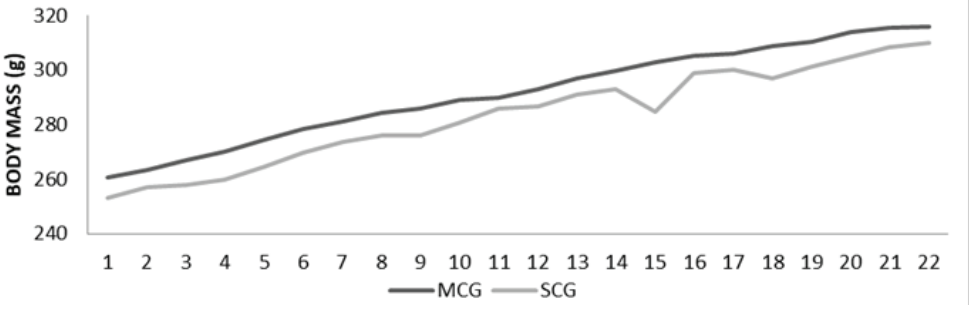

Figure 1: Body mass gain

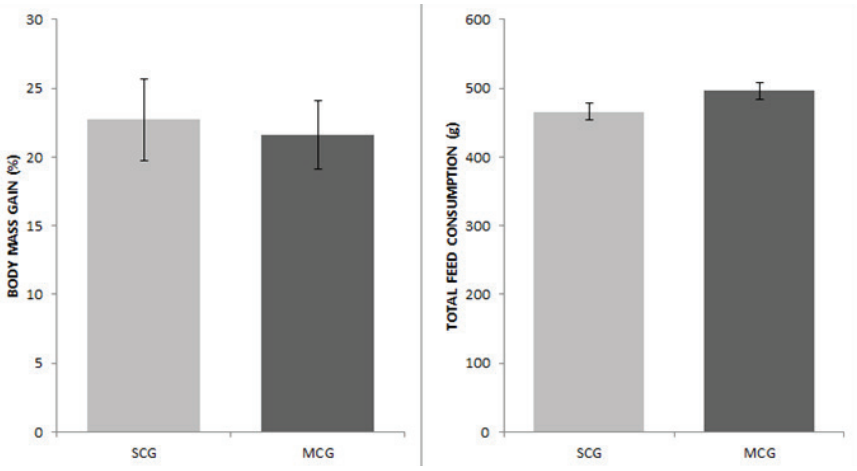

Figure 2: Total mass gain in % of initial mass and total feed consummation in grams. Each point value is a mean ± *SEM*

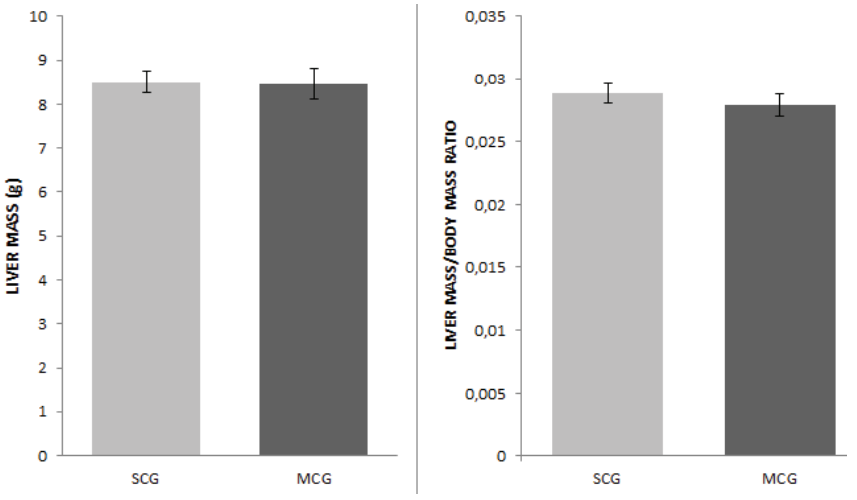

Figure 3: Liver mass and liver mass/ body mass ratio. Each point value is a mean ± *SEM*

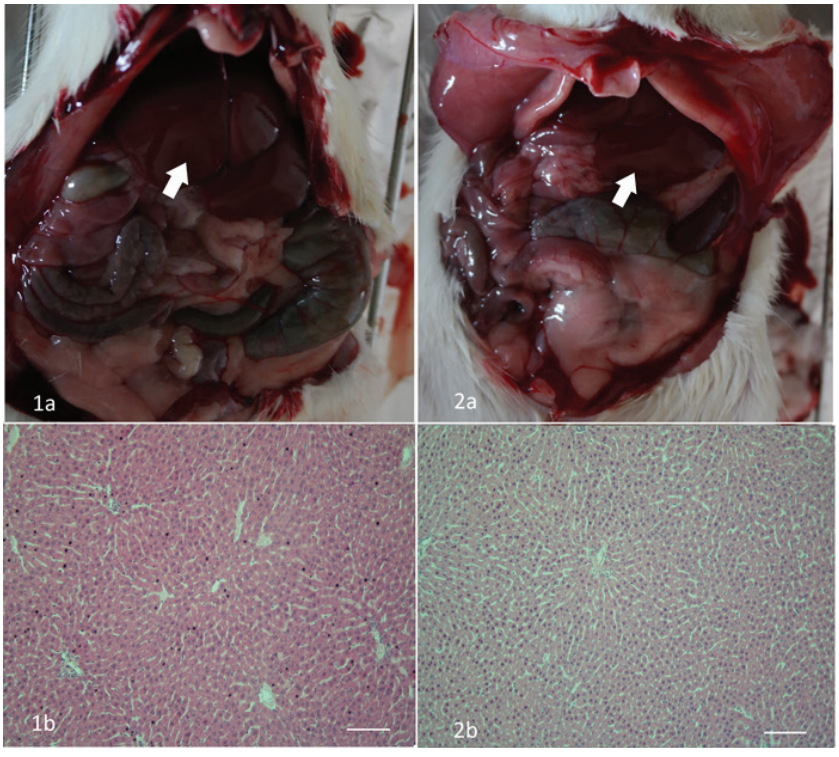

Figure 4: The viscera organs and the liver (arrow) in SCG (1a) and MCG (2a); Histological liver slide H&E, 100x magnifications, scale 100μm in SCG (1b) or MCG (2b)

Table 1: Blood lipids and glucose. Each point value is a mean ± *SEM*

	SCG	MCG
Fasting glucose (mmol/L)	6.45 ± 0.30	5.62 ± 0.77
Triacylglycerols (mmol/L)	0.88 ± 0.19	0.54 ± 0.05
Total cholesterol (mmol/L)	0.89 ± 0.04	0.95 ± 0.06
HDL-cholesterol (mmol/L)	0.58 ± 0.17	0.51 ± 0.07

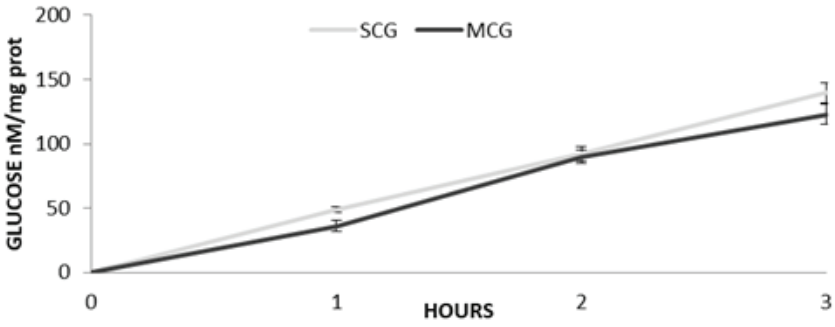

Figure 5: Glucose production (nmol/mg protein) in cultured hepatocytes in glucose-free Hanks-Hepes medium in presence of 10 mmol/L pyruvate. Each point value is a mean ± *SEM*

In the cultures from SCG and MCG groups, gluconeogenesis from pyruvate was constant (Figure 5). There were no statistically significant differences in hepatocyte glucose production isolated from animals in different housing.

Discussion

Housing an animal in a metabolic cage may result in metabolic syndrome as a consequence of restricted physical activity combined with *ad libitum* feeding and stress. In the study on housing activity, it was found that rats kept individually in smaller cages gained more body mass without the differences in feed consumption in comparison with the rats housed in groups using large pens (9). In our study, animals in an MCG and SCG group had the same mass gain and total feed intake, indicating that the housing conditions in our experiment had similar effects on both groups on energy intake and expenditure. It was suggested that elements of housing in metabolic cages such as grit flooring can be associated with an increase in corticosterone levels and injury. Excessive cortisol as a response to environmental and psychological stressors leads to the formation of fatty liver and central accumulation of fat, which in turn leads to insulin resistance (2). Stress can cause a liver enlargement (10). In that study, rats were put into two stressful situations, forced swimming or restraint, 90 minutes every day for two weeks period. Both stress groups had elevated liver mass, but only in forced swimming group this elevation was statistically relevant. Chronic stress study on rats indicated that chronic stress that had lasted three weeks caused dyslipidemia, evident two weeks after stressful conditions (11). A murine model of acute stress demonstrated the development of insulin intolerance and hepatic insulin resistance(12). Also, it has been shown that stress can cause profound changes in hepatic gene expression leading to the development of metabolic syndrome (13). In our research, there were no signs of central or liver fat accumulation. There was no accumulation of fatty droplets in hepatocytes as both groups had a normal histology of liver. Liver mass and liver mass to body mass ratio were the same in both groups. There were no differences in the concentration of blood lipids. Liver glucose production and fasting glucose in blood were not elevated in MCG group compared to SCG group. Animals, who were housed three weeks in metabolic cage housing condition, had the same results of the tested parameters as those housed in standard cage conditions. Modified conditions of the metabolic cage did not lead to dyslipidemia, hyperglycemia or accumulation of fatty droplets in the liver and did not alter liver glucose production in young male rats.

In conclusion, although there was a very different

environment of housing the rats in the metabolic cages compared to their natural surroundings and social behavior, our results show no indication of acute or chronic stress through observed parameters which are altered in these states.

Housing a young male rat for three weeks in metabolic cage had no effect on tested parameters.

Acknowledgements

We are grateful to Prof. Dr. sc. Ljerka Banek M. D., for assistance in histology. This study was supported by the grant No. 065-0982464-0189 from the Croatian Ministry of Science and Technology. This work represents an essential portion of the doctoral research by AUTHOR for the Ph.D. degree at the University of Zagreb.

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VPLIV BIVANJA V PRESNOVNI KLETKI NA SPREMEMBE V PRESNOVI JETER PRI MLADIH PODGANJIH SAMCIH

I. Marolt Banek, J. Roša, D. Ježek, I. Delaš

Povzetek: Cilj študije je bil raziskati ali je bivanje v presnovnih kletkah primerno za proučevanje presnovnih sprememb v podganjih jetrih. Presnovne kletke so namenjene ločenemu zbiranju živalskega urina in blata ter merjenju porabe krme in vode. Pogosto se uporabljajo v biomedicinskih raziskavah, zlasti v študijah presnove in prehrane, kjer je potrebno nadzorovati skupni vnos krme in vode. V številnih študijah so raziskovalci ugotovili, da lahko bivanje v pogojih, ki so podobni metabolnim kletkam tj. na tleh, ki so sestavljena iz mrež, pomanjkanju nastilja, pomanjkanju gibanja in posamičnem bivanju živali povzroča stres in lahko vpliva na rezultate raziskav. Dva meseca stare samce podgan Wistar smo naključno razporedili v dva različna sistema za nastanitev za obdobje treh tednov. Ena skupina podgan je bila nameščena posamično v presnovnih kletkah, podgane druge skupine pa so bile nastanjene v parih v klasičnih polikarbonatnih kletkah tipa IV s steljo iz lesnih ostružkov. Dnevno smo spremljali njihovo telesno maso in porabo krme. Na koncu študije smo štiri podgane iz vsake skupine anestezirali in odvzeli vzorce jeter in krvi. Preostale podgane smo uporabili za pridobivanje primarnih jetrnih celic z namenom določanja proizvodnje glukoze. Pogoji bivanja v presnovnih kletkah v študiji niso povzročili stresa, ki bi imel značilen vpliv na proučevane spremenljivke.

Ključne besede: presnovna kletka; jetrne celice; odpornost na inzulin; plazemske maščobe

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Original Research Article

EFFECTS OF FEEDING GRAINS NATURALLY CONTAMINATED WITH *Fusarium* TOXINS ON SELECTED HAEMATOLOGICAL PARAMETERS AND LYMPHOCYTE SUBSETS IN PRIMIPAROUS SOWS

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Abstract: In a field experiment (54±1 days), 20 primiparous sows (day 89±2 of gestation) were randomly divided into two equal groups. The sows were fed diets naturally containing 0.3 mg/kg of the mycotoxin deoxynivalenol (DON) for the control group and 5.1 mg/kg DON, 0.1 mg/kg zearalenone and 21.6 mg/kg fusaric acid for the experimental group. In the control group, the concentrations of zearalenone and fusaric acid were under detection limit. The sows from the experimental group consumed significantly less feed during gestation (P=0.002), during lactation (P=0.027) and in the weaning to oestrus interval (P<0.001) than control sows. Blood samples were taken four times during the experiment (day 0, 17, 42 and 52). There were no differences in total and differential blood leukocyte count, with the exception of neutrophils on day 52, which reached 42.22±9.02% in the experimental group and 32.10±10.65% in the control group (P=0.040). Flow cytometric analysis of peripheral blood T lymphocytes with monoclonal antibodies against CD3, CD4 and CD8 revealed the percent of both $CD3^+CD4^+$ and $CD3^+CD8^+$ cells. We calculated the absolute number of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells (10^9/L), which showed a decreasing trend in the experimental group, with 2.40±0.63 and 3.83±1.15, respectively, at the beginning and 1.78±0.39 and 2.74±0.89, respectively, at the end of experiment. In the control group, these values were 2.19±0.70 and 3.41±0.87 and finally 2.12±0.69 and 3.11±1.12 x 10^9/L, respectively. The obtained results suggest that feed naturally contaminated with Fusarium toxins reduces the feed intake, influences the neutrophil count, and has immunomodulatory effect on T lymphocyte numbers.

Key words: leukocytes; T lymphocytes; mycotoxins; deoxynivalenol; flow cytometry; sow

Introduction

Trichothecene mycotoxins are a group of over 200 structurally related compounds produced primarily by *Fusarium* species and related fungi. Most studies of trichothecenes have examined deoxynivalenol (DON, vomitoxin) (1). Although DON is not as toxic as other trichothecenes, such as T-2 toxin, HT-2 toxin, or fusarenon-X, it is one of the most common contaminants of wheat, corn and barley (2).

Pigs show the greatest sensitivity to DON compared to poultry, ruminants and laboratory animals. Reduced feed consumption and decreased weight gain are the principal clinical effects observed following ingestion of DON in naturally contaminated feedstuffs (1-3 mg/kg DON in feed) (1). As a result, DON is considered to be a major cause of economic losses due to reduced production performance (3).

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The ability of DON to enhance or inhibit immune function has been well established in mouse, rat and human lymphocytes (4, 5), but there are only a few reports concerning the effects of feeding grains naturally contaminated with DON to first-time pregnant sows, which represent a particularly sensitive category among pigs. Previous reports mostly investigated the feed consumption and the effects on newborn piglets (6-10) and the transfer of DON from naturally contaminated feed from the sow to the piglets (11). Nevertheless, some studies examined the effects of DON on immune response in other categories of pigs using classical immunological methods, such as lymphocyte proliferation assays and analysis of metabolic activity with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (12-17).

Flow cytometry has become increasingly important in analysing immune responses in animals. The recent development of monoclonal antibodies (mAb) directed against cluster of differentiation (CD) and other membrane molecules of porcine leukocytes has improved phenotypic characterisation and functional analysis of various porcine leukocyte populations (18). Within a diverse panel of T lymphocyte-specific surface antigens, CD3 molecules were shown to be most potent marker for the characterization and definition of this leukocyte population (19). CD4 is a cell surface protein characteristic of T helper cells (Th), and expression of CD8 is associated with cytotoxic T lymphocytes (Tc) (20).

$CD4^+$ and $CD8^+$ lymphocytes play a crucial role in immune responses. Therefore, the present study aimed to establish the effects of feedstuff containing DON, zearalenone (ZEN) and fusaric acid (FA) on two lymphocyte subpopulations, the double labelled lymphocytes $CD3^+CD4^+$ and $CD3^+CD8^+$. The Commission recommendations on the presence of DON, ZEN, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal consumption (2006) suggest that complementary and complete feedstuffs for pigs do not exceed 0.9 mg/kg DON and 0.1 mg/kg ZEN. There is no recommendation for FA. FA is probably the most widely distributed mycotoxin produced by *Fusarium* species, and according to Bacon et al. (21), FA may serve as a presumptive indicator of *Fusarium* contamination in food and grain. In addition, FA is known to have synergistic effects with other fusariotoxins. In our study, only the concentration of DON was above the recommended concentration; thus, we primarily focused on DON.

The effects of such feed on feed consumption and reproduction parameters (duration of parturition, number and weight of newborns and weanling piglets) are described elsewhere (22).

Material and methods

Experimental animals, breeding environment and feeding

The experiment was performed on a large pig farm in Slovenia with 3,600 sows, where there was a farrow-to-finish operation (22). The Veterinary Administration of the Republic of Slovenia approved realization of the experiment.

Briefly, twenty pregnant primiparous sows (Landras x Large White, day 89±2 of gestation) were randomly chosen and divided into two equal groups: the control and experimental. The sows participated in the trial 22 to 26 days before farrowing, during the lactation period (21 days) and in the period between weaning and reinsemination (5-8 days) for a total of 54±1 days.

During gestation, the sows were fed 3.5 kg/day of diet divided into two meals (6 a.m. and 1 p.m.). The feed allowance from the day of farrowing until weaning (at day 21) was 6.0 kg per sow a day. From weaning the piglets to reinsemination, the sows were fed again with 3.5 kg of feed per day, divided into two meals. To obtain relevant information about feed consumption, we collected the remaining feed and weighed it daily.

The mycotoxin content of the experimental diets

The feed was composed of 50% maize, 19% soybean meal, 8% barley, 7% beet pulp, 4.5% sunflower seed oil, 3% wheat feed, 3% dehydrated lucerne, 3% fish meal, 0.5% molasses and 2% vitamin and mineral mix. All components in the feed were the same for both groups; only the maize used in the experimental diet was naturally contaminated with DON.

The feedstuffs for both groups of sows were analysed as previously described (22). The nutritional value corresponded to all the nutritional requirements for gestating and lactating sows (23).

Sample collection

One hour after the morning feeding, sterile blood samples from sows were taken (Vacutainer systems with a luer, Vacutainer® Brand Pronto™ Holder, Becton Dickinson) from the *cranial vena cava* on days 0, 17, 42 and 52 of the experiment and transferred into tubes containing lithium (Li) heparin.

White blood cell (WBC) count

The blood samples were analysed immediately after blood collection using standard haematological equipment. Values of leukocytes (WBC) were measured by Coulter Counter ZF_6 (Coulter Electronic, UK). Differential leukocyte counts, neutrophils (Ne), eosinophils (Eo), basophils (Ba), lymphocytes (Ly), band neutrophils (Nb) and monocytes (Mo) were determined microscopically on blood smears stained by Pappenheim; 100 leukocytes were identified and differential leukocyte counts were calculated.

Flow cytometry assay

For phenotyping, mAb against various cell surface molecules were used: mouse anti-pig CD3ε conjugated with fluorescein isothiocyanate (FITC), mouse anti-pig CD4a conjugated with R-phycoerythrin (R-PE) and mouse anti-pig CD8a (R-PE). mAb were purchased from BD Bioscience. Two-colour immunofluorescence analyses by flow cytometry were performed for the identification of CD4 ($CD3^-CD4^-$, $CD3^+CD4^-$, $CD3^-CD4^+$, $CD3^+CD4^+$) and CD8 ($CD3^-CD8^-$, $CD3^+CD8^-$, $CD3^-CD8^+$, $CD3^+CD8^+$). Optimal dilutions of mAb were standardized in previous experiments. Then, 100 µl of anticoagulated blood was incubated with 2.5 µl of CD3ε mAb and either 5 µl of CD4a mAb or 5 µl CD8a mAb for 20 min at room temperature in the dark. After incubation, erythrocytes were lysed with FACS Lysing Solution (Becton Dickinson). Cells were washed twice in PBS (FACSFlow, Becton Dickinson). Acquisition of data was performed using a FACSCalibur flow cytometer, and analysis was performed with CellQuest software (both Becton Dickinson). A lymphocyte gate was defined according to the position in the forward scatter/side scatter distribution, and the percentage of marked lymphocytes was determined in each sample. At least 10,000 cells were analysed for each sample.

Statistical analysis

The data obtained in the study was statistically analysed, and the significance between groups was determined by paired t-tests. The difference was considered statistically significant when $P<0.05$. Pearson's correlation was used to determine the correlation between continuous variables (consumption of feed and values of $CD3^+CD4^+$, $CD3^+CD8^+$ and neutrophils). The SPSS statistical programme (Statistical Package for Social Sciences, Version 15, 2006) was used.

Results

The chemical analysis and mycotoxin content of diets

The chemical composition and mycotoxin content of both diets are presented in Table 1.

In both feeds, aflatoxin B_1 (<0.20 µg/kg), ochratoxin A (<0.01 mg/kg), 15 A-DON (<0.05 mg/kg), nivalenol (<0.05 mg/kg), fusarenon-X (<0.05 mg/kg), DAS (<0.03 mg/kg), T-2 toxin (<0.03 mg/kg), HT-2 toxin (<0.03 mg/kg) and fumonisins B_1, B_2, and B_3 (<0.10 mg/kg) were under the detection limits.

Feed intake and body weight change of sows

Sows in the experimental group consumed statistically less feedstuff than the sows in the control group (22). The mean weight loss in the experimental group was 21.5 kg (11.2%), from 191.3±12.5 kg at the beginning of the experiment down to 169.8±9.29 kg at piglet weaning. However, in the control group the mean weight loss was only 15.5 kg (8.1%), from 192.0±12.68 kg down to 176.5±12.28 kg. However, the differences between the groups were not statistically significant.

WBC count

As demonstrated in Table 2, the *Fusarium* toxin-contaminated feed did not alter WBC count or differential leukocyte counts in general. However,

Table 1: Analysed composition and mycotoxins content of diets

Parameter (unit)	Diet	
	Control	Experimental
dry matter (g/kg)	889.7	889.8
moisture (g/kg)	110.3	110.2
crude protein (g/kg)	162.5	162.5
crude fibre (g/kg)	53.0	49.4
crude fat (g/kg)	74.0	72.0
ash (g/kg)	72.1	68.2
ME (MJ/kg dry matter)[1]	13.4	13.5
deoxynivalenol, DON (mg/kg)	0.3	5.1
zearalenone, ZEN (mg/kg)	< 0.02	0.1
fusaric acid, FA (mg/kg)	< 0.77	21.6

[1]*Metabolic Energy*

Table 2: The effects of *Fusarium* mycotoxin intoxication on the total and differential leukocyte count (mean and SD)

Experimental day	Group	Parameters						
		WBCx10^9/L	Ne %	Nb %	Eo %	Ly %	Mo%	Ba %
day 0	control	14.14 (3.07)	28.40 (8.42)	0.00 (0.00)	2.50 (1.35)	68.90 (7.70)	0.00 (0.00)	0.10 (0.32)
	experimental	17.54 (4.67)	30.78 (11.49)	0.00 (0.00)	2.44 (1.13)	66.44 (10.99)	0.11 (0.33)	0.22 (0.44)
	P	0.75	0.61	-	0.92	0.57	0.30	0.49
day 17	control	15.12 (4.10)	38.60 (10.28)	0.00 (0.00)	5.40 (3.02)	56.00 (10.55)	0.00 (0.00)	0.00 (0.00)
	experimental	15.41 (4.35)	36.78 (5.71)	0.00 (0.00)	4.70 (2.58)	58.00 (6.67)	0.11 (0.33)	0.00 (0.00)
	P	0.88	0.64	-	0.82	0.63	0.30	-
day 42	control	16.80 (5.44)	49.10 (9.96)	0.00 (0.00)	4.70 (2.21)	44.90 (9.74)	1.30 (1.41)	0.00 (0.00)
	experimental	15.61 (1.72)	40.67 (7.90)	0.00 (0.00)	6.89 (5.11)	52.11 (7.84)	0.33 (0.70)	0.00 (0.00)
	P	0.54	0.06	-	0.23	0.09	0.08	-
day 52	control	14.19 (4.85)	32.10 (10.65)	0.60 (0.84)	7.20 (4.49)	59.30 (10.97)	0.60 (0.84)	0.20 (0.42)
	experimental	15.48 (3.92)	42.22 (9.02)	0.20 (0.42)	4.78 (2.99)	52.33 (7.10)	0.44 (0.72)	0.11 (0.33)
	P	0.53	0.04	0.12	0.19	0.12	0.67	0.62

WBC, leukocytes; Ne, neutrophils; Eo, eosinophils; Ba, basophils; Ly, lymphocytes, Nb, band neutrophils; Mo, monocytes; the difference is statistically significant when $P<0.05$; experimental group: 5.1 mg/kg DON, 0.1 mg/kg ZEN and 21.6 mg/kg FA

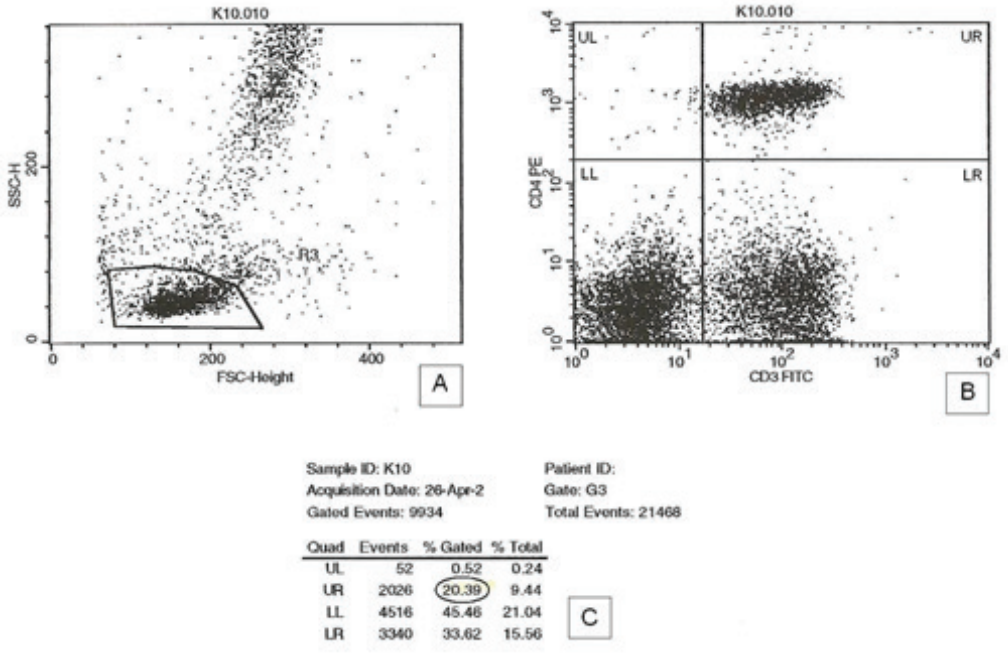

Figure 1: Dot plots of flow cytometry analysis. The lymphocytes are gated for further analysis (A). The marked lymphocytes show green (FITC) and red (PE) fluorescence (B). The percent of lymphocytes in individual quadrants (C). The percent of $CD3^+CD4^+$ lymphocytes is circled in the table below the graphics

Table 3: Effects of consumption of *Fusarium* toxins on blood lymphocyte subsets

Experimental day	group	Absolute number of lymphocytes $\bar{x}$ (SD) x 10^9/L	% of cells, obtained with flow cytometry $\bar{x}$ (SD)		Absolute number $\bar{x}$ (SD) x 10^9/L	
			$CD3^+CD4^+$	$CD3^+CD8^+$	$CD3^+CD4^+$	$CD3^+CD8^+$
day 0	control	9.74 (2.44)	22.47 (4.73)	35.27 (6.63)	2.19 (0.70)	3.41 (0.87)
	experim.	11.54 (3.03)	20.96 (2.53)	33.04 (4.07)	2.40 (0.63)	3.83 (1.15)
	P	0.169			0.491	0.374
day 17	control	8.33 (2.41)	22.88 (3.77)	34.38 (3.88)	1.89 (0.58)	2.87 (0.89)
	experim.	9.04 (2.91)	20.25 (1.99)	33.54 (7.90)	1.81 (0.55)	3.12 (1.20)
	P	0.570			0.755	0.608
day 42	control	7.23 (1.43)	27.59 (4.81)	38.59 (5.03)	1.96 (0.37)	2.78 (0.57)
	experim.	8.17 (1.71)	23.37 (3.70)	33.66 (4.77)	1.89 (0.42)	2.72 (0.59)
	P	0.210			0.697	0.842
day 52	control	8.20 (2.61)	26.03 (4.58)	37.57 (3.03)	2.12 (0.69)	3.11 (1.12)
	experim.	8.07 (81.98)	22.57 (3.43)	33.94 (6.17)	1.78 (0.39)	2.74 (0.89)
	P	0.907			0.222	0.440

experim., experimental group (5.1 mg/kg deoxynivalenol, 0.1 mg/kg zearalenone and 21.6 mg/kg fusaric acid; the difference is statistically significant when $P<0.05$)

at the end of the experiment, there was a higher percentage of neutrophils in the experimental group of sows consuming feed contaminated with 5.1 mg/kg DON (P=0.040) than the control group.

Pearson's correlation shows that the feedstuff consumed did not correlate with the percentage of neutrophils at day 52 in the control group (r=0.524, P=0.120) and the experimental group (r=-0.172, P=0.658).

Flow cytometry

The percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells were determined by flow cytometry. The model for flow cytometric analysis of the $CD3^+CD4^+$ lymphocyte subpopulation, expressed as a relative proportion of gated lymphocytes, is presented in Figure 1 (A). Diagram B shows both fluorescence intensities applied on the x- and y-axes. The percentages of marked cells in individual quadrants are shown in Figure 1 (C), under diagram A and B. For further analyses, only the values from the UR quadrants were used and compared between the groups.

The same procedure was followed to determine the percentage of $CD3^+CD8^+$ cells, and the results are given in Table 3. From these values and from the lymphocyte numbers obtained at the differential leukocyte count, the absolute numbers of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells were calculated (Table 3). A notable difference between groups was observed towards the end of the experiment, when absolute numbers of $CD3^+CD4^+$ and $CD3^+CD8^+$ in the control group showed greater increases (2.12±0.69 x 10^9/L and 3.11±1.12 x 10^9/L, respectively) than the experimental group (1.78±0.39 x 10^9/L and 2.74±0.89 x 10^9/L, respectively) (P=0.222 and P=0.440, respectively).

Pearson's correlation showed that the feedstuff consumed did not correlate with the values of $CD3^+CD4^+$ cells on day 52 in the control group (r=-0.216, P=0.549) and the experimental group (r=0.143, P=0.713). Similar results were observed for $CD3^+CD8^+$ cells in the control (r=-0.279, P=0.405) and experimental group (r=0.272, P=0.479).

Discussion

The comparison between the control group and the experimental group confirmed previous results that DON reduces feed consumption. Notably, in addition to DON, ZEN (0.1 mg/kg) and FA (21.6 mg/kg) were present in the diet for the experimental group, and consequently, they may have had an effect on the results as well. In many trials, the FA concentrations were not analysed, although synergistic interactions between FA and DON have been reported. FA was shown to increase the toxicity of DON in starter pigs (24). FA concentration, however, is seldom determined in swine feeds due to its low toxicity when consumed in the absence of other toxins (25, 26).

The percentage of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells, representing the Th and Tc, obtained with flow cytometry and their calculated absolute numbers indicated that the feed may have immunomodulatory effects and suppress the immune response of sows in the perinatal period. Recently, Ferrari et al. (27) used flow cytometric analysis to characterize and quantify the lymphocyte subsets $CD3^-CD8^+$, $CD4^+CD8^-$, $CD4^-CD8^+$, $CD8^{high}$, $CD4^+CD8^+$ and TCRγ/δ+ in 8-week-old pigs that received 0.5 mg/kg DON in the first week and 1 mg/kg for the next 5 weeks. Although increased mean absolute values for Tc ($CD4^-CD8^+$, $CD8^{high}$) were observed in the control group in the last experimental weeks, the DON treatment did not significantly influence the levels of lymphocyte subpopulations, which is similar to the findings in our study.

Mycotoxins, DON among them, are believed to be one of the most immunosuppressive factors in animal diets. For the vast majority of mycotoxicoses, which are chronic, the signs of disease are generally subtle and unspecific. Thus, it is difficult to establish a cause-effect relationship to contaminated feedstuffs (3). Our experiment had similar results: the reduced feed consumption was the only clinically observed difference between the groups. However, the analysis of WBCs showed that towards the end of the experiment, sows that consumed 5.1 mg/kg DON per feed had a significantly increased relative number of neutrophils in their blood. The increased number of neutrophils occurs in the early stage of bacterial infection, which could lead to three conclusions: the sows from the experimental group are more susceptible than those from the control group and defend themselves from subclinical infections, this is their prolonged reaction to parturition, or DON influences the secretion of immune system mediators, which increase the number of neutrophils. An increased number of neutrophils

was also by reported Rotter et al. (12) in pigs that consumed feed with 0.75-3 mg/kg DON for 29 days. Other authors did not find these results (14).

A recent report (28) found that low concentrations of DON can alter the immune functions of pig polymorphonuclear cells, the first line of defence against infection, which suggests the involvement of p38 mitogen-activated protein kinase in the signal transduction pathway. These immunosuppressive effects of DON may have implications for humans and/or animals when eating contaminated food/feed.

The absolute numbers of both the $CD3^+CD4^+$ and $CD3^+CD8^+$ subsets towards the end of the experiment increased more obviously in the control group. These changes might be caused by damage of macrophages or T regulatory cell activities. Chen et al. (29), working with pigs fed DON, described the decreased mRNA expression levels of IFN-γ, TNF-α and IL-2, which could be a possible explanation for the decreased number of Tc ($CD3^+CD8^+$) cells in the experimental group.

The characteristics of porcine lymphocytes forced us to take into consideration not only Th lymphocytes ($CD3^+CD4^+$) and Tc ($CD3^+CD8^+$) cells but also the double positive cells $CD4^+CD8^+$ cells. These cells can be found in extrathymic sites in healthy pigs, while in humans and mice, this population is found only in some physiological disorders (30). Porcine $CD4^+CD8^+$ double positive lymphocytes were shown to increase gradually with age (30-55% by 3 years of age). Cells could proliferate in response to stimulation with recall viral antigen, consistent with the hypothesis that this population in swine includes memory/effector T cells (18, 31, 32). This cell function obscurity is acceptable in our experiment because the sows were young, and the focus of our study was not to define the exact number of Th and Tc cells but to establish the differences between groups.

Dabrowski et al. (33) studied the in vivo effect of low doses of ZEN and DON, administered individually or in combination, on immune system function based on the subpopulations of $CD4^+8^-$, $CD4^+8^+$ and $CD4^-8^+$ lymphocytes in the peripheral blood of pigs. The results revealed that long-term exposure to low doses of ZEN, DON and ZEN+DON disrupted linear proliferation of $CD4^+8^+$ cells. Co-contamination of feed with both mycotoxins had a stronger effect on the immune system and led to a transient decrease in the percentage of $CD4^+8^+$ lymphocytes in week 5 of exposure. Another study (34) also suggested that prolonged exposure to low doses of DON can change the proportions of immunocompetent cells (a shift towards humoural immunity), without affecting their overall counts.

In experiments with *Fusarium* mycotoxins on animal models, it is important to evaluate correlation between described changes and decreased feed consumption. By using Pearson's correlation, we demonstrated that the results for the absolute number of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells and the percentage of neutrophils at the end of the experiment did not have any correlation with sow consumption.

In conclusion, the results of this study could be useful for further elucidation of the cellular basis of immune responses to *Fusarium* toxins in pigs and consequently in humans, since pigs are used as animal models for human diseases. In addition, humans, as well as animals, are exposed to mycotoxins by consumption of contaminated grains.

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UČINEK KRME, NARAVNO KONTAMINIRANE S TOKSINI PLESNI *Fusarium* sp., NA IZBRANE HEMATOLOŠKE PARAMETRE IN LIMFOCITNE PODVRSTE PRVIČ BREJIH SVINJ

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Povzetek: V nadzorovanem poskusu, ki je na veliki farmi prašičev trajal 54±1 dan, smo 20 mladic (breje 89±2 dni) naključno razdelili v dve enaki skupini. Živali so zauživale krmo, ki je bila v kontrolni skupini naravno kontaminirana z mikotoksinom deoksinivalenolom v koncentraciji 0,3 mg/kg krme. Krma za poskusno skupino je vsebovala 5,1 mg/kg deoksinivalenola, 0,1 mg/kg zearalenona in 21,6 mg/kg fuzarne kisline. Mladice iz poskusne skupine so v obdobju brejosti (P = 0,002), laktacije (P = 0,027) in v času od odstavitve pujskov do ponovne osemenitve (P < 0.001) zaužile statistično značilno manjšo količino krme kot živali iz kontrolne skupine. Med poskusom smo mladicam štirikrat odvzeli kri (dan 0, 17, 42 in 52). V skupnem številu levkocitov in diferencialni krvni sliki med skupinama ni bilo statistično značilnih razlik, razen v številu nevtrofilcev 52. dan poskusa, ko je bila vrednost v poskusni skupini 42.22±9.02 % in v kontrolni skupini 32.10±10.65 % (P = 0.040). S pretočno citometrijo smo določili delež limfocitov T in njihovih podvrst, celic T pomagalk ($CD3^+CD4^+$) in citotoksičnih celic T ($CD3^+CD8^+$), ki smo jih označili z monoklonskimi protitelesi. Iz dobljenih podatkov smo izračunali absolutno število $CD3^+$ $CD4^+$ in $CD3^+$ $CD8^+$ (10^9/L), ki se je v poskusni skupini zmanjševalo od 2.40±0.63 in 3.83±1.15 na začetku poskusa do 1.78±0.39 in 2.74±0.89 na koncu poskusa. Vrednosti v kontrolni skupini so bile na začetku poskusa 2.19±0.70 in 3.41±0.87 ter na koncu 2.12±0.69 in 3.11±1.12 x 10^9/L. Rezultati kažejo, da zauživanje krme, naravno kontaminirane s toksini plesni vrste *Fusarium*, vpliva na ješčnost živali, število nevtrofilcev in ima imunomodulatorni učinek na število limfocitov T.

Ključne besede: levkociti; limfociti T; mikotoksini; deoksinivalenol; pretočna citometrija; prašiči

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Original Research Article

ANTIBACTERIAL ACTIVITY OF CLOVE ESSENTIAL OIL AND EUGENOL AGAINST FISH PATHOGENIC BACTERIA ISOLATED FROM CULTURED OLIVE FLOUNDER (*Paralichthys olivaceus*)

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Abstract: The antibacterial activity of clove (*Syzygium aromaticum*) essential oil (CEO) and eugenol was tested against seven Gram-negative and nine Gram-positive fish pathogenic bacteria isolated from cultured olive flounder (*Paralichthys olivaceus*) in Korea. Eugenol was >99% concentrated and CEO consisted of seven chemical compounds including 83.63% of eugenol. Disk diffusion assay, MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) tests showed CEO and eugenol inhibited the growth of both Gram-negative and Gram-positive bacteria. CEO (MBC/MIC= 1-2) and eugenol (MBC/MIC= 1-4) were bactericidal for all tested strains. The percentage of relative inhibition zone diameter exhibited high values at higher concentration of both CEO and eugenol. Positive correlation was observed between MAR index values and MIC values of CEO (r =0.61) and eugenol (r =0.15). Since antibacterial activity of CEO and eugenol were effective against fish pathogenic bacteria, the use of CEO and eugenol could be advantageous to treat bacterial infections in aquaculture.

Key words: antibacterial activity; clove essential oil (CEO); eugenol; fish pathogenic bacteria; olive flounder

Introduction

Bacterial diseases pose one of the major threats to aquaculture industry worldwide. The crucial bacterial diseases of marine fish in Korea are edwardsiellosis caused by *Edwardsiella tarda*; streptococcosis caused by *Streptococcus iniae*, *S. parauberis*, and *Lactococcus garvieae*; and vibriosis caused by *Vibrio harveyi, V. ichthyoenteri,* and *Photobacterium damselae*, which have recently increased in cultured fish populations (1, 2). Disease outbreaks are responsible for elevated mortality rates and decrease of the productivity, causing a high economic loss in olive flounder (*Paralichthys olivaceus*) aquaculture in Korea (3).

Antibiotics are widely used to treat bacterial infections in fish. However, misuse of antibiotics leads to drug resistance and thereby to the reduced efficacy of the drugs (4). Antibiotics accumulate in the environment and fish, hence pose a potential risk to humans and the environment (5). Therefore, it is essential to develop antibacterial treatments that are made from natural substances. Natural products especially from plants have been investigated for their therapeutic and prophylactic

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effects on several fish diseases (6). Essential oils are one kind of plant products which have been used with their aromatic, flavor, bactericidal, preservative and medicinal properties (7).

One of the most influential antimicrobial medicinal herbs is clove (*Syzygium aromaticum*) bud belonging to Myrtaceae family, indigenous to India, Indonesia, Zanzibar, Mauritius and Sri Lanka (8). Clove essential oil (CEO) was reported to possess antibacterial, antifungal, insecticidal, and antioxidant properties (9-11). These activities of CEO are mainly due to its high content of phenolic derivatives. Eugenol, a phenylpropene compound with strong biological and antimicrobial activities, is the major constituent of CEO. Eugenol was reported to play an important role in inhibiting the growth of bacteria (12). It can denature protein and react with phospholipids in the cell membrane. It also affects the transport of ions and ATP and changes the fatty acid profile of different bacteria (13, 14). Antimicrobial mechanism of eugenol has been found, affecting not only the membrane but also the envelope of fungal and bacterial cells (15).

However, until now no study has been conducted to investigate the antimicrobial property of CEO and its major component against fish pathogenic bacteria isolated from olive flounder. Therefore, this study was carried out to examine the potential of CEO as well as eugenol as alternatives to commercial antibiotics in aquaculture use.

Materials and methods

As test strains, seven Gram-negative and nine Gram-positive bacterial strains isolated from Korean cultured olive flounder were used. The Gram-negative strains were *E. tarda* (FP5060, ED47, Yoshida and ED45), *P. damselae* (FP4101), *V. harveyi* (FP 8370) and *V. ichthyoenteri* (FP 4004), and the Gram-positive strains were *L. garvieae* (FP5245), *S. iniae* (FP5228, S186, S530 and S131) and *S. parauberis* (FP5228, S124, S527 and S1466).They were obtained from Geyongsang National University (Jinju, Korea) and National Institute of Fisheries Science (Busan, Korea). The 100% pure CEO (Aromarant Co. Ltd., Rottingen, Germany) purified from the bud of clove grown in Zanzibar and the commercial eugenol (>99%) (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) were purchased.

The disk diffusion assay with CEO and eugenol was conducted to detect the antimicrobial activity. Sterile disks (Advantec Toyo Kaisha, Ltd., Japan) were impregnated with 20 μL of CEO and eugenol with different dilutions; [1%, 5%, 10%, 25%, 50%, 100 % (V/V)] and each disk was placed on a Mueller Hinton agar (MB Cell, LA, CA) plate smeared with the test organism. Plates were incubated for 24 h at 27 °C to determine the antimicrobial effect. Antibacterial activity was determined by measuring the inhibition zone diameter (IZD) (mm) against each test organism. The antimicrobial activity expressed as percentage of relative inhibition zone diameter (RIZD %) and it was calculated according to Njau et al. (16) using amoxicillin as a standard antibiotic. The determination of MIC was done using broth micro dilution method with some modifications using different concentrations which 5% DMSO was used to dissolve CEO and eugenol. The MIC was measured after 24 h incubation and each test was conducted in triplicates. In order to determine the MBC, the cultured medium from wells which have higher concentration of CEO or eugenol than MIC was smeared on separate Tryptic Soy Agar (TSA) (MB Cell, LA, CA) plates and incubated for 24 h at 27 °C (17). The concentration, at which no growth was observed on TSA plate, was determined as the MBC.

Antibiotic susceptibility was determined by disk diffusion method using sixteen antibiotics. Resistance profiles (resistant, intermediate or susceptible) were assigned using criteria described by Clinical and Laboratory Standards Institute (CLSI) (18). Each test was repeated three times. Following the disc diffusion test results of antibiotics, multiple antibiotic resistance (MAR) index was calculated as the ratio of the number of antibiotics to which bacteria was resistant to the total number of antibiotics to which the bacteria was exposed. For the statistical analysis, differences were considered significant at $p<0.05$. The correlation indices were calculated using the Pearson coefficient (r).

Results

The CEO obtained in the present study constituted 83.63% of eugenol and other six chemical compounds (Table 1).

Table 1: Composition of clove essential oil used in this study

Compound name	Composition %*
Eugenol	83.63
β-Caryophyllene	10.5
α-Humulen	2.78
Eugenyl-acetate	1.02
caryophyllene Oxide	0.39
Cadinene	0.37
Cadalene	0.35

*Composition of the essential oil was analysed by Neumond GmbH, Raisting, Germany

IZD of Gram-negative bacteria ranged from 19 to 27 mm and IZD of Gram-positive bacteria ranged from 15 to 25 mm in 100% (V/V) of eugenol while IZD of Gram-negative bacteria ranged from 16 to 20 mm and IZD of Gram-positive bacteria ranged from 14 to 22 mm at 100% (V/V) of CEO. RIZD % exhibits high values at higher concentration of both eugenol and CEO (Table 2).

MIC values of the CEO for Gram-negative bacterial strains *ranged* from 0.125 to 0.5% (V/V) and for Gram-positive strains *ranged* from 0.25 to 0.5% (V/V). MIC of eugenol for Gram negative bacteria ranged from 0.0312 to 0.0125% (V/V) and for Gram-positive bacteria it was 0.125% to 1 % (V/V). Mean MBC/MIC for CEO was 1 to 2 and for eugenol it was 1to 4 (Table 3).

The MAR index of four strains [one *E. tarda* (ED47), one *L. garvieae* (FP5245), one *S. iniae* (FP3287) and one *S. parauberis* (S124)] have been calculated as ≥ 0.2. The observed Pearson coefficient (r) between the MAR index values and MIC values of eugenol and CEO were r =0.15 and r =0.61, respectively (Figure 1).

Discussion

Many reports have claimed that eugenol is the major compound in the CEO. However, inhibitory activity of clove is due to the presence of several constituents, mainly eugenol, eugenyl acetate and β- caryophyllene (19, 20). Sohilait (21) reported same composition range of eugenol in clove bud (81.13 - 84.44%).

According to disk diffusion test result, CEO inhibited the growth of all test bacteria at every concentration except 1 % (Table 2). Chaieb, (22) observed that each bacterial strain demonstrated a significant degree of sensitivity to the CEO, and extensive activity against Gram-positive bacteria, producing a clear zone of inhibition against the majority of the tested strains.

Meanwhile, eugenol was found to be effective against almost all of Gram-negative strains except *E. tarda* (ED45) strain at every concentration, and the widest IZD was 27 mm against *V. ichthyoenteri*. Eugenol exhibited higher activity against Gram-negative strains than Gram-positive strains and several studies have reported similar results against pathogenic bacteria (12, 20). In another study (23), eugenol showed the highest effect against *S. aureus* compared to the Gram-negative bacterial strains in contrast to the other previous studies.

In comparison, eugenol exhibited highest IZD (27 mm) against Gram-negative [*V. ichthyoenteri* (FP 4004)] bacterial strain and CEO exhibited highest IZD (22 mm) against Gram-positive [*S. iniae* (S186)] bacterial strain. The inhibition zones induced by the CEO or eugenol were relative to the concentration of eugenol. CEO contains 86.63% of eugenol as well as six other components, whereas concentration of commercial eugenol was >99%. The different components of essential oils can act on bacterial proteins using several mechanisms and may affect cell division (14). However, eugenol and CEO were effective against both Gram-positive and Gram-negative microorganisms. In every tested fish pathogenic bacteria, the IZD increased in proportion to the eugenol or CEO concentration and the maximum effect was found at 100% (V/V) concentration of both

Table 2: Inhibition zone diameter and the percentage of relative inhibition zone diameter (RIZD %) values of clove essential oil (CEO) and eugenol against fish pathogenic bacteria

Bacteria	Inhibition zone diameter (mm) and RIZD% *																							
	1 % (V/V)				5 % (V/V)				10 % (V/V)				25 % (V/V)				50 % (V/V)				100 % (V/V)			
	CI	CR	EI	ER	CI	CR	EI	ER	CI	CR	EI	ER	CI	CR	EI	ER	CI	CR	EI	ER	CI	CR	EI	ER
Gram-negative																								
Vibrio harvey (FP8370)	0	0	8	33.3	10	41.7	14	58.3	12	50	17	70.8	15	62.5	20	83.3	18	75	23	95.8	20	83.3	24	100
V. ichthyoenteri (FP4004)	0	0	8	0.21	11	28.9	13	34.2	12	31.6	15	39.5	15	39.5	18	47.4	19	50	21	55.3	20	52.6	27	71.1
Photobacterium damselae (FP4101)	0	0	7	25.9	8	29.6	11	40.7	11	40.7	17	63	14	51.9	18	47.4	17	62.9	19	70.4	19	70.4	20	74
Edwardsiella tarda (FP5060)	0	0	8	29.6	8	29.6	13	48.1	10	37	14	51.9	12	44.4	20	74.1	13	48.1	21	77.8	16	59.3	22	81.5
E. tarda (ED47)	0	0	7	29.2	8	33.3	10	41.7	10	41.7	16	66.7	13	54.2	18	75	15	62.5	20	83.3	16	66.7	22	91.7
E. tarda (Yoshida)	0	0	7	25.9	10	37	10	37	11	40.7	12	44.4	14	51.9	15	55.6	16	59.3	17	63	18	66.7	19	70.4
E. tarda (ED45)	0	0	0	0	10	41.7	13	54.2	12	50	17	70.8	15	62.5	19	79.2	17	70.8	20	83.3	18	75	21	87.5
Gram-positive																								
Lactococcus garvieae (FP5245)	0	0	0	0	9	36	11	44	11	44	12	48	13	52	13	52	15	60	14	56	17	68	15	60
Streptococcus iniae (FP3287)	0	0	0	0	7	25	8	28.6	8	28.6	11	39.3	12	42.9	12	42.9	13	46.4	13	46.4	16	57.1	15	53.6
S. iniae (S186)	0	0	0	0	12	34.3	14	40	15	42.9	15	42.9	17	48.6	17	48.6	19	54.3	19	54.3	22	62.9	22	62.9
S. iniae (S530)	0	0	0	0	9	23.7	10	26.3	11	28.9	20	52.6	15	39.5	21	55.3	17	44.7	23	60.5	19	50	25	65.8
S. iniae (S131)	0	0	0	0	7	20	9	25.7	10	28.6	16	45.7	18	51.4	17	48.6	19	54.3	18	51.4	21	60	20	57.1
Streptococcus parauberis (FP5228)	0	0	0	0	7	25	9	32.1	9	32.1	10	35.7	10	35.7	15	53.6	12	42.9	16	57.1	14	50	17	60.7
S. parauberis (S124)	0	0	0	0	8	32	12	48	11	44	15	60	15	60	16	64	16	64	17	68	17	68	18	72
S. parauberis (S527)	0	0	0	0	7	24.1	8	27.6	13	44.8	11	37.9	15	51.7	14	48.3	18	62.1	16	55.2	19	65.5	18	62.1
S. parauberis (S1466)	0	0	0	0	10	37	11	40.7	12	44.4	15	55.6	15	55.6	16	59.3	16	59.3	17	63	19	70.3	19	70.4

*CI- CEO IZD, CR-CEO RIZD, EI- Eugenol IZD, ER- Eugenol RIZD

Table 3: Susceptibility pattern of clove essential oil and eugenol against fish pathogenic bacteria

Species	Clove essential oil			Eugenol		
	MIC (V/V)	MBC (V/V)	MBC/ MIC	MIC (V/V)	MBC (V/V)	MBC/ MIC
Gram-negative bacteria						
Vibrio harveyi (FP8370)	0.125	0.25	2	0.0312	0.125	4
V. ichthyoenteri (FP4004)	0.125	0.25	2	0.0312	0.125	4
Photobacterium damselae (FP4101)	0.125	0.25	2	0.125	0.25	2
Edwardsiella tarda (FP5060)	0.25	0.5	2	0.0625	0.25	4
E. tarda (ED47)	0.5	0.5	1	0.125	0.5	4
E. tarda (Yoshida)	0.125	0.25	2	0.125	0.25	2
E. tarda (ED45)	0.5	0.5	1	0.125	0.5	4
Gram-positive bacteria						
Lactococcus garvieae (FP5245)	0.5	1	1	1	1	1
Streptococcus iniae (FP3287)	0.5	1	2	0.5	1	2
S. iniae (S186)	0.25	0.25	1	0.5	0.5	1
S. iniae (S530)	0.25	0.5	2	0.5	0.5	1
S. iniae (S131)	0.25	0.25	2	0.125	0.5	4
Streptococcus parauberis (FP5228)	0.5	0.5	1	1	1	1
S. parauberis (S124)	0.5	0.5	1	0.5	0.5	1
S. parauberis (S527)	0.5	0.5	1	1	1	1
S. parauberis (S1466)	0.25	0.5	2	0.5	0.5	1

Antimicrobial properties (MIC and MBC) were mentioned. MBC/MIC results were used to interpret bacteriostatic or bactericidal activity of oil

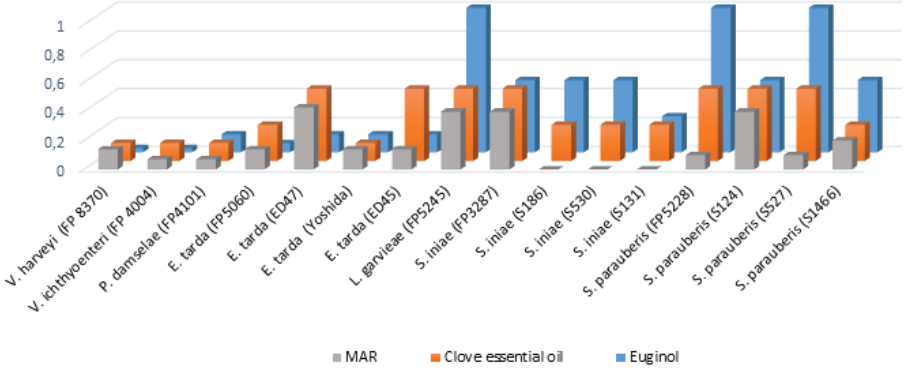

Figure 1: Comparative expression of MAR index values and MICs of clove essential oil and eugenol detected for sixteen strains of fish pathogenic bacteria

CEO and eugenol. Previous study has reported highest diameter of zone of inhibition of against *Staphylococcus aureus* followed by *Escherichia coli* and *Listeria monocytogens* which were food borne pathogens (24). Fagere (2016) has reported that CEO showed higher IZD against *Bacillus subtilis* and *S. aureus* (25).

RIZD % exhibits high values at higher concentrations. All the Gram-negative strains had higher RIZD % at every concentration of eugenol except *E. tarda* (ED45). It was observed that eugenol had zero RIZD % against Gram-positive strains at 1 % (V/V) concentration. This indicates that Gram-positive bacteria were not susceptible at every tested concentration of eugenol.

In the present study, the *MIC* of CEO for Gram-negative bacterial strains *ranged* from 0.125 to 0.5% (V/V) and for Gram-positive strains *ranged* from 0.25 to 0.5% (V/V) (Table 3). Eugenol showed lower MIC values than CEO against Gram-negative bacteria which range from 0.0312 to 0.0125% (V/V). According to the MBC/MIC ratio, both CEO (MBC/MIC 1-2) and eugenol (MBC/MIC 1-4) demonstrated bactericidal activity against 16 fish pathogenic bacteria. In a previous study, MBC and MIC values were identical, indicating that the effect of CEO was mainly bactericidal and not bacteriostatic (26) and eugenol demonstrated bacteriostatic activity at lower concentrations and bactericidal activity at slightly higher concentrations against *Salmonella* (27, 28).

The antibiotic resistance profile indicated that all the bacterial strains excluding three strain of *S. iniae* (S186, S530, and S131) have shown resistance to one or more antibiotics. This was facilitated by MAR index values where all the strains except the three strains mentioned above have been calculated as ≥ 0.07. The MAR index which is higher than 0.2 (>0.2) identifies bacteria isolated from a source with a high risk of contamination where antibiotics have been often used (28). The MAR index of four strains [one *E. tarda* (ED47), one *L. garvieae* (FP5245), one *S. iniae* (FP3287) and one *S. parauberis* (S124)] have been calculated as ≥ 0.2 were noted as comparatively high-risk strains. Finally, the observed Pearson coefficient (r) between the MAR index values and MIC values of eugenol (r =0.15) and CEO (r =0.61) indicated that there is a positive correlation between the CEO or eugenol efficacy and the multidrug-resistance profile of fish pathogenic bacteria (Figure 1).

Although both CEO and eugenol inhibited the growth of tested fish pathogenic bacteria, eugenol could inhibit the growth of bacteria at lower concentrations than CEO. The present study established that both CEO and eugenol are effective against fish pathogenic bacteria isolated from olive flounder and are good candidate for further research to develop a new antibacterial drug against fish pathogenic bacteria. CEO and eugenol can be used to prevent fish diseases by adding to fish feed or immersion treatment. Moreover, in order to apply CEO and eugenol for treatment of bacterial diseases in aquaculture, the stability of them in the aquatic environment, their digestibility and the toxicity in fish should be further investigated.

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PROTIBAKTERIJSKA AKTIVNOST ETERIČNEGA OLJA NAGELJNOVIH ŽBIC IN EVGENOLA PROTI PATOGENIM BAKTERIJAM RIB, IZOLIRANIH IZ GOJENEGA MORSKEGA LISTA (*Paralichthys olivaceus*)

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Povzetek: Protibakterijsko delovanje eteričnega olja nageljnovih žbic (*Syzygium aromaticum*) (CEO) in evgenola je bilo testirano pri sedmih vrstah po Gramu negativnih in devetih vrstah po Gramu pozitivnih patogenih bakterij, ki so bile izolirane iz gojenega morskega lista (*Paralichthys olivaceus*) v Južni Koreji. Evgenol je bil 99-odstotno koncentriran, CEO pa je sestavljalo sedem kemičnih spojin, med njimi je bilo največ evgenola, ki je predstavljal 83,odstotka eteričnega olja.
Metoda difuzije v trdem gojišču, minimalna zaviralna koncentracija (MIC) in minimalna baktericidna koncentracija (MBC) so pokazale, da sta CEO in evgenol zavirala rast tako po Gramu negativnih kot po Gramu pozitivnih bakterij. CEO (MBC/MIC = 1-2) in evgenol (MBC/MIC = 1-4) sta bila baktericidna za vse testirane seve. Odstotek velikosti relativne zaviralne cone je bil visok pri višji koncentraciji CEO in evgenola. Pozitivna soodvisnost je bila opažena med vrednostmi indeksa MAR in vrednostmi MIC pri CEO (r = 0,61) in evgenolu (r = 0,15). Ker sta CEO in evgenol pokazala učinkovito delovanje proti patogenim bakterijam rib, bi bila uporaba CEO in evgenola lahko uporabna za zdravljenje bakterijskih okužb v ribogojništvu.

Ključne besede: antibakterijsko delovanje; eterično olje klinčkov (CEO); evgenol; patogene bakterije rib; morski list

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Slov Vet Res 2019; 56 (1)

Review Article

Jazbec K, Jež M, Justin M, Rožman P. Molecular mechanisms of stem cell aging.....5

Original Research Articles

Marolt Banek I, Roša J, Ježek D, Delaš I. Effect of metabolic cage housing on metabolic changes in the liver of young male laboratory rats.....13

Ujčič-Vrhovnik I, Nataša Kopitar A, Malovrh T, Ježek J, Jakovac-Strajn B. Effects of feeding grains naturally contaminated with *Fusarium* toxins on selected haematological parameters and lymphocyte subsets in primiparous sows 21

Pathirana HNKS, Wimalasena SHMP, De Silva BCJ, Hossain S, Gang-Joon H. Antibacterial activity of clove essential oil and eugenol against fish pathogenic bacteria isolated from cultured olive flounder (*Paralichthys olivaceus*) 31