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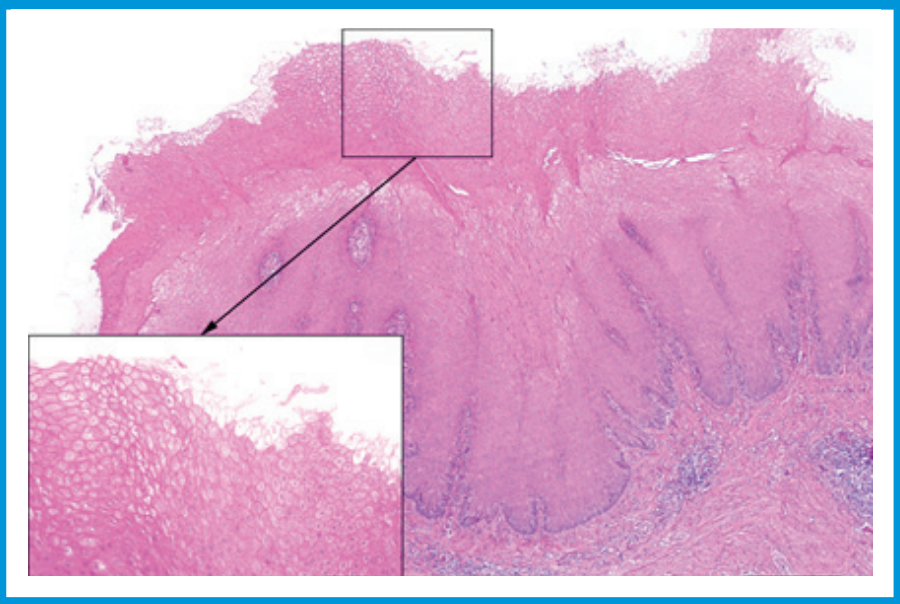

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Address: Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia
Naslov: Veterinarska fakulteta, Gerbičeva 60, 1000 Ljubljana, Slovenija
Tel.: +386 (0)1 47 79 100, 47 79 129, Fax: +386 (0)1 28 32 243
E-mail: slovetres@vf.uni-lj.si

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Review Article

CRITICAL APPROACH TO THE ALTERNATIVE TREATMENT OF CHRONIC KIDNEY DISEASE IN DOGS AND CATS

Jeff M. Pérez[1,2]*, Chiara Alessi[1]

[1]Department of Animal health, University of Caldas, C.P: 170004, Street 65, No 26-10, Manizales, [2]Cuor-Tech™ Sport Medicine and Veterinary Cardiology Service, C.P 660006, Pereira, Colombia

*Corresponding author, E-mail: jeff.mp@outlook.com

Abstract: Chronic kidney disease (CKD) is common in dogs and cats and can occur at any age, especially in geriatric animals. The various presentations of the disease and their different hemodynamic and metabolic alterations are issues of profound research. Currently clinicians improvements of the comprehensive management of chronic kidney disease focuses on the delay of the progression of clinical signs of the disease and there now are numerous novel methods that also were proposed to slow the progression of the disease, with the possibility of use in non-referral centers. The aim of this critical approach is to provide an overview of the comprehensive treatment of chronic kidney disease, expose new treatments that could improve the intervention of dogs and cats with chronic kidney disease and reevaluate the usefulness of some existing drugs.

Key words: chronic kidney disease; cardiorenal syndrome; glomerulonephritis; dog; cat

Introduction

Chronic kidney disease (CKD) is a common disease in dogs and cats with greater prevalence in geriatric animals, although it can occur at any age and its incidence in the general dogs and cats population is approximately 0.5 and 4.5%, respectively (1-3). CKD is defined as an structural and/or functional disability in one or both kidneys present for more than 3 months (2, 4) and their main features are deteriorated renal excretion and decreased glomerular filtration rate (GFR) (5). In addition, endocrine and biosynthetic functions are progressively altered (3). Dialysis and kidney transplantation are still only performed in reference centers and sometimes are not feasible procedures for the owner. Despite medical treatment continues to be invaluable to slow the progression of the disease, nutritional modification is one of the most important factors that influence the metabolic imbalances and is a determining factor in the health-related quality of life of affected patients (6). Thus, in human and veterinary medicine, poor body condition has been associated with decreased health-related quality of life and survival time (7, 8).

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The clinical presentation of the disease, the early diagnosis and the comprehensive treatment are a medical challenge that needs individual strategies due to the great variability between different patients and diverse clinical presentation. The goals of medical treatment of CKD are to slow down the progression of the disease, prevent complications caused by decreased renal function and control the clinical signs of uremia. It is also critical to stabilize the biosynthetic process and correct non-renal diseases that may decompensate patients with CKD such as diarrhea, vomiting and dehydration. Sometimes secondary endocrine disruption of CKD is neglected because of purely renal approaches that usually take place (2, 3). The aim of this critical approach is to present the progress in recent years in the comprehensive treatment of CKD. In addition, it aims to provide a different perspective, in terms of treatments that have existed during the past decade and those medical and / or pharmacological implications that might have changed.

Nutritional Intervention

The Critical Role of Nutrition

One of the main signs that present dogs and especially cats with kidney dysfunction is the loss of appetite. Thus, nutritional intervention is always required, considering that decreasing the presentation of many complications related to functional deficiency of the kidney (p.e uremic syndrome, hyperparathyroidism and anemia), improve coming through in health-related quality of life and survival time (8). The specific targets of the nutritional modification are to prevent uremic syndrome, maintain acid-base control, correct electrolyte imbalances and slow down the progression of CKD. In addition, this diets provide water-soluble vitamins and minerals supplement for maintain a balance between nutritional requirements and the necessary dietary restrictions (2, 3, 9). Nevertheless, the primary goal of nutritional therapy is to maintain stable body condition and muscle mass. These therapeutic strategies are more effective combined with focused therapies that directly correct the progression of specified etiology of CKD (such as nephrotic syndrome, hypercalcemia, renal disease, urinary tract infection, chronic urinary tract obstruction, glomerulonephropathies and immune-mediated disease, etc.)(9, 10).

To initiate the nutritional modification, experts have recommended correcting first the clinical signs of uremia (11). The evidence-based medicine is the most important factor in the management of the CKD (12), many studies have demonstrated the implications of nutritional therapy and renal diets typically have low levels of protein, sodium, and phosphorus. But also, high levels of antioxidants, polyunsaturated fatty acids (Omega 3 and 6) and water soluble vitamins, fiber and more energy density (13-16). Renal diets statistically decrease 72% of the relative risk of uremic crisis in cats (9). In the same way, renal diets in canines allows to remain 2.5 times longer without showing signs of uremia in this species (7), demonstrating that only with renal diet, disease progression is slower (6, 13). Although there are few research about this topic, the health-related quality of life and life expectancy significantly improves with the nutritional management (16).

Modulation of inflammation and Oxidative stress

A variety of benefits have been attributed to supplementation with polyunsaturated fatty acids (PUFA's), including its tendency to decrease cholesterol, modulation of inflammation and control of blood pressure (17, 18). In human patients with renal disease, improvement in glomerular renal circulation and decreased calcification has also been recognized with this supplementation (19). One review published by Roudebush and colleagues (9) found that injuries such as glomerulosclerosis, tubulointerstitial fibrosis and infiltration of pro-inflammatory cells decreased significantly in dogs diets supplemented with PUFA's.

Oxidative stress caused by free radicals generation and reactive oxygen species can cause kidney damage accelerate the progression of CKD (20). Yu et al (21) found that antioxidant supplementation significantly reduced oxidative stress and plasma creatinine levels compared with dogs receiving a diet without antioxidants. Other research in dogs with induced CKD showed that the nutritional supplementation with omega-3 and antioxidants (p.e vitamin E,

carotenoids and lutein) were independently reno-protective and its effect was additive when used together (22). However, at the time meta-analysis or cohort studies were not published to determine if PUFA´s have clinical value in representative populations of dogs or cats and further investigations are expected.

Chronic Kidney Disease-mineral Bone Disorder

The mechanism of excretion and control of phosphorus is normally composed of glomerular filtration and tubular reabsorption (23, 24). During CKD glomerular filtration rate decreases and phosphorus is retained, then tubular reabsorption of Phosphorus increases as a countervailing mechanism of "false" decreasing in the amount of phosphorus reaching the tubule lumen, which ends with the development of hyperphosphatemia and all its related aberrations, especially hormonal imbalance responsible of mineral equilibrium (see figure 1) (25). Hyperphosphatemia plays an important role in the development of secondary hyperparathyroidism in CKD. There is now appropriated evidence to recommend diets with restriction of phosphorus in kidney-affected patients (10, 26, 27).

In patients with advanced stages of renal dysfunction (IRIS III-IV), the restriction of phosphorus in the diet alone does not prevent hyperphosphatemia (28). Using phosphorus binders (p.e hydroxide aluminum) would be of help at least in dogs. It should be said that some patients cannot tolerate the intake of hydroxide aluminum and no serious investigations were published with a good level of evidence that allow its recommendation in clinical practice. A new oral phosphate binder SBR759 (Lenziaren®) was developed and evaluated by King (28) with favorable results in acceptability, clinical improves, tolerability and safety in cats. However, further evidence is needed to prove the real clinical benefit.

Calcitriol is the hormone responsible of renal calcium metabolism (23). Kidney converts the 25-hydroxycholecalciferol by the enzyme hydroxylase 1 alpha, to the active metabolite 1, 25-Dihidroxicolecalficerol (Calcitriol). Thus, in turn has a relationship with the modulation of parathyroid hormone. Because the CKD may be associated with decreased production of 1, 25-dihydroxycholecalciferol, this deficiency can promote hyperplasia of the parathyroid gland, alteration known as secondary hyperparathyroidism to CKD (27). Calcitriol supplementation may decrease a variety of complications attributed to the excess parathyroid hormone (PTH) levels in kidney-affected patients (26) and research suggests that calcitriol therapy prolongs survival time in dogs (26, 27, 29). The authors recommend caution in this topic and waited for further research to define a possible overall conclusion.

Hyperkalemia in CKD

The electrolytes disturbance, especially alteration of potassium (Hyperkalemia) is common in dogs with CKD. Although they are of common incidence in acute complications it has a critical role in the final stages of the disease (30). In addition, there is the possibility that the amount of potassium in the diet exceeds renal excretion resulting in hyperkalemia in some patients. In humans with primary renal disease, the use of angiotensin converting enzyme inhibitors (ACEI's) can have complications such as hyperkalemia (31). Although disagreement exist and little evidence in canine and feline patients, there may be also a moderate or similar risk to present hyperkalemia.

One Research done by Segev and colleagues (32) showed that hyperkalemia is a potential complication of CKD in dogs that consumed a commercial therapeutic diet. Nevertheless, this investigation apparently found no relationship between the administration of ACEI's and serum potassium levels in the study population. In addition, canines that require hemodialysis are prone to develop severe hyperkalemia and the point-of-care testing of electrolytes should be applied during the procedure (33). Finally, it is important to take into account that depending on the stage of the disease, each patient should receive an individual treatment and nutritional therapy corresponding to renal function, considering that one diet for a patient with stage IRIS II-III, cannot necessarily be identical to an IRIS patient IV (see table 1)(6).

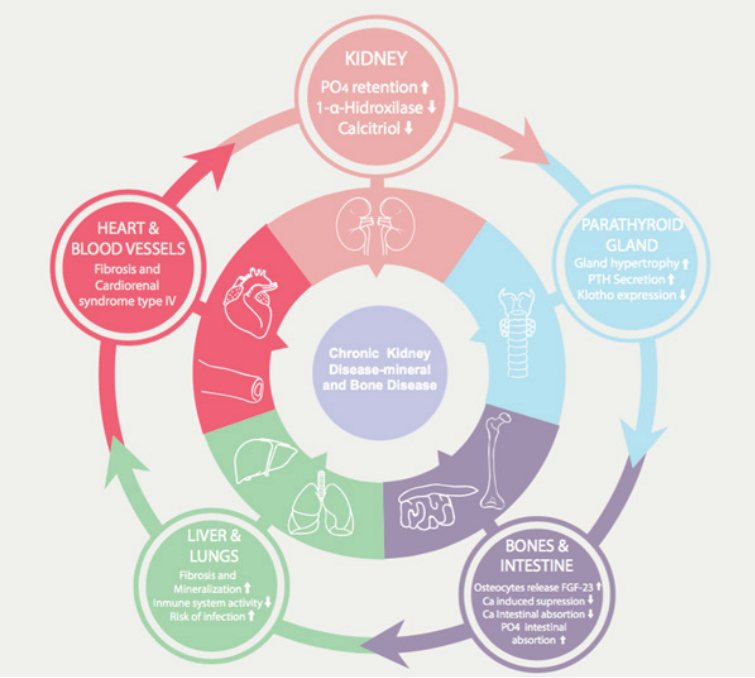

Figure 1: Chronic kidney disease-mineral and bone disorder is charactericed by PO4 retention as a consequence of declining GFR. FGF-23 increases in response to PO4 accumulation, but inhibits renal 1-α-hydroxilase and then decreases calcitriol levels. Hight calcitriol levels inhibit PTH synthesis and without this feedback inhibition, PTH increases. Other consequences of lower calcitriol levels are the increase of skeletal resistance to PTH action and the limitation of the Ca-induced suppression of PTH secretion. PTH secretion continue due to the fact that coreceptors klotho are diminished in the parathyroid gland. Impaired gastro-intestinal absorption of Ca due to low levels of calcitriol is another negative consequence. The liver, lung, heart and blood vessels are prone to the development of calcinosis and loss of function. The cardiorenal syndrome type IV is known as the impact of CKD on heart and vessels function and is recently reviewed in small animals. (Figure credit: Chiara Alessi)

Table 1: The CKD stratification system has been proposed by the International Renal Interest Society (IRIS) to help provide guidelines for clinical management of CKD. Staging is based on serum creatinine values, with substages identified for blood pressure and proteinuria. Taken and modified from (Foster JD. Canine chronic kidney disease current diagnostic and long-term management. Today's Veterinary Practice, September/October 2013)

CKD IRIS Staging System		
Serum Creatinine mg/dl	**Dogs**	**Cats**
Stage I	< 1.4	< 1.6
Stage II	1.4 – 2	1.6 – 2.8
Stage III	2.1 – 5	2 - 5
Stage IV	> 5	> 5

Getting to the extremes

Both anorexia and malnutrition are the most critical clinical complications in CKD of dogs and cats if it takes take into account their contribution on morbidity and mortality (6, 7). Sometimes assisted feeding should be provided in order to minimize the risk of uremic crisis or death because it allows the feeding and prevents long periods without nutrition or liquid consumption (34, 35). The first step is to ensure that lost of appetite is due to syndromes that increase metabolic complications such as dehydration states, gastrointestinal bleeding, metabolic acidosis, hypokalemia or urinary tract infection. When these possible causes have been excluded or corrected and no improvement is seen in appetite, it is recommended to intervene. For example gastrointestinal complications are routinely treated with the administration of H2 antagonists (p.e famotidine 0.5 mg/kg q12h PO), antiemetics (Maropitant citrate 1 mg/kg q24h S.C) and gastric mucous protectors (sucralfate 40 – 250 mg/kg q12h P.O). Isolated reports suggest that the placement of a feeding tube can reduce weight loss associated with CKD (34). However, their implications for health-related quality of life and long-term outcomes are still undefined.

Endocrine disturbances and conservative advanced therapies

Hormone replacement and Anemia

The decrease in red blood cells count, percentage of hematocrit and hemoglobin is common in dogs and cats with CKD in stage IRIS III-IV (2). In kidney-affected patients the main cause of anemia is hypoplasia of erythroid elements secondary to inadequate production of erythropoietin (EPO) in the renal marrow and is exacerbated by the short life of the erythrocytes due to azotemia, acid-base disorders, the premature apoptosis, gastrointestinal bleeding, nutritional disorders, iron deficiency and bone marrow fibrosis (36-38). Erythropoietin hormone replacement usually leads to develop with recombinant human alpha EPO in dogs (rHuEPO; Epogen® Amgen, Thousand Oaks, CA, USA). This is highly effective but with short-live in bloodstream (39). Recent research has revealed that dogs with CKD and anemia treated with rHuEPO stabilize the hematocrit levels, the augmentation in appetite was also evidenced and health-related quality of life then improving (9). Unfortunately, the development of antibodies against rHuEPO in dogs and cats has limited its use to a minor amount of the population and therefore, the use of rHuEPO should be limited to patients with less than 22 or 20% hematocrit and signs of anemia like hypoxemia, exercise intolerance, cyanosis or heart murmurs.

Although little research has been done in veterinary medicine, darbepoetin alpha it may be less immunogenic compared with rHuEPO (40). Lu and colleagues (41) evaluated the efficacy and safety of recombinant canine erythropoietin (rCaEPO) in dogs with non-regenerative anemia secondary to CKD. They found that in dogs receiving rCaEPO, a slightly increased erythrocyte production was evident. In addition, there was hyperplasia in bone marrow, reticulocytosis, normal levels of hematocrit and direct improving of health-related quality of life. In contrast, complications related to the creation of anti-rCaEPO antibodies were very low. Furthermore, it should be known that hypertension is a complication in some human patients that are treated with rCaEPO, but there is still no evidence that support this in animals. Thus, there is disagreement on whether it is preferable to improve anemia partially or completely to avoid adverse effects like cardiovascular events, coagulation abnormalities and metabolic complications related to the use of these drugs, their dose and increased erythrocyte production and hemoglobin as a direct consequence (39). Respected to animal patients it is not clear whereas the complete correction of anemia represents a negative effect on CKD.

Other methods have shown interesting results in correcting anemia caused by CKD. Erythropoietin gene therapy has proven to be able to correct anemia in mice with induced anemia (42, 43). The peginesatide is a synthetic peptide that is not homologous to human erythropoietin, representing an absence of cross-reaction and production of anti-EPO (39). However, this drug has not been demonstrated superiority in the control of anemia, compared with the other drugs discussed above (44).

Based therapy in Mesenchymal Stem Cells

Based therapy in mesenchymal stem cells (MSC) offered protection in different forms of acute kidney injury in humans (45) and there is good evidence of the positive effects of MSCs in reducing the loss of function of kidney in the early stages of CKD (46). Therapy with MSC has particular functions related to the release of growths factors and cytokines with a paracrine action (47, 48). Bi et al (49) demonstrated in a murine model of acute kidney injury that administration of conditioned medium derived from bone marrow mesenchymal stem cells (CM-MSC) increased the survival time and decreases progression of renal injury. The protective effect of CM-MSC apparently are related to the action of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and insulin growth factor (IGF) (50). Exosomes and microvesicles may have a similar effect (51, 52).

The research developed by Van Koppen and colleagues (53) found a significant decrease in systolic blood pressure in rats with induced CKD treated with CM-MSC compared to the group of rats that not receiving CM-MSC (146 ± 17 vs 163 ± 21 mmHg; $p < 0.05$). They also found a decrease in proteinuria in rats with CKD receiving CM-MSC ($p = 0.007$). However, they found no difference in renal clearance of creatinine and urea. In contrast, plasma creatinine in rats that did not receive

CM-MSC was always higher compared with the group receiving treatment with CM-MSC ($p < 0.05$). The most important result of their research was that the higher percentage of glomerular endothelial cells present in rats receiving CM-MSC and rats that did not receive CM-MSC, presented a percentage of 31 and 26% of not sclerotic glomeruli respectively. Interstitial fibrosis and tubular atrophy was lower due to decreased deposition of collagen type I and III in rats receiving CM-MSC demonstrating that repeated administration of CM-MSC as rescue intervention of kidney function is probably due to an increase in long-term endothelial regeneration and recovery.

In summary, this novel treatment could slow down the loss of glomeruli, decrease fibrosis and alterations in glomerular function. As well as help in adjusting changes in vascular pressure, considering this latter like one important factors for life expectancy of patients with CKD and impacting the velocity of degeneration and the associated clinical signs. Furthermore, it should be necessary to review more investigation in the future to assess the impact on pets with diseases of natural progression and the evaluation of large cohorts of patients could be of help to determine the impact on life expectancy and clinical usefulness in reducing the progression of CKD and recognize the real effect of CM-MSC.

Based therapy in Electroporation of Plasmid Growth Hormone

CKD in humans can affect growth hormone-release hormone; growth hormone; insulin-like growth factor I axis (GHRH/GH/IGF-I) (54, 55), which may affect disease progression and cell metabolism. Although little research exist in veterinary medicine about the axis (GHRH/GH/IGF-I) in CKD of natural progression; gene therapy based in plasmids growth hormone is a possible treatment in canine and cats, mainly for its influence on bone metabolism, proteins and glucose (56). GH and their mediator, IGF-I are growth regulators and maintain homeostasis in body weight and renal function. Some changes seen in patients with CKD are caused by the imbalance of the (GHRH/GH/IGF-I) axis (57, 58).

Therapy with GH produces a positive anabolic effect and helps maintain a physiological balance of renal function in people with CKD (59, 60). Electroporation (EP) is a very important technique in gene therapy because it allows a single dose with a long duration. This is a key factor to commensurate the impact of this type of drugs on the commodity of patients and owners that have complications receiving the medication or when owners cannot administer medication at intervals or schedules required by the clinician. The study published by Brown and colleagues (61) developed a multicenter control group study using gene therapy with GH and EP in dogs and cats with CKD as an animal model for CKD on humans. Acording to the results, they found out an increase in survival time and increased body weight and muscle mass. In addition, health-related quality of life was improved in the group of patients treated with GHEP. Particularly dogs treated with GHEP increased physical activity and appetite, compared with the control group ($p = 0.001$).

At the biological level increased blood-levels of IGF-I in patients treated with GHEP slightly improved hematologic parameters were found. The main reason for using these therapeutic options is to increase synthesis and bioavailability of IGF-I. This study suggests that clinical intervention with GHEP can preserve kidney function and reduce the negative effects of CKD in the health-related quality of life, especially decreasing the amount of times that the drug must be administered and the need for recurrent hospitalization. Furthermore, research are needed for recognize the long-term impact in the disease.

Tanshinone IIA as an alternative therapeutic option in CKD

Red sage (*Salvia miltiorrhiza*) is a perennial plant, valued as one of the most important ancient medicines, describe in the Shen-Nung's Pen-Ts'ao book of herbal medicine and agriculture of traditional Chinese medicine (62). Their chemical component (salvianolic acid, Dihidrotanshinone, Tanshinone I and Tanshinone IIA) has been also studied for years, especially for its effects against cancer and degenerative inflammatory diseases. Tanshinone IIA is the most abundant chemical component of the rhizome of the plant and its particularly evaluated for the treatment of arthritis, hepatitis, acute coronary syndrome, neuropathic pain, bone tumors and Alzheimer's

disease (63-67). This molecule has antioxidant properties and protects against oxidative stress. But also, chemical component alows vasodilation by stimulating the release of Nitrous Oxide (NO) on consequent decrease in blood pressure in animal models of hypertension and with the same protective effects in people with diabetic nephropathy (68, 69).

The investigation published by Ahn et al (70) evaluated the influence of Tanshinone IIA in renal function, proteinuria and expression of angiotensin II (Ang II), transforming growth factor beta I (TGF-B1) and type IV collagen in an experimental murine model. Taking as a hypothesis the harmful relationship of the Ang II, TGF-B1 and collagen type IV in the pathogenesis of CKD and the development of glomerulosclerosis (GC), the results found that prolonged use of Tanshinone IIA (8 to 12 weeks), the proteinuria significantly decreased. Moreover, glomerulosclerosis evaluated histologically was more widespread in the group that cannot receive Tanshinone IIA (3.2 ± 0.2 vs 2.1 ± 0.1; $p < 0.05$). The protein expression analysis showed that the use of Tanshinone IIA prevents expression of (TGF-B1) (33.8 ± 4.8 vs 20.2 ± 2.9; $p < 0.05$). In contrast, the expression of type IV collagen in patients receiving Tanshinone IIA was significantly decreased (0.45 ± 0.04ng/ml, $p < 0.05$).

In conclusion these results could be demonstrate that Tanshinone IIA decreases the progression of CKD, attenuating the pathological structural manifestation of chronic kidney disease and the progressive characteristic fibrosis. Furthermore, these results must be interpreted with caution and more investigations are expected with other animal models to deeply assess their true benefit in CKD of natural progression. Nevertheless, the reason for exposing here this plant is mainly the approach of a possible integration between modern medicine and traditional medicine, due the potential beneficial impact of combined treatment with standard therapy. Taking into account, that actually different efforts exist to create the critical approach that aims to integrate alternative therapies for CKD, but with evidence-base medicine and long-term results.

Thinking outside the box "ACE inhibitor"

In addition to the nutritional intervention and its benefits in renal protection and improvement of the health-related quality of life in dogs and cats with CKD, of all drugs studied in the last decade, angiotensin converting enzyme inhibitors (ACEI`s) have shown to be statistically the most effective. An early research with these molecules in humans was done by Lewis and colleagues (71), which evaluated the efficacy of ACEI's in treating diabetic nephropathy and initially, they efficacy was attributed to the antihypertensive effects "hemodynamic effects". However, at the time, it is well-know that their effects are not restricted to pressure. The ACEI´s are frequently used in veterinary medicine for the treatment of heart failure in dogs and cats (72, 73). These molecules are also key drugs in CKD in dogs, cats and humans.

The Renin-angiotensin-aldosterone system (RAAS), partially blocked by these ACEI's drugs, is traditionally known as an endocrine axis of control of water and salt reabsorption, commonly characterized by initial activation of renin in the kidney. Nevertheless, the production of all components of the RAAS can be synthesized in the absence of this release of renin by the kidney and could be synthesized in any organ, especially the heart, lung, brain, placenta, pancreas, adipose tissue and vascular bed (74). The difference between systemic RAAS and renal RAAS activation has a clinical importance related to ACEI's drugs use, specifically in the inhibition of tissue and plasmatic ACE. In the latter, inhibition is much lower and was confirmed by investigations of Lavoie et al (74). Moreover, the activity and concentrations of ACE, Angiotensin II and its receptor (AT-1) in the kidney are different from other organs. The renin is synthesized by cells of the juxtaglomerular cortex; angiotensinogen is secreted in the proximal tubule cells, ACE is found in large quantities in the brush border of the cell membranes of the proximal tubule and this promotes rapid conversion and levels of Angiotensin II (Ang II) rise about 1000 times more than in the general circulation (74, 75).

Angiotensin converting enzyme (ACE), not only converts Ang I to Ang II, also degrades bradykinin, with important effects on natriuresis and control of the balance between vasoconstriction and vasodilatation (76). Furthermore, Ang II is also converted by specific tissue enzymes (chymase, cathepsin G and chymostatin) (77). Angiotensin II is the most powerful end effector systems, which control blood pressure through the reabsorption

of sodium and water, stimulating receptors (AT-1 and AT-2) in the kidney or indirectly stimulating the production of aldosterone by the adrenal glands. Although ACEI's inhibit the conversion of Ang I to Ang II, these do not inhibit conversion mechanisms not ACE (chymase, cathepsin G and chymostatin) and then, Ang II levels cannot completely be suppressed. This are defined as "angiotensin breakthrough". Lantis and colleagues (77) found that aldosterone levels do not appear to decrease in patients treated with ACEI's, this also known as aldosterone breakthrough. The implications of both “breakthroughs" in the pathophysiology of cardiovascular and kidney disease as we knew are enormous and perhaps revolutionary.

Exposures to high and prolonged concentrations of aldosterone result in sodium retention, expansion of extracellular volume and fibrosis contributing to endothelial damage (75). Possible options to avoid this breakthrough are the use of drugs as Ang II receptor blockers “ARBs”, aldosterone receptor blockers or direct renin inhibitors (77). Regardless of the etiology of CKD, glomerulosclerosis and interstitial fibrosis are characteristic pathologic findings in the final stages of CKD (IRIS III-IV) (78). The RAAS activation has proven to be the cause of progressive hypertension, fibrosis, tubulointerstitial injury and increased pro-inflammatory cells in the kidney in different species including dogs and cats (74). Specifically Ang II increases the formation of Tissue Growth Factor type B1 (TGF-B1) a fibrogenic cytokine responsible not only for the increase in the synthesis but also the decreased degradation of extracellular matrix (ECM) by the expression of fibronectin, laminin, collagen, proteoglycans and entactines that develop interstitial fibrosis.

The (TGF-B1) also stimulates contraction of vascular smooth muscle and mesangial cells, the latter involved in the development of glomerulosclerosis (11). Serpin E1 (inhibitor of plasminogen activator type 1 or PAI-1) are directly related to the pathophysiology of thromboembolic events and arterial fibrotic; it’s a protein responsible for preventing fibrinolysis, by inhibiting urokinase and tissue plasminogen activator (uPA and tPA). The production of this protein increases due to the Ang II (79) causing inhibition of proteases that regulate the formation of ECM, increasing progression of glomerulosclerosis, tubulointerstitial fibrosis and increased inflammatory cells in kidney, especially macrophages (80). Another mechanism that increases the formation of ECM is the inability to self-regulate glomerular pressure. In physiological situations, variations in systemic blood pressure have little influence on glomerular arteriolar pressure, as CKD progresses this ability is lost, leaving the glomeruli exposed to very marked changes in systemic pressure and then distend the glomerular capillaries with subsequent stimulation of mesangial cell and production of TGF-B1. Thus, CKD progresses to chronic renal failure (CRF), but the authors recommend that indiscriminately both terms have been used to refer to the same concept. There may be chronic kidney disease without renal failure. However, renal failure cannot exist without chronic or acute kidney disease.

The ACEI's lower blood pressure experimentally when inhibit Ang II production and help decreasing the formation of ECM (production of TGF-B1) and these effects have been study in rodent models. One research develop by Brown and colleagues (81) started administrating Benazepril (0.25mg / kg V.O SID) for 6.5 months to cats with induced CKD, showing a decrease in glomerular pressure (< 12-14 mmHg). The relation between afferent arteriolar and efferent arteriolar resistance decreased in the cats receiving Benazepril. Other studies also confirmed these results (82). In dogs with induced CKD treated with ACEI’s, experimentally a decrease of 30% in efferent arteriolar resistance was evident, while the afferent remained unchanged (83).

Hypertension is associated with kidney damage and progressive loss of kidney filtration in people (44). Although not widely investigated in dogs and cats, there are enough reasons to extrapolate this from humans to pets with CKD. Considering the above, the use of ACEI’s as hypotensive and antisclerotic drug can benefit patients with CKD. However, the decrease in blood pressure is not evident in a percentage of the population by the breakthrough of angiotensin, the decreased plasma renin activity in some patients and high circulating levels of sodium (78). The decrease in blood pressure is dose dependent especially in cats. Using ACEI’s as Enalapril to 0.25 mg/ kg once a day, the blood pressure decrease 23%, whereas if the same dose is administered twice a day, blood pressure decreases near 41% (84). The calcium channel blockers such as amlodipine, belonging to the dihydropyridine drugs, is the first

choice in the treatment of high blood pressure in cats and can be used in a joint with ACEI's therapy to treat patients with uncontrolled hypertension and CKD (85).

Proteinuria is caused by alterations in the permeability and selectivity of the glomerular membrane, such as mechanical factors (excessive pressure), incomplete coverage of the glomerular podocytes caused by hypertrophy through toxins or immune complexes in the surface. The ACEI's are protective for future increases of proteinuria, despite the molecular and cellular mechanisms under its antiproteinuric effect at the time cannot yet fully elucidated. Several hypotheses exist and one is the molecular modulation of podocytes. Podocytes are visceral glomerular cells, lining the outside of the glomerular basement membrane; this being the final barrier to the loss of protein (86, 87). The author Asanuma and colleagues (86) recently reviewed the importance of podocytes in the progression of damage, glomerulosclerosis and progression of renal disease. The first decrease in proteinuria appears to be development by decreasing blood pressure (78, 88).

Enalapril and benazepril has been the most ACEI drugs documented to decrease proteinuria. Urinary Protein creatinine ratio (UP/C) decreases approximately 4.2 times in patients treated with Enalapril vs 1.9 times in feline patients treated with placebo (83, 88). In summary, the use of ACEI's in patients with CKD modulate blood pressure, renal excretion of protein and slows the rise on blood creatinine levels. Moreover, reduction of the incidence of decompensation in the final stages of the disease is one of the critical benefit (78, 88, 89). The effectiveness is dependent when ACEI treatment starts (90). Overall, it has been admitted that the administration of ACEI's early in the course of CKD improve the antihypertensive, anti-proteinuric and antiesclerotic effects (11, 75, 90).

The ACEI's has been shown to increase survival time by 36% in patients with CKD (88). Moreover, changes in diet and simultaneously medication with ACEI's produces an increase of 53% life expectancy (73). CKD has the consequences that decrease the concentration of plasma proteins due to their loss through the affected glomeruli (90) and the use of ACEI's showed that in cats with CKD also contributes to maintaining stable levels of protein in plasma. However, further research is recommended to interpret some missing issues of the mechanism of action of these drugs. Finally, it is unquestionable that the ACEI's remain the critical drugs of choice in CKD for dogs and cats.

Conclusions

After discussing about different treatment options, it is clear that further research is needed to develop better treatments and optimize the use of current therapy. Furthermore, the integrated management of proteinuria, clinical symptoms and the changes in biosynthesis, are unquestionably, factors influencing the health-related quality of life, survival time and disease progression. Recommended here, treatments are part of that integrated management, although individually reported each effects on the CKD, the joint use of these therapies and drugs may have superior effects. Nevertheless, it is important to recommend that every patient is different, their status, initial condition, the variability of disease and its approach are factors to be necessarily taken into account and the need to treat several patients in a specific way not will necessarily be the same in another.

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KRITIČEN PRISTOP K ALTERNATIVNI OBRAVNAVI KRONIČNE ODPOVEDI LEDVIC PSOV IN MAČK

J. M. Pérez, C. Alessi

Povzetek: Kronična odpoved ledvic (CKD – iz angl. chronic kidney disease) se pogosto pojavlja pri psih in mačkah katerekoli starosti, pogostejša je pri starih pacientih. Z različnimi oblikami bolezni in njihovimi raznolikimi hemodinamskimi in presnovnimi različicami se ukvarja veliko raziskav. Trenutno izboljšanje celostnega zdravljenja kronične odpovedi ledvic se osredotoča na upočasnitev napredovanja kliničnih simptomov bolezni, obstaja pa vedno več novih metod za ta način zdravljenja v nereferenčnih centrih. Cilj predlaganega kritičnega pristopa je zagotoviti celovito obravnavo kronične odpovedi ledvic ter izpostaviti nove načine zdravljenja, ki bi lahko izboljšali poseganje v zdravje psov in mačk s kronično odpovedjo ledvic in ponovno oceno uporabnosti nekaterih obstoječih zdravil.

Ključne besede: kronična odpoved ledvic; kardiorenalni sindrom; glomerulonefritis; pes; mačka

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Original Research Article

POTENTIAL APPLICATION OF LYOPHILIZATION IN COMMERCIAL USE OF BACTERIOPHAGE PREPARATIONS IN VETERINARY MEDICINE

Aneta Skaradzińska[1]*, Grzegorz Skaradziński[2], Anna Choińska-Pulit[1], Paulina Śliwka[1], Wojciech Łaba[1], Paweł Mituła[3], Maciej Żaczek[4], Beata Weber-Dąbrowska[4]

[1]Department of Biotechnology and Food Microbiology, [2]Department of Fermentation and Cereal Technology, Faculty of Biotechnology and Food Science, Wroclaw University of Environmental and Life Sciences; Chełmońskiego 37, 51-630 Wrocław, [3]Institute of Environmental Engineering, Faculty of Environmental Engineering and Geodesy, Wroclaw University of Environmental and Life Sciences, Grunwaldzki Sq. 24, 50-363, Wrocław, [4]Laboratory of Bacteriophages, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy (IIET), Polish Academy of Sciences; R. Weigla 12, 53-114 Wrocław, Poland

*Corresponding author, E-mail: aneta.skaradzinska@upwr.edu.pl

Abstract: Microbial contamination in livestock, resulting in foodborne illnesses, poses a serious problem for veterinary medicine. Rapidly growing number of antibiotic-resistant bacterial strains triggers increased interest in phage therapy, which has already been tested against zoonotic pathogens in animals.

The aim of the study was to investigate the potential application of lyophilization process in commercial processing of phages. We lyophilized three phages active against common animal pathogens *Escherichia coli*, *Salmonella* sp. and *Enterococcus faecalis*, in the presence of three different cryoprotectants. The activity of phages was determined after 10 days, 1 month, 3 months, 6 months and 1 year storage at room temperature. All conditions of storage were established to consider practical aspects of phages processing.

Skim milk appeared to be the most effective cryoprotectant, however the obtained results varied for different phages, suggesting that efficiency of this process was phage-dependent. This in turn, may be problematic during optimization of phage lyophilization for commercial processing. Nevertheless, further commercialization of phage preparations seems to be unavoidable and the development of new methods for phage processing and storage is required.

Key words: bacteriophages; lyophilization; freeze-drying; long-term preservation of bacteriophages

Introduction

Bacteriophages (phages) have been used as antibacterial agents almost since their discovery at the beginning of the 20th century. The discovery of penicillin and subsequently other antibiotics, accompanied by scarce knowledge regarding the nature of these viruses, caused significant limitation of phage application. Nowadays, increasing inefficiency of chemical agents in treatment and prevention of bacterial infections is the main cause of renewed interest in bacteriophages (1).

Microbial contamination of livestock, resulting in foodborne illnesses, is a serious problem for the food industry. Bovines are the main animal reservoir of enterohemorrhagic *E. coli* (EHEC), which is considered the major cause of hemolytic-uremic syndrome (HUS) worldwide (2-4). In turn, contaminated poultry and swine are the two major reservoirs of *Salmonella*. Increasing multi-

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drug resistance of *Salmonella enterica* strains is due to use of antibiotics as growth promoters in food animal production (5,6).

The rising importance of phage preparations is reflected by the increasing number of commercial products based on phages appearing on the market. Although phage therapy in humans has been practiced only as an experimental form of treatment, in the last decade, approvals for commercial use of phage products in the area of veterinary medicine and food safety have been granted increasingly often. An important advantage is the possibility of using phage preparations for reducing bacterial contamination in the entire food chain, from reducing pathogen colonization in livestock, through decontamination of raw meats, to expanding shelf life of ready products (7). Nowadays, several phage products are available on the market, including the following: (i) LMP-102TM (Listshield) produced by Intralytix Inc. (USA), which is used to control *Listeria monocytogenes* in ready-to-eat meat and poultry products before packaging; (ii) ECP-100TM (Ecoshield) against *E. coli* O157:H7 i.a. in ground beef (Intralytix Inc.) (USA); (iii) Listex, effective against *L. monocytogenes*, and (iv) SALMONELEX against *Salmonella*, both produced by EBI Food Safety (Netherlands); and (v) BacWash, which is in the form of two separate products targeting *Salmonella* and *E. coli* O157:H7 on animal hides prior to slaughter (Omnilytics, Inc) (USA) (8). As the further commercialization of phage preparations seems to be unavoidable, there is an obvious need to search for methods of phage processing and long-term storage.

The aim of our studies was to investigate the potential application of lyophilization in commercial use of phage preparations as components of feed additives in veterinary medicine. We compared the activity of three bacteriophages from three different groups of phages related to animal infections (specific to *E. coli*, *Salmonella* sp. and *Enterococcus* sp.), lyophilized with different cryoprotectants and stored for one year. In our studies we used bacteriophage T4 (specific to *E. coli*), which is one of the best known bacteriophages, and the two bacteriophages Salm G713 and Entc 24 with high lytic activity (specific to *Salmonella* sp. and *Enterococcus faecalis*, respectively). As the cryoprotectants we used skim milk, as it is one of the most frequently used cryoprotectants (9), and sucrose, which according to previous reports may be useful in stabilization of freeze-dried bacteriophages (10). Considering potential application of phages as components of feed additives for farm animals, we also used commercial feed additive (Skotan S.A., Poland) as a potential stabilizer of phages during lyophilization. This additive is based on the yeast *Yarrowia lipolytica*, the cell wall of which, like the cell walls of all the yeasts, is built from different polysaccharides. This might suggest the potential role of this yeast in phage protection during lyophilization and would be a practical solution in commercial processing of phages. The phage activity was determined after 10 days, 1 month, 3 months, 6 months and 1 year storage at room temperature (RT). Time and conditions of storage were also established taking into consideration practical processing of phages.

Material and methods

Bacteriophages and bacterial strains

Bacteriophages T4, Salm G713 and Entc 24 were obtained from the Polish Collection of Microorganisms in the Institute of Immunology and Experimental Therapy (IIET), Polish Academy of Sciences (PAS). Phages were propagated respectively with *Escherichia coli* B, *Salmonella* 7116 and *Enterococcus faecalis* 661 strains (IIET Bacterial Collection). Bacteriophage lysates were obtained according to the method described by Carlson and Miller 1994 (11). The titer of phages used in the experiments was: T4 – 3 x 10^9 pfu/mL; Salm G713 – 4.50 x 10^9 pfu/mL; Entc 24 – 2.5 x 10^{10} pfu/mL.

Cryoprotectants

Three cryoprotectants were used: (i) skim milk (Sigma-Aldrich, Germany) with the final concentration 10% w/v, (ii) sucrose (Sigma-Aldrich, Germany) with the final concentration 17% w/v, and (iii) commercial animal feed additive for farm animals, with great nutritional value, based on *Yarrowia lipolytica* yeast (Yarrowia GoodStart, Skotan S.A., Poland), with the final concentration 10% w/v. The feed additive was disrupted by sonification (5 min, ice cooling, P = 70 W, f = 20 kHz (Sonoplus HD 2200, Bandelin electronic GmbH & Co. KG, Germany).

Twice concentrated solutions of skim milk and yeast feed additive were sterilized by autoclaving (117 °C, 0.8 atm, 20 min). To confirm the efficiency of this sterilization process, we prepared 72 h cultures of the *Bacillus cereus* (ATCC 14579) and *Bacillus subtilis* (ATCC 6633) in LB and sterilized it under the above conditions. Then we induced the germination of the spores with a heat shock (80°C, 20 min.) and determined the number of bacteria with plate count method. For both bacterial species we observed 100% inactivation of bacteria compared to control cultures, which were not sterilized.

Twice concentrated solution of sucrose was sterilized by filtration (membrane pore size: 0.22 μm). Phage lysates were mixed with cryoprotectants (1:1 v/v) before lyophilization.

Lyophilization

The phage lysate was mixed with protective agents (1:1 v/v). The samples of 1 mL in vials of 2 cm diameter were placed on the shelf in the Labconco Triad freeze-drier and prefrozen to -36 °C at 0.5 °C/min. The process of freeze-drying was carried out at -36 °C, 0.20 mbar for 19 hours, followed by a temperature shift to -10 °C and pressure 0.18 mbar for 1.5 hours and finally +5 °C, 0.16 mbar for 4 hours. The vials were sealed under vacuum and stored.

Storage of the samples

Samples were stored at RT and the temperature of the storage was controlled twice a day each day of the experiment. Total storage time of the samples was 1 year as the term of validity for the tested feed additive was 12 months.

Stability tests of the lyophilized bacteriophages

After 10 days, 1 month, 3 months, 6 months and 1 year, the lyophilized samples were hydrated by the addition of 1 mL of sterile, 0.9% NaCl solution. Then, the samples were incubated at 37 °C with shaking (200 rpm) for 1 hour. Titer of phages was determined with routine test dilution (RTD) and subsequently with the double-layer agar plates method (12). The stability tests for each variant were performed in triplicate.

Statistical analysis

Two-way ANOVA was performed for the stability test results and significant differences were determined according to Duncan's test, at P = 0.05. Interaction graphs were plotted and standard error bars were provided to data points (Statistica 10, StatSoft).

Results

The following samples were prepared: (i) bacteriophage lysate stored at 4°C; (ii) bacteriophage lysate stored at RT; (iii) lyophilized bacteriophage lysate with no cryoprotectants stored at RT; (iv-vi) lyophilized bacteriophage lysates with different cryoprotectants stored at RT.

Number of viable phages T4, Salm G713 and Entc 24 per mL stored for 10 days, 1 month, 3 months, 6 months and 1 year of storage at RT are presented in Tables 1-3.

Discussion

Lyophilization is commonly used for immobilization of different biological particles in industrial applications, as it facilitates transport of the product and reduces the space needed for its storage. Although lyophilization is considered one of the most effective methods of stabilization of various biological particles, in the context of commercial phage usage its application might be rather limited.

In our studies, phages retained the highest activity when stored at 4 °C (as lysates); however, the storage of the product at this temperature is not convenient for the consumer, especially in farms. A slight decrease in phage activity (compared to the initial preparation and to the preparation stored at 4 °C) was observed for phage preparations stored at RT, suggesting that this temperature of storage might also be applied. The drying method was not satisfactory, as phages stored in freeze-dried form significantly lost their titer compared to phages stored at 4 °C and RT. It is noticeable that the obtained results differed for different phages.

These results confirm previous observations of Clark (1962), who compared different methods of storage of phages specific to nine different bacterial species. He showed that phage storage in broth

Table 1: The number of viable phages T4 per ml during storage of lyophilized products. The initial phage titer was 9.48 log pfu/mL (3 x 10^9 pfu/mL)

T4	10 days (log pfu/mL)	1 month (log pfu/mL)	3 months (log pfu/mL)	6 months (log pfu/mL)	1 year (log pfu/mL)
Phage lysate (4°C)	9.73^{a}	9.91^{a}	9.86^{a}	9.87^{a}	9.58^{a}
Phage lysate (RT)	8.22^{b}	9.91^{a}	9.75^{a}	9.43^{a}	8.89^{i}
Lyophilized phage lysate	9.77^{a}	8.20^{b}	$5.16^{e.f}$	3.02^{h}	ud.
Phage lysate lyophilized with skim milk	$6.21^{c.d}$	$5.96^{c.d}$	$5.68^{c.f}$	4.90^{e}	4.26^{j}
Phage lysate lyophilized with sucrose	$5.23^{e.f}$	$5.99^{c.d}$	5.09^{e}	7.43^{g}	$6.18^{c.d}$
Phage lysate lyophilized with yeast feed additive	$7.73^{b.g}$	$7.65^{b.g}$	6.55^{d}	3.41^{h}	ud.

The same superscript letters designate homogenous groups.
ud. - undetected

Table 2: The number of viable phages Salm 713 per ml during storage of lyophilized products. The initial phage titer was 4.50 x 10^9 pfu/mL (9.65 log pfu//mL)

Salm 713	10 days (log pfu/mL)	1 month (log pfu/mL)	3 months (log pfu/mL)	6 months (log pfu/mL)	1 year (log pfu/mL)
Phage lysate (4°C)	9.73^{a}	9.80^{a}	9.70^{a}	$9.65^{a.b}$	$9.17^{b.d}$
Phage lysate (RT)	$9.65^{a.b}$	$9.60^{a.b}$	$9.51^{a.b}$	$9.48^{a.b}$	$8.76^{c.d}$
Lyophilized phage lysate	$8.79^{c.d}$	$8.37^{c.f.g}$	$7.98^{f.j}$	6.89^{l}	5.00^{i}
Phage lysate lyophilized-with skim milk	$8.83^{c.d}$	$8.52^{c.g}$	$8.34^{c.f.g}$	$8.15^{f.g.j}$	$7.31^{k.l}$
Phage lysate lyophilized with sucrose	6.27^{e}	$4.60^{h.i}$	6.35^{e}	$4.69^{h.i}$	5.63^{m}
Phage lysate lyophilized with yeast feed additive	$8.27^{f.g}$	$8.14^{f.g.j}$	$7.74^{j.k}$	6.32^{e}	4.29^{h}

The same superscript letters designate homogenous groups

Table 3: The number of viable phages Entc 24 per ml during storage of lyophilized products. The initial phage titer was 2.5 x 10^{10} pfu/mL (10.40 log pfu/mL)

Entc 24	10 days (log pfu/mL)	1 month (log pfu/mL)	3 months (log pfu/mL)	6 months (log pfu/mL)	1 year (log pfu/mL)
Phage lysate (4°C)	$10.22^{a.b}$	$10.27^{b.h}$	10.38^{h}	$9.75^{c.e}$	$9.63^{e.f}$
Phage lysate (RT)	$10.10^{a.c.d}$	9.99^{d}	9.85^{c}	9.27^{g}	7.88^{m}
Lyophilized phage lysate	$9.71^{c.e}$	9.50^{f}	$9.62^{e.f}$	8.49^{j}	7.83^{m}
Phage lysate lyophilized with skim milk	9.52^{f}	9.54^{f}	9.79^{c}	9.18^{g}	8.72^{k}
Phage lysate lyophilized with sucrose	9.50^{f}	$9.63^{e.f}$	9.82^{c}	$8.9^{i.k}$	$8.64^{k.l}$
Phage lysate lyophilized with yeast feed additive	9.18^{g}	8.93^{i}	9.19^{g}	$8.51^{j.l}$	7.20^{n}

The same superscript letters designate homogenous groups

at 4 °C was the most efficient method for phage preservation, while freeze-drying (with skim milk as a cryoprotectant) was damaging for all studied viruses. Considering both prestorage treatment and the storage (2 years, 4 °C), of twenty phages, only two showed the best recovery in lyophilized form (13).

We showed that skim milk and sucrose may be potential cryoprotectants in the lyophilization process. However, optimization of the method in order to increase phage survival is required. This applies in particular to lyophilization with sucrose, as the results of the studies were particularly inconsistent. Yeast feed additive was the least efficient cryoprotectant, but it is necessary to emphasize that the results were significantly different for different phages. Radical differences were observed in the case of phages lyophilized with no cryoprotectant. Phage Entc 24 specific to *Enterococcus faecalis* retained its activity compared to phage stored at RT after 12 months, while phage T4 specific to *E. coli* completely lost its activity when stored in this form. Phage Salm G713 specific to *Salmonella* sp. showed intermediate loss of its activity.

In studies presented by Dini and de Urraza (2013), phages lyophilized with skim milk lost 1.2 log pfu/mL after the process (6% phage survival) (14). This is also in accordance with the aforementioned publication of Clark (1962), who observed that the survival of two coliphages lyophilized with skim milk was in the range 25% - less than 1%.

Although disaccharides are expected to increase the protection of the particles during lyophilization (15), application of sucrose as the cryoprotectant of phages gives ambiguous results. Data regarding usage of sucrose as a cryoprotectant are variable and show that in each case, optimization of the method is needed. In studies presented by Dini and Urraza (2013) disaccharides did not improve the stability of phages during freeze-drying compared to PBS. It is worth mentioning that the addition of 0.3 M sucrose provided a protective effect on the lyophilized phage during storage (120 days) compared to that obtained immediately after lyophilization. Phages lyophilized in SM buffer showed significantly increased stability compared to PBS (phage survival range 1-15% after lyophilization).The addition of 0.1 M sucrose significantly increased the number of active phages after lyophilization compared with the number of phages lyophilized in SM buffer alone. The stability was also improved compared to skim milk. Phage preparations lyophilized in SM buffer with 0.1 M sucrose also showed great stability, retaining almost full phage activity during 120 days of storage compared to the phage titer immediately after lyophilization. The addition of higher sucrose concentrations (0.3 M; 0.5 M) was detrimental for phage stability compared to samples lyophilized without sucrose (14).

Dini and de Urraza (2013) analyzed each step of the freeze-drying process (PBS with 0.5 M sucrose), showing that the main phage loss occurs during the drying step (decrease of 1.58 log pfu/mL). If lyophilized with 0.1 M concentration of sucrose, phage viability was almost completely retained during the freeze-thaw step, and the observed loss of phage titer (0.56 log pfu/mL) was attributed to the drying step. The analysis of the storage of lyophilized preparations (4 °C, 120 days) showed the stabilizing effect of sucrose (PBS with 0.3 M sucrose and SM with 0.1 M sucrose) (14).

In another study, the authors optimized the lyophilization process by testing four different concentrations of trehalose and sucrose, as they seemed to be the best cryoprotectants of *Staphylococcus* phage ISP. The immediate decrease in titer after lyophilization varied between 0.6 and 1.4 log/mL, and the best results were obtained in the case of 0.8 M and 1.0 M sucrose, with loss of only 0.4-0.5 log/mL. During the 27-month storage period, the activity of ISP remained stable, with variations within one log/mL in all preparations, except for 0.3 M trehalose (10). Also, in studies presented by Puapermpoonsiri et al. (2010), higher loss of activity was observed for phages lyophilized with high concentrations of sucrose (0.5 M) than with lower concentrations (0.1 M) (16).

Also potential application of other cryoprotectants in lyophilization of phages has been studied. PEG6000 was used for stabilization of *Staphylococcus aureus* phage. Immediately after immobilization, phages showed a titer loss of 1.8 log/mL and 5.0 log/mL for 1% and 5% PEG6000 concentrations respectively. Titer loss was also observed after storage for 37 months (loss of 3 log/mL for 1% PEG6000 and 1.7 log/mL of the titer for 5% PEG6000) (10). These observations were also confirmed by Puapermpoonsiri et al. (2010), who showed that PEG6000 as a cryoprotectant at concentrations of 1% and 5% was detrimental for phage specific to *Staphylococcus aureus* but also for phage specific

to *Pseudomonas aeruginosa* (16). The same phages lyophilized in SM medium with or without gelatin began to lose their activity after the 30th day of storage. Also glycine and polyvinylpyrrolidone (PVP) were detrimental for phages. Glycine caused their inactivation immediately after lyophilization and PVP inactivated phages completely at both concentrations (1% and 5%) even before lyophilization (10). Also mannitol at a concentration 0.1 M caused total inactivation of phages after lyophilization. At concentration of 0.5 M, a 4 log/mL decrease in phage titer was observed (10). Alfadhel et al. (2011) used a lyophilized viscous solution of hydroxypropyl methylcellulose (HPMC) in concentration 1-2% (w/v) with and without addition of 1% (w/v) mannitol to prepare inserts for nasal administration of phage against *Staphylococcus aureus*. Phage preparations in HPMC/mannitol gel formulations were lyophilized, which resulted in 90% loss of phage titer, but still 10^8 pfu/mL (1 log/mL decrease from 10^9 pfu/mL). After 1-12 months of storage at 4 °C, lytic activity of phages decreased 10-1000 fold (from 10^8 pfu/mL on day 1 to around 10^7, 10^6, 10^5 pfu/mL after 1, 2 and 5 months respectively), which still was a therapeutic dose (17).

Although there have already been published several reports regarding the influence of the freeze drying process on activity of phages, still the knowledge on this topic may be considered scarce. The results of our studies confirmed previous observations that different phages may show different tolerance to lyophilization, which may be one of the most important obstacles in optimization of the process. Even though freeze drying in general seems to be detrimental for phages, optimization of the method may allow improvement of phage survival, which may contribute to its practical usage. It is worth noting that application of lyophilization may not be limited to phage storage but also may precede (18) or follow (19) the process of phage encapsulation. Although encapsulation methods also need to be optimized, they are necessary for improved survival of phages in animals' digestive systems, as phages applied orally are often destroyed in low stomach pH.

Phage potential in prevention and treatment of farm animal bacterial infections has been proved. According to several reports, phages were successfully applied against *E. coli* in calves, pigs, lams and poultry, against *Campylobacter jejuni* in chicken and against *Salmonella* in pigs (20-22). It was shown that bacteriophages biocontrol of *S. enterica* in dried pet food is effective and technically feasible (23). These promising results translate into a tendency toward commercialization of phage products in the fields of agriculture and veterinary medicine. Nonetheless, as phage application seems to be inevitable, research regarding biology of these viruses must be combined with development of technological processes associated with their processing.

Conclusion

Lyophilization is one of the most commonly used methods for stabilization of biological particles, however its application for bacteriophages might be rather limited. Our studies confirmed previous observations, that efficiency of the freeze-drying process depends on the applied cryoprotectant, as well as on the type of bacteriophage. Optimization of the method generally for all bacteriophages for commercial purposes is problematic, however, it is possible for single phages. In this case, lyophilization of phages may be a good solution for preservation of phage viability in time, especially considering the convenience of dried form of the preparation in contrast to a liquid lysate. As an interest in phage application significantly grows, further studies regarding the development of new methods for phage processing and storage are required.

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Ethics approval: not required

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MOŽNOSTI UPORABE LIOFILIZACIJE V KOMERCIALNE NAMENE ZA PRIPRAVO BAKTERIOFAGOV V VETERINARSKI MEDICINI

A. Skaradzińska, G. Skaradziński, A. Choińska-Pulit, P. Śliwka, W. Łaba, P. Mituła, M. Żaczek, B. Weber-Dąbrowska

Povzetek: Mikrobiološka kontaminacija hrane v živinoreji, ki povzroča bolezni, predstavlja resen problem v veterinarski medicini. Hitro naraščajoče število bakterijskih sevov, odpornih proti antibiotikom vzbuja vedno večje zanimanje za uporabo t.i. fagoterapije, zdravljenja z bakterijskimi virusi bakteriofagi, ki je bila že preizkušena kot metoda za preprečevanje okužb pri živalih. Cilj raziskave je bil proučiti potencialno uporabo postopka liofilizacije za komercialno obdelavo fagov. Liofilizirali smo tri fage, aktivne proti skupnim živalskim patogenom *Escherichia coli*, *Salmonella* sp. in *Enterococcus faecalis* v prisotnosti treh različnih krioprotektantov. Dejavnost fagov je bila določena po 10 dneh, 1 mesecu, 3 mesecih, 6 mesecih in 1 letu skladiščenja pri sobni temperaturi. Vzpostavljeni so bili vsi pogoji shranjevanja, z namenom upoštevanja praktičnih vidikov obdelave fagov. Posneto mleko je bilo najučinkovitejši krioprotektant, vendar so se dobljeni rezultati razlikovali pri različnih fagih, kar kaže na to, da je učinkovitost postopka odvisna od vrste faga. To bi lahko predstavljalo težavo pri optimizaciji liofilizacije fagov v komercialne namene. Kljub temu je nadaljnja komercialna priprava preparatov s fagi neizogibna in bo potrebno razvijati nove metode za obdelavo in shranjevanje fagov.

Ključne besede: bakteriofagi; liofilizacija; sušenje z zamrzovanjem; dolgoročno ohranjanje bakteriofagov

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Original Research Article

SEX DIFFERENCES IN LIVER GENE EXPRESSION IN WT AND SF-1 KNOCKOUT MICE

Katja Kozinc Klenovsek,[1] Tanja Spanic [1], Gregor Majdic [1,2]

[1]Institute for preclinical studies, Department for Biochemistry, Molecular Biology and Genetics, Veterinary Faculty, University of Ljubljana, Ljubljana, [2]Institute of Physiology, Medical School, University of Maribor, Maribor, Slovenia

*Corresponding author, E-mail: gregor.majdic@vf.uni-lj.si

Abstract: Liver development and function are dependent on specific gene expression profile. Many genes in the liver are differentially expressed between females and males and these sex differences are thought to be at least partially influenced by sex specific patterns of growth hormone secretion. The aim of this study was to examine whether sex chromosomes contribute to sex differences in the liver gene expression. Expression of *Cyp4a10*, *Cyp2u1*, *Cux2* and *Hsd3b5*, which are known to be differentially expressed between sexes in adulthood, were studied in WT and SF-1 knockout mice. Steroidogenic factor 1 knockout (SF-1 KO) mice that are born without gonads were used to determine whether there are any sex differences in the gene expression even in the absence of exposure to sex steroid hormones. Gonadectomised mice were also compared to gonadally intact mice and gene expression of studied genes was examined during estrous cycle in gonadally intact female mice. Higher expression of *Cux2* and *Cyp4a10* was detected in gonadally intact WT females in comparison to gonadally intact WT males and higher expression of *Cyp2u1* and *Hsd3b5* was detected in gonadally intact WT males in comparison to gonadally intact WT females. There were no sex differences in the expression of studied genes between WT gonadectomised and SF-1 KO mice. The results of our study therefore suggest that sex differences in the liver gene expression of the four studied genes are solely dependent on sex hormones and are not influenced by sex chromosome complement.

Key words: sex difference; liver; gene expression; sex steroid hormones; sex chromosomes

Introduction

Liver is one of the largest and the most important organs in the body that performs numbers of essential functions for life. It is involved in a variety of physiological functions including metabolizing carbohydrates, proteins and lipids, producing bile acids, forming plasma proteins and filtering harmful substances from the blood. Development and functioning of such a complex organ is dependent on specific gene expression profile (1). Several studies have shown that mammalian liver is also a sexually dimorphic organ, exhibiting major sex differences in drug and lipid metabolism and some other functions (2-5). Sex differences in liver function are present in the expression of cytochromes P450 (*Cyp*) and other drug-metabolizing enzymes (5). Over 1000 genes were shown to be differentially expressed between male and female liver by microarray studies and these sex differences are thought to be regulated primarily by sex specific patterns of pituitary growth hormone (GH) secretion (6, 7), which is regulated by estrogens' secretion in adult females and by androgens neonatally and during adulthood in males (8).

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GH acts through signal transducer and activator of transcription STAT5b (3) and hepatocyte enriched nuclear factor HNF4 (7). Both these transcription factors are involved in the regulation of expression of many genes expressed in the liver and most likely contribute to the sexually dimorphic gene expression in the liver (7, 9).

Steroidogenic factor 1 (SF-1), officially designated NR5a1, is a nuclear receptor that was initially discovered as a regulator of the cytochrome P450 steroid hydroxylase family of enzymes (10). SF-1 plays a role in the regulation of a number of genes involved in gonadal and adrenal development, reproduction and steroidogenesis (11). Mice lacking SF-1 gene, SF-1 knockout (KO) mice, are born without adrenal glands and gonads, and exhibit male to female sex reversal (12, 13). Both male and female SF-1 KO mice are thus phenotypically females, although with different sex chromosome complement. Due to adrenal agenesis, SF-1 KO mice die shortly after birth, but could be rescued by corticosteroid replacement therapy followed by adrenal transplantation (14). Because SF-1 KO mice are born without gonads, they represent an important tool that allows studies of gonadal hormone-independent sexual differentiation in different tissues, including liver gene expression.

In the present study sex specific gene expression of two cytochrome P450 genes (*Cyp4a10* and *Cyp2u1*), transcriptional regulator *Cux2* and enzyme *Hsd3b5* was examined in agonadal SF-1 KO mice to establish whether sex differences in liver gene expression are solely dependent on the secretion of gonadal hormones, or are some differences caused by sex chromosome complement. All studied genes have been shown before to have significant sex specific differences in the expression in adult mice. *Cyp4a10* (6, 15-17) and *Cux2* (6, 18) are thus expressed at the higher levels in female livers while *Cyp2u1* (6, 16) and *Hsd3b5* (6, 19) show male specific expression pattern.

Materials and methods

Animals

All animal experiments were done according to ethical principles and in accordance to the EU directive (2010/63/EU) and were approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection (license number U34401-22/2015/4).

Mice were bred and housed in the animal facility at Institute for preclinical studies, Department for Biochemistry, Molecular Biology and Genetics, Veterinary Faculty, University of Ljubljana under standard laboratory conditions (temperature 20-25 °C, humidity 40-60 %) and 12:12 dark/light cycle. Mice were housed in plastic cages on bedding of wood chips (Lignocel, Rosenberg, Germany) with phytoestrogens-free diet (No. 2916; Harlan Teklad, Milan, Italy) and water *ad libitum*.

Heterozygous mice with a disrupted *Sf-1* (*Nr5a1*) allele (D2.129P2(B6)-*Nr5a1;* SF-$1^{+/-}$) were mated to produce SF-1 KO (SF-$1^{-/-}$) and control WT (SF-$1^{+/+}$) offspring. To ensure survival of SF-1 KO mice, all newborn pups were genotyped and rescued as described before (12, 14).

After weaning, some WT mice were castrated (CAS) or ovariectomized (OVX) prior to puberty between postnatal days 21-25. For gonadectomy, WT mice were anesthetized with mixture of ketamine (Vetoquinol Biowet, Gorzów Wielkopolski, Poland; 100 μg/g body weight (BW)), xylazine (Bioveta, Ivanovice na Hané, Czech Republic; 10 μg/g BW) and acepromazine (Vetoquinol, Lure, France; 2 μg/g BW) subcutaneously. Testes were removed through bilateral incisions (CAS), and ovaries through a single medial incision (OVX). After gonadectomy, animals received 2 injections of analgesic butorphanol (Richter Pharma AG, Wels, Austria; 3 mg/kg BW) in 4-hour interval. For control to gonadectomy, SF-1 KO animals were sham operated at the same age. After surgery animals of the same chromosomal sex were housed together until the time of sacrifice. Gonadally intact WT females were sacrificed at different stages of estrous cycle (proestrus, estrus, metestrus and diestrus), which were determined by vaginal smear cytology.

Mice 5 to 7 months old were euthanized to obtain the liver tissue. Previous studies have shown that there is small effect of age on liver gene expression in adult mice and rats (20, 21) and therefore, we predicted that 5 to 7 months span should not affect the results. Animals were euthanized with CO_2 and a sample of right hepatic lobe was taken. Livers were snap frozen in liquid nitrogen and then stored at -80 °C until RNA isolation.

RNA isolation and quantitative RT PCR

Total RNA was isolated from frozen individual liver tissue samples using TRIzol® reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, California, USA). RNA concentration and quality were determined on Nanodrop™ Lite Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). RNA was transcribed to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, California, USA) and then stored at -20 °C until next use. Dilution of cDNA was prepared with nuclease free water. Quantitative real-time PCR (RT-qPCR) was done on the ABI 7300 Real-Time PCR System (Applied Biosystems) using TaqMan® Universal Master Mix II, with UNG (Applied Biosystems) and with ROX reference dye. RT-qPCR reactions were conducted in triplicates. Ten nanograms of cDNAs were used with commercial TaqMan Gene Expression Assays for genes: *Hsd3b5*, *Cyp2u1*, *Cyp4a10* and *Cux2*, and *Gapdh* and *Actb* as internal controls (Applied Biosystems). All probes had a FAM™ dye label on the 5' end and minor groove binder (MGB) and nonfluorescent quencher (NFQ) on the 3' end. The cycling conditions were as follows: an initial 50 °C for 2 minutes and then 90 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 sec, 60 °C for 1 minute. To determine the Ct (threshold cycle) for each amplification reaction 7300 System Software was used.

All data were analyzed using the Pfaffl method as previously described (22). Two reference genes (housekeeping genes: *Gapdh* and *Actb*) were used to determine the normalization factor from geometric mean of their expression levels for every sample (23). To calculate normalized expression level of all four genes of interest, relative quantity for every sample of every gene were divided with corresponding normalization factor.

Statistical analysis

Statistical analyses were performed using the NCSS software (NCSS, Kaysville, Utah, USA). Results of gene expression study were compared using planned comparison ANOVA, followed by posthoc Fisher LSD test. All data are presented as mean $\pm$ S.E.M. and statistical significance was considered at $p < 0.05$.

Results

Cux2

As expected, the expression of *Cux2* was higher in gonadally intact WT females than in gonadally intact WT males ($p < 0.001$). The expression of *Cux2* did not differ significantly between different stages of the estrous cycle in gonadally intact WT females. Absence of gonads significantly reduced the level of expression in both WT ovariectomized females and SF-1 KO females ($p < 0.001$) with no significant difference between WT OVX and SF-1 KO females. Interestingly, there was a significant difference between gonadally intact WT males, WT castrated males and SF-1 KO males with gonadally intact WT males showing lower expression in comparison to both agonadal groups ($p < 0.05$). There was no significant sex difference between gonadectomised WT males and females or SF-1 KO males and females (Figure 1).

Cyp4a10

As expected, the expression of *Cyp4a10* was higher in livers from gonadally intact WT females in comparison to gonadally intact WT males ($p < 0.01$), but there was no difference in the expression in female livers during different stages of the estrous cycle. Absence of gonads in WT females and agonadal SF-1 KO mice resulted in the expression of *Cyp4a10* similar to the levels observed in livers of all three groups of males and was significantly lower in comparison to the expression in gonadally intact WT females ($p < 0.05$). There was no significant difference between all three groups of male mice and there was no significant sex difference between gonadectomised WT males and females or SF-1 KO males and females. (Figure 2).

Cyp2u1

As expected the expression of *Cyp2u1* was significantly higher in gonadally intact WT males in comparison to gonadally intact WT females regardless of the females' estrous cycle ($p < 0.001$) and there was no significant difference between females during different stages of the estrous cycle. Absence of gonads in females (in

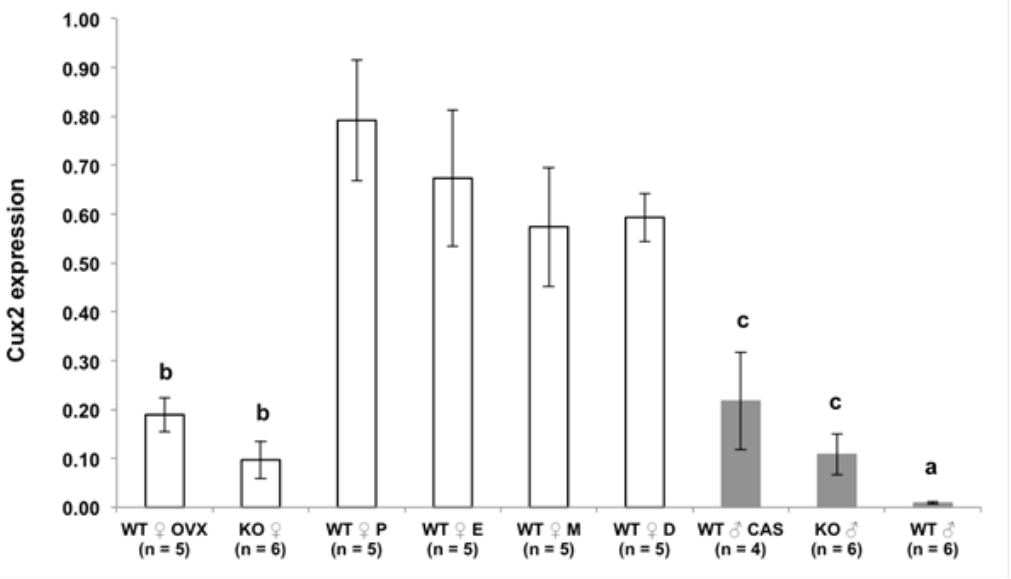

Figure 1: Expression of *Cux2* was higher in gonadally intact WT females in comparison to gonadally intact WT males (a, $p < 0.001$) and to WT ovariectomized females and SF-1 KO females (b, $p < 0.001$) There was no significant difference between different stages of estrous cycle, however, expression in gonadally intact WT males was lower in comparison to WT castrated males and SF-1 KO males (c, $p < 0.05$; mean + S.E.M.; WT ♀ OVX - WT ovariectomized females, KO ♀ - SF-1 KO females, WT ♀ P – gonadally intact WT females in proestrus, WT ♀ E - gonadally intact WT females in estrus, WT ♀ M - gonadally intact WT females in metestrus, WT ♀ D - gonadally intact WT females in diestrus, WT ♂ CAS - WT castrated males, KO ♂ - SF-1 KO males, WT ♂ - gonadally intact WT males).

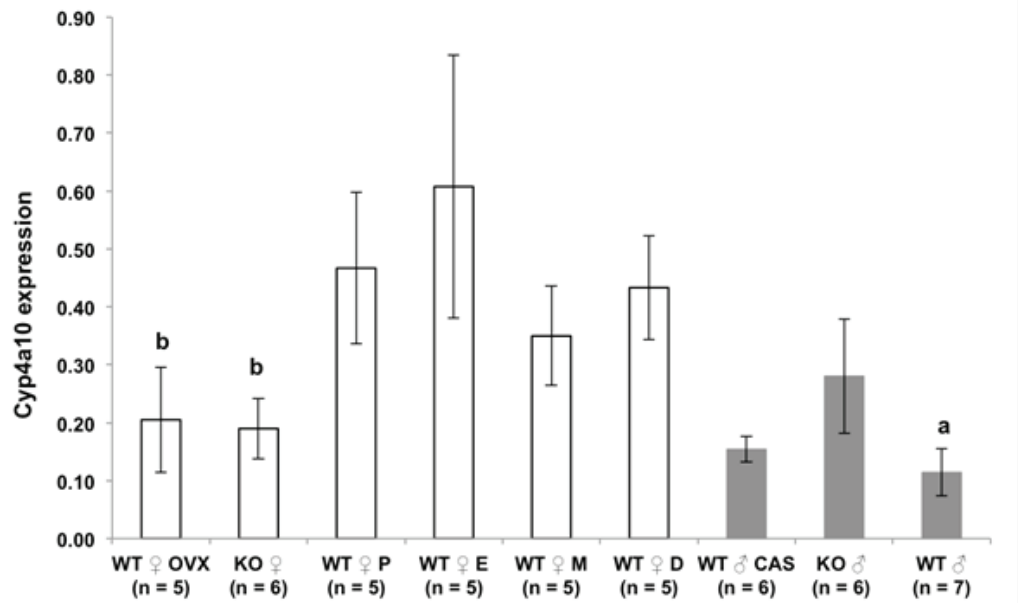

Figure 2: Expression of Cyp4a10 was higher in gonadally intact WT females in comparison to gonadally intact WT males (a, $p < 0.01$). There was no significant difference between females during different stages of the estrous cycle. The expression was significantly lower in both ovariectomized WT females and agonadal SF-1 KO females in comparison to the expression in gonadally intact WT females (b, $p < 0.05$), and was similar to the expression level observed in all three groups of males. There was no significant difference between all three groups of males, or between WT gonadectomised males and females and SF-1 KO males and females (mean + S.E.M.; WT ♀ OVX - WT ovariectomized females, KO ♀ - SF-1 KO females, WT ♀ P - gonadally intact WT females in proestrus, WT ♀ E - gonadally intact WT females in estrus, WT ♀ M - gonadally intact WT females in metestrus, WT ♀ D - gonadally intact WT females in diestrus, WT ♂ CAS - WT castrated males, KO ♂ - SF-1 KO males, WT ♂ - gonadally intact WT males).

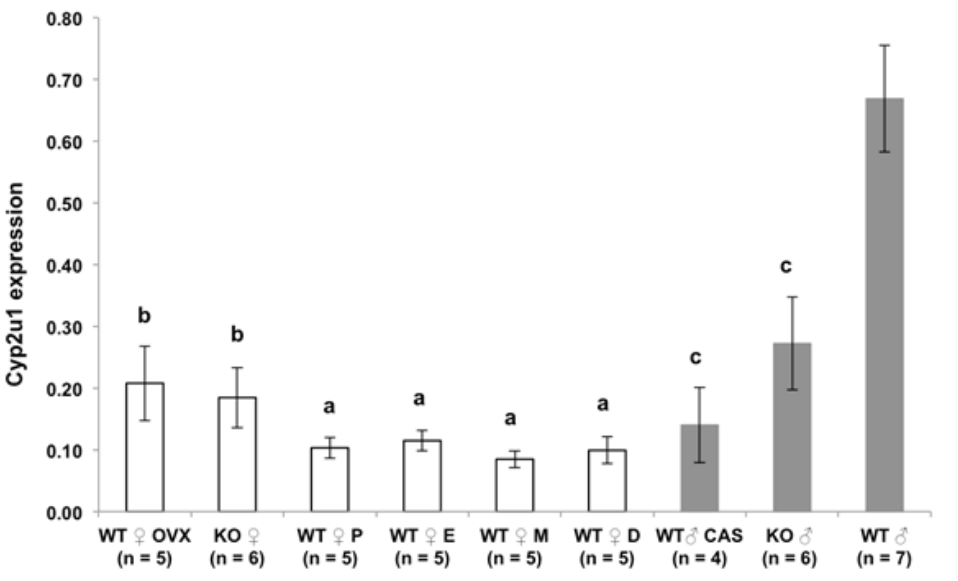

Figure 3: Expression of *Cyp2u1* was higher in gonadally intact WT males in comparison to gonadally intact WT females regardless of the estrous cycle (a, $p < 0.001$), and there was no significant difference between different stages of the estrous cycle. WT ovariectomized females and SF-1 KO females showed significantly increased expression levels in comparison to gonadally intact WT females (b, $p < 0.05$) but lower in comparison to gonadally intact WT males. Expression in gonadally intact WT males was significantly higher in comparison to expression in both agonadal groups of males (WT castrated and SF-1 KO males, c, $p < 0.001$; mean + S.E.M.; WT ♀ OVX - WT ovariectomized females, KO ♀ - SF-1 KO females, WT ♀ P - gonadally intact WT females in proestrus, WT ♀ E - gonadally intact WT females in estrus, WT ♀ M - gonadally intact WT females in metestrus, WT ♀ D - gonadally intact WT females in diestrus, WT ♂ CAS - WT castrated males, KO ♂ - SF-1 KO males, WT ♂ - gonadally intact WT males)

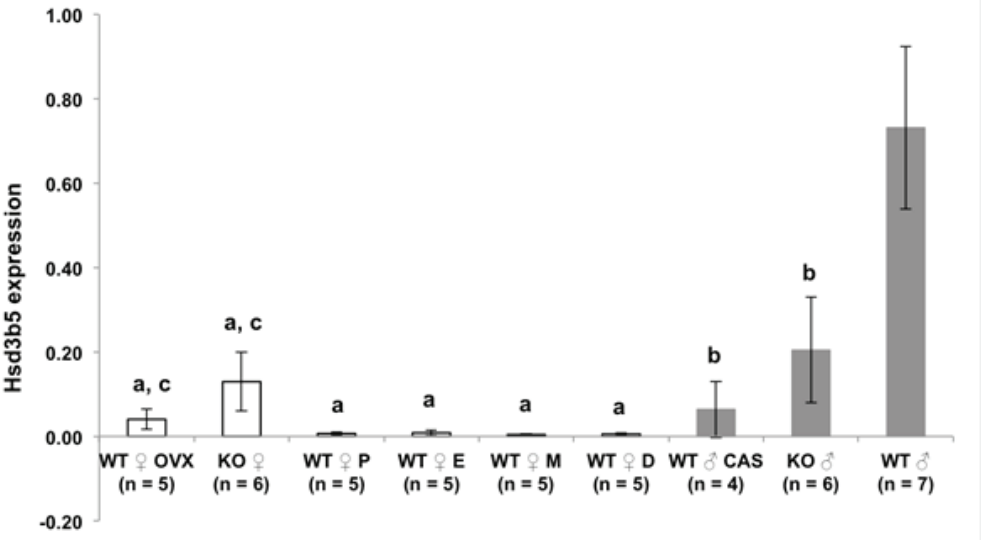

Figure 4: Expression of *Hsd3b5* was significantly higher in gonadally intact WT males in comparison to all groups of females regardless of the estrous cycle (a, $p < 0.001$) and to both groups of agonadal males (WT castrated and SF-1 KO males; b, $p < 0.05$). In gonadally intact females, there was no significant difference between stages of the estrous cycle, however, the expression was significantly higher in both ovariectomized WT and SF-1 KO females in comparison to the gonadally intact WT females (c, $p < 0.01$; mean + S.E.M.; WT ♀ OVX - WT ovariectomized females, KO ♀ - SF-1 KO females, WT ♀ P - gonadally intact WT females in proestrus, WT ♀ E - gonadally intact WT females in estrus, WT ♀ M - gonadally intact WT females in metestrus, WT ♀ D - gonadally intact WT females in diestrus, WT ♂ CAS - WT castrated males, KO ♂ - SF-1 KO males, WT ♂ - gonadally intact WT males)

both WT ovariectomized females and SF-1 KO females) significantly increased the expression in comparison to gonadally intact females ($p < 0.05$), but did not reach the levels observed in gonadally intact WT males. In males, there was significantly higher expression in gonadally intact WT males in comparison to both agonadal groups of males (WT castrated and SF-1 KO males, $p < 0.001$;). There was no significant sex difference between gonadectomised WT males and females or SF-1 KO males and females (Figure 3).

Hsd3b5

In accordance with previous studies, expression of *Hsd3b5* was the highest in gonadally intact WT males and was significantly higher than in gonadally intact WT females regardless of females' estrous cycle ($p < 0.001$). There was no significant difference between females during the estrous cycle, but there was a significant difference between agonadal female mice (both WT ovariectomized and SF-1 KO) and gonadally intact female mice with agonadal WT ovariectomized and SF-1 KO female mice having higher expression of *Hsd3b5* ($p < 0.01$). In male mice, the expression was significantly lower in SF-1 KO males and WT castrated males in comparison to gonadally intact WT males ($p < 0.05$). There was no significant difference between gonadectomised WT males and females or SF-1 KO males and females (Figure 4).

Discussion

In the present study, the influence of sex hormones and sex chromosomes on mouse liver gene expression was examined. SF-1 KO mice that are born without gonads were used to investigate whether sex chromosomes play a role in the regulation of liver gene expression in addition to sex hormones. Results revealed significant differences in liver gene expression between sexes in gonadally intact WT animals, but not between SF-1 KO and WT GDX females and males. These findings therefore suggest that sex differences in the liver gene expressions are primarily influenced by sex hormones and not by sex chromosomes.

Many studies in the past have shown that gene expression in the liver is sexually dimorphic (6, 24). It is believed that these sex differences in liver gene expression mainly arise as a consequence of sexually dimorphic patterns of pituitary GH secretion at least in mice and rats (8, 7). GH is secreted in more pulsatile manner in males while its secretion from the pituitary in females is steadier. These differences in the secretion of GH are thought to be influenced by sex steroid hormones, namely estrogens in females and androgens in males (8). In males neonatal exposure to testosterone imprints the male pattern of the pulsatile pituitary GH secretion that is first seen at puberty and continues through adulthood. However, the presence of androgens is also necessary during adult life as in the absence of androgens, secretion pattern of growth hormone will show a feminine pattern in gonadectomised males (8).

Both estrogens and androgens can influence GH centrally by regulating pituitary GH secretion, but they can also influence the action of growth hormone peripherally by modulating the level of growth hormone receptor (GHR) expression and its signaling (25, 26).

Despite the established role for sex steroid hormones in the regulation of sex specific gene expression in the liver, possible influence of genes present on sex chromosomes on sex differences in the expression have not been reported. In the present study we have therefore chosen 4 genes, whose expression has been previously reported to be strongly sexually dimorphic, and examine their expression with regard to the exposure to sex steroid hormones during development and/or in adult life. Two of these genes, *Cux2* and *Cyp4a10* are expressed at the higher levels in females (6, 18, 15-17) and *Cyp2u1* and *Hsd3b5* are expressed at the higher levels in male mice (6, 16, 19). *Cyp2u1* and *Cyp4a10* are members of the cytochrome P450 family, which is comprised of hem-containing enzymes involved in metabolism of endogenous steroids and fatty acids, as well as detoxification of many drugs and environmental compounds (27). Similarly, like *Cyp* genes, *Hsd3b5* gene is involved in steroid hormone metabolism (28), whereas the role of transcription factor *Cux2* in the liver is to repress male-biased gene expression and induce female-biased gene expression (29).

Cux2 gene is a transcription factor that is believed to have a regulatory role in the sexually dimorphic liver gene expression. Previous studies have shown, that *Cux2* directly regulates expression of many sexually dimorphic genes in

the liver and is thus responsible for many sexual dimorphic gene expression patterns (18). In the present study, the expression of *Cux2* was higher in WT gonadally intact females in comparison to WT gonadally intact males, as expected. However, *Cux2* gene expression did not differ during the estrous cycle as females in proestrus, estrus, diestrus and metestrus all show similarly high levels of expression of *Cux2*. Absence of gonads either through prepubertal gonadectomy of WT mice at 3 weeks of age, or in developmentally agonadal SF-1 KO mice reduced the level of expression in both agonadal males and females, suggesting that high expression in gonadally intact females is influenced by circulating female gonadal sexual hormones, estrogens and progestins. However, as there is no difference in *Cux2* gene expression during the estrous cycle, this suggests that effects of estrogens (or possibly progestins) on liver gene expression of *Cux2* gene are organizational and not activational. There was no difference in the expression of *Cux2* between males and females in WT gonadectomised and in SF-1 KO mice, further confirming that gonadal hormones are necessary for sexually dimorphic expression of *Cux2*, and there is no influence of sex chromosome genes on this expression. However, since WT males and females were gonadectomised only at three weeks of age, but there is no difference in the expression within the estrous cycle, this suggest that activational effects of gonadal hormones must not occur during classical organizational periods but later, during puberty or in early adulthood. Several studies indeed suggest that puberty is another period important for organizational effects of gonadal hormones in the brain (30). Although there are no reports about organizational effect of gonadal hormones on GH secretion patterns during puberty, it is possible that sexually dimorphic GH secretion pattern might develop during puberty and this could then influence sexually dimorphic liver gene expression. Interestingly, we also observed a significant difference in *Cux2* expression in liver between WT gonadally intact males and both WT castrated males and SF-1 KO males. The expression was significantly lower in WT gonadally intact males in comparison to both agonadal males. This suggests that not only estrogens are involved in the regulation of *Cux2* expression, but most likely testosterone further suppress this gene's expression levels. Whether this difference is due to differences in GH secretion patterns or due to direct effects of testosterone will have to be determined in the future studies.

Cyp4a10 is a member of cytochrome P450 family with the function in xenobiotic metabolism (15). As with *Cux2*, we observed sexually dimorphic pattern of expression in gonadally intact WT mice only, and there was also no difference in the expression during the estrous cycle. Furthermore, expression of *Cyp4a10* was reduced in both gonadectomised WT male and female mice, and agonadal SF-1 KO male and female mice, and there was no difference between these groups, again suggesting that sex chromosomes do not have a role in the sexually dimorphic expression. However, in contrast to *Cux2* expression, expression levels of *Cyp4a10* were similar between all three groups of males, suggesting that estrogens have the primary role in establishing sexually dimorphic gene expression pattern. Again, these roles must be primarily organizational as there was no difference between the females in different estrous cycle stage, but there is probably no influence of testosterone as there was no difference present between three groups of males.

As expected in accordance to previous results (6, 16), the expression of *Cyp2u1* was significantly higher in gonadally intact males in comparison to gonadally intact females during different stages of the estrous cycle. This suggests that the main hormone regulating the expression of *Cyp2u1* is testosterone, increasing expression of this gene either directly or through regulating pituitary GH secretion. In comparison to WT gonadally intact males, expression of *Cyp2u1* was lower in both WT castrated males and SF-1 KO males. This suggests, that testosterone exposure after prepubertal development is responsible for higher expression of *Cyp2u1* in gonadally intact WT males. Whether this higher expression is the result of direct activational effects by testosterone (directly or through GH secretion) in adult life, or perhaps through the organizational effects during puberty, remain to be determined in the future studies and was beyond the scope of this study. As with the other genes studied, there were no differences between gonadally intact WT females during the estrous cycle, again suggesting that estrogens or progestins do not have a direct, activational role in the regulation of expression of this gene in adult female mice. Interestingly, the expression in both WT ovariectomized females

and SF-1 KO females was higher in comparison to gonadally intact females, suggesting that exposure to estrogens are also involved in the regulation of sexually dimorphic expression of *Cyp2u1* by reducing the levels of expression of this gene.

The expression of *Hsd3b5* was higher in WT gonadally intact males in comparison to gonadally intact females regardless of the estrous cycle, and to gonadectomised WT males and SF-1 KO males, again suggesting the direct role of testosterone in the regulation of sex specific expression of *Hsd3b5*. Interestingly, in female mice, similar to *Cyp2u1*, expression in WT gonadectomised and SF-1 KO females was significantly higher in comparison to gonadally intact females, again suggesting additional role of estrogens in the regulation of the expression of this gene, although direct regulation is unlikely, as there were no differences in the expression during the estrous cycle.

In general, results from this study suggest that sex differences in liver gene expression are consequence of different exposure to sex steroids and there is little, if any, role for the genes present on the sex chromosomes. Although we have studied only four genes, and therefore this does not rule out the possibility that the expression of some other genes might be influenced also by sex chromosomes, this is probably unlikely, firstly, because all the genes studied in the present study, showed somewhat similar patterns of expression with regard to sex and gonadal status, and secondly, because one of the studied genes, *Cux2*, is thought to be the regulator of male specific gene expression in the liver. Furthermore, results of the present study suggest, that sex differences are influenced by exposure to sex steroid hormones not during early developmental periods, similarly to numerous sex differences in the brain, but rather depend on adult activational, or perhaps pubertal organizational effects of sex steroid hormones. Although effects of testosterone on male specific expression of the studied genes might be purely activational, it is unlikely that effects of estrogens are purely activational, as we did not observe difference in the expression of any of the studied genes during the estrous cycle. This suggest that effects of estrogens on the expression of these four genes are not direct and acute, but rather some kind of organizational effects must be involved, either during pubertal period or in adult life.

In conclusion, the results of this study reveal that sex difference in the liver gene expression are dependent on adult (and possibly pubertal) exposure to sex steroid hormones in both sexes. For most of the genes studied, perhaps with the exception in *Cyp4a10* expression in males, it also seems that both testosterone and estrogens are involved in the sex specific regulation, as gonadless mice from both sexes differ from the gonadally intact WT mice of respective sex. Since there were no sex differences in gene expression in either SF-1 KO mice or in prepubertally gonadectomised mice it seems that sex chromosomes, as well as early developmental exposure to sex steroid hormones, do not play a role in the regulation of sex difference in liver gene expression.

Acknowledgements

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RAZLIKE MED SPOLOMA V IZRAŽENOSTI GENOV PRI NAVADNIH MIŠIH IN MIŠIH Z IZBITIM GENOM SF-1

K. Kozinc Klenovsek, T. Spanic, G. Majdic

Povzetek: Razvoj in delovanje jeter sta odvisna od natančne izraženosti genov. Predhodne raziskave so pokazale, da so številni geni v jetrih različno izraženi med spoloma, te razlike pa naj bi bile vsaj delno posledica spolnih razlik v izločanju rastnega hormona. Cilj opisane raziskave je bil proučiti, ali tudi spolni kromosomi vplivajo na spolne razlike v izraženosti genov v jetrih. V raziskavi smo pri navadnih miših in miših z izbitim genom SF-1 (SF-1 KO) preverili izraženost genov *Cyp4a10*, *Cyp2u1*, *Cux2* in *Hsd3b5*, za katere je znano, da se v odraslih jetrih izražajo drugače pri samcih kot pri samicah. Miši SF-1 KO se rodijo brez spolnih žlez in zaradi tega niso nikoli izpostavljene spolnim hormonom, razlike med spoloma v izraženosti genov bi zato pri teh miših kazale na neposreden vpliv spolnih kromosomov. Dodatno smo primerjali med seboj tudi miši, ki smo jim odstranili spolne žleze, ter samice v različnih fazah spolnega cikla. Ugotovili smo višjo izraženost genov *Cux2* in *Cyp4a10* pri navadnih samicah s spolnimi žlezami v primerjavi z navadnimi samci s spolnimi žlezami. Ugotovili smo tudi višjo izraženost genov *Cyp2u1* in *Hsd3b5* pri navadnih samcih s spolnimi žlezami v primerjavi z navadnimi samicami s spolnimi žlezami. Nismo pa ugotovili vpliva odstranitve spolnih žlez ali vpliva izbitja gena SF-1 na spolne razlike, saj le-te niso bile prisotne ne med samci in samicami z odstranjenimi spolnimi žlezami kot tudi ne med samci in samicami z izbitim genom SF-1. Ti rezultati kažejo, da so vse razlike med spoloma v izraženosti genov v jetrih popolnoma odvisne od prisotnosti in vpliva spolnih hormonov, ki jih proizvajajo spolne žleze.

Ključne besede: spolne razlike; jetra; izraženost genov; spolni hormoni, spolni kromosomi

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Original Research Article

ASSESSING GASTRIC ULCERATION IN FATTENING PIGS HOUSED WITHOUT OR WITH STRAW AND ADDITIONAL SPACE – A MACROSCOPIC AND MICROSCOPIC STUDY ON A CONVENTIONAL AUSTRIAN FARM

Ines Krauss[1], Lukas Schwarz[1], Katharina Schodl[4,5], Christian Knecht[1], René Brunthaler[3], Barbara Metzler-Zebeli[2], Christine Leeb[5], Isabel Hennig-Pauka[1,6]*

[1]University Clinic for Swine, [2]Institute of Animal Nutrition and Functional Plant Compounds, Department for Farm Animals and Veterinary Public Health, [3] Institute of Pathology and Forensic Veterinary Medicine, Department of Pathobiology, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, [4]Division of Livestock Sciences, Department of Sustainable Agricultural Systems, [5]Doctoral School of Sustainable Development, University of Natural Resources and Life Sciences, Gregor-Mendel-Straße 33, 1180 Vienna, [6]Field station for Epidemiology, University of Veterinary Medicine Hannover, Büscheler Str. 9, 49456 Bakum, Germany

*Corresponding author, E-mail: isabel.hennig-pauka@vetmeduni.ac.at

Abstract: Gastric ulcerations in finishing pigs can cause growth restriction, sudden death and contamination of the carcass by invading microorganisms. The aim of the study was to compare macroscopic and histological findings of the stomach mucosa in fattening pigs kept at 1m²/pig and provided with long straw (10 groups, 113 pigs) with a control group kept at 0.7m²/pig without straw (11 groups, 120 pigs). At slaughter, the gastric health of pigs was assessed by macroscopic and histological scoring of 233 stomachs ranging from 0 (no alteration of mucosa) to 3 (ulceration). Gastric scores were correlated with organ alterations, carcass lesions and blood parameters. Based onto histological findings after gold standard sensitivity and specificity of macroscopic findings for ulceration (score 3) were 53 % and 98 %, respectively. While the extent of mucosal alterations can be assessed by macroscopic scoring easily at slaughter, histological examination reveals the depth of alterations. Median group prevalences of gastric ulcerations diagnosed by macroscopic examination were 5 % in the control group (range 0–40 %) and 18 % in the straw group (range 0–50 %), with no significant difference between both groups. Macroscopic scores were significantly higher in the straw group. Prevalence of ear-tip lesions was positively correlated with gastric health ($p < 0.05$).
Analysis of particle size distribution in feed revealed, that more than 50 % of the feed consist of particles with less than 0.5 mm in diameter. The fine-ground diet in this herd was therefore identified as an important risk factor for the development of gastric ulceration on this farm. As a conclusion, the known risk factor of a high proportion of small particles in diet was not compensated by possible positive effects of straw and more space, and should be eliminated with high priority.

Key words: ear tip lesions; histology; mucosa alterations; stomach; straw; swine

Introduction

Ulcerations of the *pars oesophagea* (PO) in the stomach of pigs are considered to be a serious health and welfare problem in pig production with varying prevalences on different farms (1,2). Although gastric ulceration (GU) is a severe disease that can even cause sudden death of the affected animal, precursors of GU such as different stages of hyperkeratosis or superficial epithelial damages do not necessarily cause clinical signs (3). Epithelial damage of the PO can occur rapidly but can also heal quickly (4,5). After an initial proliferation of the affected mucosa, hyperkeratotic layers become fissured, resulting

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in small erosions as precursors of ulceration (6). Damage of blood vessels can cause chronic bleeding and mucosal leakage with the consequence of ingesta contamination of inner body surfaces. The entire process of ulcer development can take place within less than 24 hours (5,7).
While the extent of mucosal damage can be quantified using a scoring scheme during macroscopic examination (4,7,8), the depth of the erosions in a particular small area and early pathological alterations can be assessed by histological examination (6). The two techniques provide complementary information on gastric health.

Several predisposing factors for GU have been identified so far, but the pathogenesis has not yet been completely elucidated. Diet structure and composition, as pelleted or finely ground feed, have a major impact on the development of gastric lesions and are considered to be the most important risk factors (9-13). Overcrowding, grouping, transportation, environmental changes and feed withdrawal are assessed as stressful situations predisposing for GU development (14-17). The provision of higher space per animal is expected to prevent GU development, because social stress is reduced (18,19). It was shown that enrichment of a barren environment with straw enabling foraging and rooting behaviour of pigs reduced the development of gastric lesions (18-20). The beneficial effect of straw was related to an increased structure and lower fluidity of the stomach contents, a higher concentration of short chain fatty acids and more saliva production due to increased chewing activity (21,22).

The effect of straw and a higher space allowance on the gastric health of fattening pigs was evaluated in this fattening farm. Blood parameters and clinical data were analyzed for any correlation to gastric scores. The provision of straw in racks and more space per pig was expected to result in fewer stomach alterations. In addition, sensitivity and specificity of macroscopic examination of gastric mucosa were determined in comparison to histological findings.

Materials and methods

Animals, groups and handling

All animals were raised and handled on a commercial farm adhering to Austrian Animal Welfare Legislation and feeding was in compliance with producer standards with respect to origin of protein feed (regional and GMO-free). Approval from the institutional Animal Care and Use Committee was not required as the work involved no special treatment outside of normal commercial practice.

In total, 590 Large White/German Landrace x Pietrain F2-cross-bred pigs, raised on a commercial pig fattening farm in Lower Austria, were included in the analysis of feed consumption and daily gain with feeding valve as the statistical unit. All pigs were randomly assigned to either straw group (SG, 248 pigs, 10 feeding valves) or control group (CG, 342 pigs, 11 feeding valves). Pigs were housed on fully slatted floor in groups of 16 pigs per pen (0.7 m² space per pig) in the CG and in groups of 13 pigs per pen (1 m² space per pig) in the SG. On arrival on the farm and immediately before slaughter the total weight of the pigs belonging to one feeding valve was recorded. Prior to slaughter, pigs were individually tattooed to allocate them to group and feeding valve at the abattoir. Pigs were slaughtered at the end of fattening within 3 weeks. At slaughter in total 233 pigs (114 female and 119 castrated males) were selected randomly from all feeding valve groups for evaluation of gastric health, inspection of skin, joint, lung and liver alterations and for blood sampling (CG: 120 pigs; SG: 113 pigs). The difference in gender distribution in both groups was not significant ($p=0.64$, chi-square test).

In blood samples the number of leucocytes as a marker for inflammation, as well as haematocrit and haemoglobin for anaemia diagnostic were determined. The mean corpuscular haemoglobin concentration (MCHC) was calculated by dividing haemoglobin by haematocrit. Blood samples were analysed with an automatic cell counter following the manufacturer´s instructions (IDEXX ProCyte Dx™, Idexx Laboratories, Ludwigsburg, Germany). To confirm results of differential cell counts, blood smears from all pigs were stained using HAEMA-LT-SYS® Quick-Stain (Diff-Quick) (Henry Schein, Germany) and 200 cells were differentiated at 1000-fold magnification by eye using immersion oil according to routine methods.

Feeding

Feeding technique and diet composition was the same for all pigs. Liquid feeding with 4:1 water to feed ratio (approximately 25 % dry matter

content) was provided automatically by a sensor-controlled liquid feeding system three times a day, with multiple intervals of approximately two minutes at every feeding until satiation was achieved. The pig-to-feeding-place ratio was 1:1. In the SG individual pigs had more feeding space at the trough. The composition and chemical analysis of the conventional diet for finishing pigs with 14.7 MJ ME/kg dry matter is shown in table 1. Fresh water was provided by one drinker per pen. Pigs in the SG had ad libitum access to long wheat straw, which was offered in racks above their feeding troughs. Fresh straw was provided by the farmer on a daily basis. Pigs consumed 110–150 g straw/day/pig.

Macroscopic examination

At the abattoir, stomachs were labelled individually and examined approximately 4 hours after exsanguination by opening the gastric wall at the large curvature. The stomach was emptied and the mucosal surface was cleaned with tap water. Mucosal alterations around the stomach's PO were quantified by the same person without knowledge of the group using the slightly modified macroscopic score (Table 2) of Straw et al. (4) and Große Liesner et al. (8). A macroscopic score of 3 corresponds to the clinical signs of GU and was assessed as relevant disease with a probable impact on production parameters. Figures 1a-f illustrate findings corresponding to the macroscopic and histological scores.

During slaughter also carcass inspections were made and ear and tail lesions were recorded (no lesion=0; missing tissue=1). These lesions were diagnosed to be caused by biting, but any previous primary skin alterations, e.g. necroses or scarifications, could not be excluded. Joints, skin, liver and lungs of pigs were inspected macroscopically at the abattoir for alterations to assess the overall herd health status of the pigs.

Histological examination of gastric mucosa

Immediately after macroscopic scoring of the gastric mucosa, tissue pieces containing parts of the PO were sampled and fixed in 4 % buffered formaldehyde solution. Samples were stored at room temperature for 48 h, alcohol dehydrated and embedded in paraffin wax. Paraffin sections (5 µm) were cut, stained with haematoxylin-eosin (HE) and inspected by light microscopy (Olympus CX21, Olympus Corporation, Japan) according to routine methods. Histological tissue alterations were quantified using a modified histological score (Table 2) according to Embaye et al. (6) and Eisemann and Argenzio (16).

Table 1: Composition and analysis of the extruded formulated diet

Ingredients	**% of diet formulation**
barley	13,5
corn silage	50,8
rapseed meal	12,7
Mineral and vitamin mix	6,2
H_2O	16,8
Chemical analysis	**(g/kg dry matter content)**
dry matter	1000
crude protein	145
crude fat	38
crude fibre	51
nitrogen-free extract	717
ash	48
starch	565

Table 2: Results of modified macroscopic (4,8) and histological (6,16) scores of the pars oesophagea (PO) of gastric mucosa in all examined pigs. Macroscopic and histological scores of 0 and 3 are corresponding, while the other identical macroscopic and histological scores did not refer to the same pathological alterations

Macroscopic scores		**Histological scores**						
		0	0.5	1	1.5	2	3	**Stomachs inspected**
		mucosa unaltered	slight hyperkeratosis -proliferation of stratum corneum, < 5 cell layers	moderate hyperkeratosis, proliferation of stratum corneum, 5-10 cell layers	severe hyperkeratosis, proliferation of stratum corneum, > 10 cell layers	lesions extended in maximum to stratum spinosum	ulceration -extended into stratum basale	
0	smooth and white, surface of PO unaltered	5	13	14	13	4	4	53
0.5	slight hyperkeratosis (< 25 %)	1	8	9	19	5	3	45
1	moderate hyperkeratosis (< 50 %)	0	5	15	11	4	6	41
1.5	severe hyperkeratosis (> 50 %)	0	0	3	12	4	4	23
2	severe hyperkeratosis (> 75 %) and lesions	0	1	3	10	5	14	33
3	ulceration	0	0	0	1	2	35	38
Stomachs inspected		6	27	44	66	24	66	233

Table 3: Comparison of mean health and blood parameters. Reference ranges for blood parameters in fattening pigs were shown in brackets (23)

	Straw group (n=113)	**Control group** (n=120)	
	Mean±standard deviation (range)	Mean±standard deviation (range)	p
Macroscopic gastric health score (score 0 – 3)	1.4+ 1.1 (0-3)	1.0+0.9 (0-3)	0.02
Histological gastric health score (score 0 – 3)	1.9±1.0 (0-3)	1.6+0.8 (0-3)	0.23
Leukocytes (G/l) (reference range: 12.0-24.6 G/l)	19.51±4.37 (8.75-35.84)	19.83±3.85 (11.84-32.09)	0.86
Haematocrit (l/l) (reference range: 0.3-0.4 l/l)	0.49±0.03 (0.41-0.59)	0.49±0.04 (0.32-0.59)	0.69
Haemoglobin (g/l) (reference range: 100-147 g/l)	150±82 (115-179)	148+11 (97-168)	0.69
Mean corpuscular haemoglobin concentration (MCHC), (g/l) (reference range 317-370 g/l)	302+93 (278-321)	299+8 (275-318)	<0.01

Blinded histological scoring was performed separately by a professional pathologist and by a clinician and findings were reassessed in the case of diverging scores. Histological scores 0 and 3 correspond to the macroscopic scores. Other identical macroscopic and histological scores did not refer to the same pathological alterations and could therefore not be compared.

Statistical analysis

Statistical analysis was performed using the statistical software package SPSS® version 20 (IBM Corp., Armonk, New York) as well as SAS (Version 9.4, SAS Inst. Inc., Cary, NC, USA).

Parameters were first analyzed for normality using Shapiro-Wilk test with the PROC UNIVARIATE method in SAS (Version 9.4, SAS Inst. Inc., Cary, NC, USA). Proportion of pigs with respective scores belonging to one feeding valve were used for statistical comparison of CG and SG, with n=11 for control pigs (CG) and n=10 for pigs provided with straw and more space (SG). Spearman´s rank correlation coefficients calculated between different quantitative parameters (macroscopic and histological gastric scores, leucocytes, haemoglobin, haematocrit, mean corpuscular haemoglobin concentration (MCHC), proportions of pigs with tail and ear-tip alterations) were tested for significance ($p < 0.05$).

The Cook's distance (Cook's D) test was used to determine any influential observation on the model. Data were analyzed by ANOVA using the MIXED procedure in SAS. The final model included the fixed effects group, sex and their two-way interaction and the random effect 'feeder'. The experimental unit was pig nested within number per pigs per feeder. As the pig's body weight at the start of the experiment was different in the two pig groups, pig's starting body weight was used as co-variate in the model for the weight at slaughter. As the growing days differed among pigs, they were significant for most parameters and were included as co-variate in the final model. Degrees of freedom were approximated by the method of Kenward-Roger. The Tukey-Kramer test was used for pairwise comparisons between least squares means.

Results

At the age of 12 weeks the pigs weighed 31.4 ± 1,5 kg and were slaughtered with 127± 7,9 kg body weight. The mean daily weight gain in feeding valve groups was 850g ± 140g and did not differ between pigs provided with straw and more space and control pigs. No acute gastric ulcerations were observed and all mucosal alterations were assessed as chronic as ulcers were surrounded by hyperkeratotic regions. Long unchewed straw stems were a frequent finding in the stomach content of the SG pigs. Macroscopic and microscopic scores are summarized in table 2. The sensitivity of macroscopic detection of GU (score 3) was 53 % allocated to histological findings, while the specificity was 98 %.

The median prevalence of pigs in one feeding valve group with score 3 was 18 % (range 0–50 %) in the SG compared to 5 % (0–40 %) in the CG ($p=0.61$). The median prevalence of pigs with histological score 3 was 34 % (range 0–64 %) in the SG, compared to 11 % (range 0–62 %) in the CG ($p=0.25$). Statistical mixed model calculations resulted in higher macroscopic scores in pigs provided with straw compared to control pigs ($p=0.02$), whereby male pigs had a higher macroscopic score than female pigs ($p=0.02$). Histological scores did not differ between the two groups, but were generally greater in males compared to female pigs ($p=0.01$). Haematocrit and haemoglobin were similar between pig groups and sexes. Pigs provided with straw had an increased MCHC (standard calculation: Haemoglobin/ Haematocrit), compared to control pigs. The MCHC was similar between both sexes. Ranges of red blood parameters measured within the groups were beyond the published reference ranges (Table 3). There were no correlations between haematocrit, haemoglobin concentration or MCHC with the gastric health scores. The statistical comparison of pigs with macroscopically or histologically defined GU (score 3) with pigs with lower gastric scores resulted in no differences in the red blood parameters. The mean carcass weight after the slaughtering process was slightly higher ($p=0.03$) in the straw group (105.3 kg) compared to the control group (101.6 kg). Pigs provided with straw and more space had a higher mean total feed consumption (268 kg) compared to control pigs during the fattening period (259 kg, $p < 0.01$).

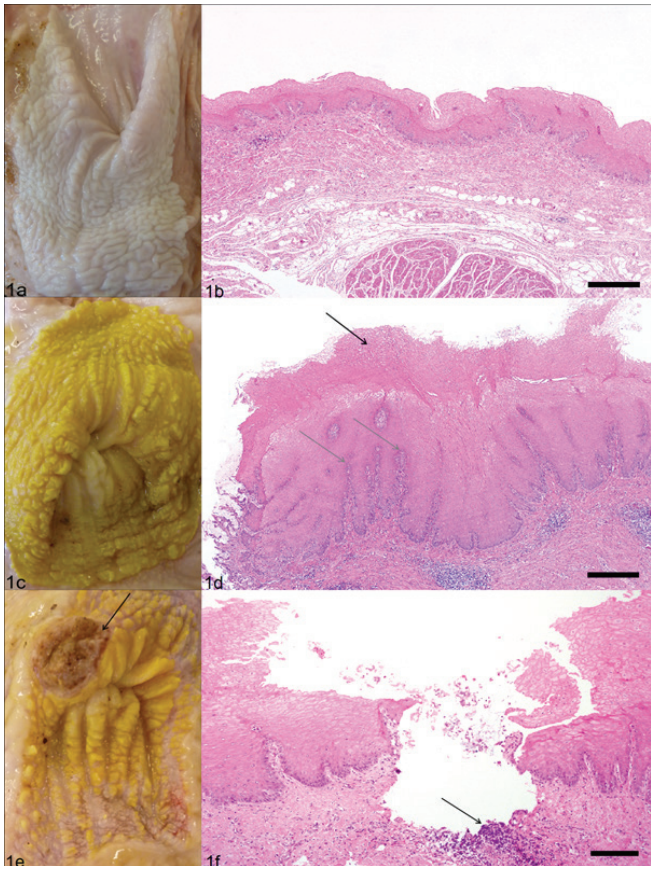

Figure 1a-f: Pictures of stomachs *pars oesophagea* (PO) scored using the modified macroscopic score (MS 0-3) according to Straw et al. (4) and Grosse Liesner et al. (8) and corresponding histological slides scored using the modified histological score (HS 0-3) according to Embaye et al. (6) and Eisemann and Argenzio (16)

Fig. 1a. Unaltered PO with smooth and white surface, MS 0. Fig. 1b. Unaltered PO, HS 0. Haematoxylin and eosin (HE), scale bar length 400µm. Fig. 1c. Severe Hyperkeratosis > 50 % of PO, MS 1.5. Fig. 1d. Severe Hyperkeratosis > 50 %, cells with pallor cytoplasma (black arrow) and extended papillae of lamina propria (grey arrow), HS 1.5, HE, scale bar length 400µm. Fig. 1e. Hyperkeratosis of PO, ulcer (black arrow), MS 3. Fig. 1f. Hyperkeratosis of PO, ulceration extending throughout the basal membrane (black arrow), HS 3, HE, scale bar length 150µm.

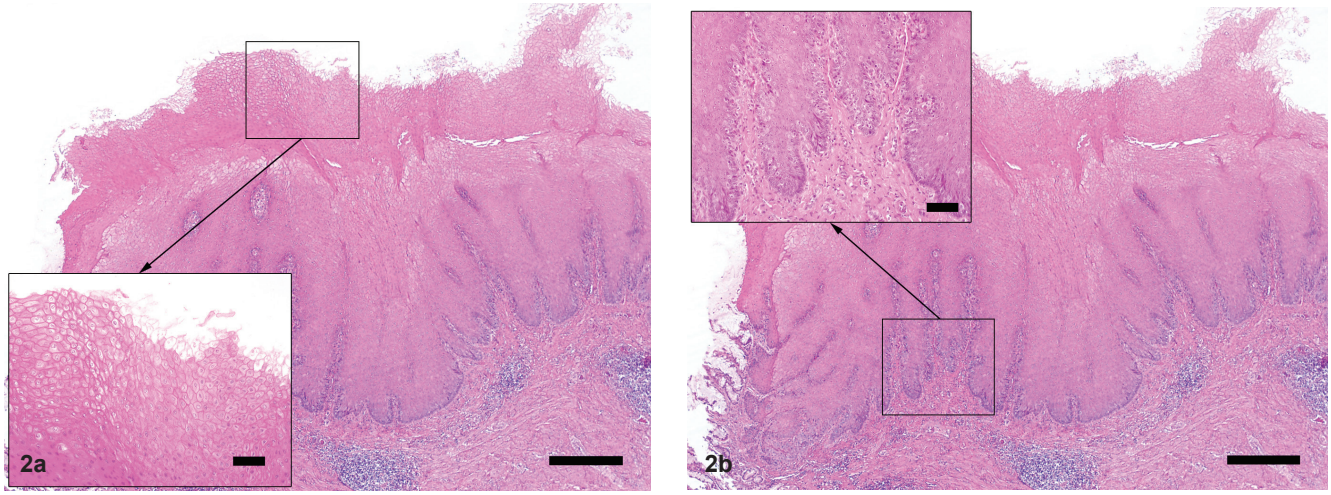

Figure 2a-b: Higher magnification of Fig. 1d to illustrate cells with pallor cytoplasma (Fig. 2a.) and enlarged papillae (Fig. 2b.), scale bar length 400 µm and for higher magnification 80µm

The overall average prevalence of ear-tip lesions was 10 ± 14 % and was positively correlated with the prevalence of GUs (macroscopic GU: r_{sp}=0.55, p= 0.016, histological GU: r_{sp}=0.67, p=0.002) and with the gastric health scores (macroscopic score: r_{sp}=0.51, p=0.02, histological score: r_{sp}= 0.58, p=0.009). No additional correlations between gastric health scores and other parameters were found. Lung health status in the herd was assessed to be acceptable, because no clinical signs of disease were observed during the study period and only slight lung alterations were found

at slaughter in 22 % of the pigs. In 5 % of the animals few milk spots were detected in the liver.

Discussion

The prevalence of GU on this farm is comparable to those reported in other European countries (1,21). Compared to histological findings, macroscopic examination at the abattoir resulted in a high specificity of 98 % but a low sensitivity for the detection of GU (score 3). This is in accordance with the study of Embaye et al. (6), who reported a moderate to poor correlation between gross pathology and histological findings. A comparison between gastric lesion scores determined by gastroscopy using a flexible videoscope, by necropsy and by histopathology resulted in a poor (gastroscopy versus necropsy) or moderate (necropsy versus histopathology) agreement between the methods (24). Both scoring methods provide different information on the mucosal alterations: while the macroscopic score allows the assessment of the spatial extension of lesions, the histological score reveals the depth of tissue layers affected. Pigs with deep but small ulcerations can appear to be healthy if the blood loss is minimal (25), but negative effects on growth performance can be expected (7). While GU is of high clinical relevance, slight histological alterations characterized by a proliferation of the *Stratum corneum* are often found in healthy pigs (4).

In either groups mucosal alterations of varying severity were found and prevalence varied between feeding valve groups. Inflammatory or anaemic conditions were assessed to be of minor impact in this herd for the overall health status. This was deduced from the mainly physiological findings for white and red blood cells in majority of the pigs in both groups. In addition, examination of carcasses at the slaughterhouse revealed slight pneumonic lesions in 22 % of slaughter pigs, which is in accordance with other reports of organ findings at slaughter in Austria (26). From the slightly higher MCHC in SG pigs, which is a calculated parameter useful to diagnose anaemia due to iron deficiency in pigs, no conclusion can be drawn, because haemoglobin and haematocrit did not differ between groups.

Although all groups were fed the same diet and all pens were equal, other unknown predisposing factors within groups, e.g. social stress, cannot be excluded (22). Gender was included as a fixed effect in the statistical model and had a significant influence on the severity of gastric alterations, but not on weight or blood parameters. Pigs provided with straw and more space had slightly higher feed consumption during fattening and therefor also a higher uptake of fine-ground diet. This increased exposition to a major risk factor might have been decisive for the outcome of this study, because one of the most important predisposing factors for GU is feed with a high percentage of small particles (9,12,13). A high proportion (> 36 %) of very fine particles (< 0.4mm) was associated with a high risk of GU development (12) and therefore an appropriate parameter for risk assessment. Grosse Liesner et al. (8) described a higher risk for GU if feed contains more than 20 % of particles smaller than 0.4 mm. In this study the fraction of particles < 0.5 mm was higher than 50 % in the fattening diet, which is comparable to ulcerogenic diets in other trials (13). Particle size distribution on this farm can be considered as an important predisposing factor for GU, so that even enhanced conditions such as the provision of straw and the allocation of increased space were not able not improve gastric health in this study. In the study of Eisemann and Argenzio (16) only a beneficial effect of adequate diet structure but not of space per pig was stated. In contrast, floor type and bedding material especially in the lying area were found to have high impact on gastric health (19). Nielsen and Ingvartsen (27) reported a preventive effect of straw when a finely ground diet was fed. In some stomach contents they also found long straw stems, suggesting that pigs had swallowed straw without chewing it. In the study of Herskin et al. (28) only higher amounts of straw (500g/pig/day) were able to reduce GU prevalences, while gastric scores did not differ between pigs provided with different amounts of straw (10-1000g/pig/day). Although feasable, the amount of up to 150 g straw per pig and day provided in racks and not on the floor as described in this study, might not be adequate to reduce GU prevalence. In a recent study of Jensen et al. (22) up to 300 g straw per pig and day provided on the floor decreased the risk of GU development. Also in that study GU development was not eliminated completely by straw provision, which was supposed to be due to a high proportion of pelleted and finely-ground diet as a pre-disposing risk factor.

There are some reports from other species, that unchewed straw stems mechanically irritate an

already pre-damaged mucosa of the PO. In horses, Luthersson et al. (29) found significantly more GU when horses were fed with straw, which was deduced to the fact, that the straw was had not been chewed thoroughly and led to mechanical irritation of the gastric mucosa (29). In veal calves fed with wheat straw more erosions and ulcers in the abomasum were found (30). The authors hypothesized a partial blockage of the pyloric exit by straw and a mucosal damage by a mechanical abrasive effect of straw.

The correlation between the prevalence of ear-tip lesions, which were diagnosed to be the consequence of ear biting, and GU on this farm revealed that gastric health and other health parameters might influence one another. Whether pigs with GU had previously suffered from stress (e.g. social stress) or whether primary skin lesions at the ears as necroses or scarifications had been the original trigger factors for ear-biting, could not be assessed. Ear lesions with their relatively low prevalence and mild form on this farm had not been realized as a herd health problem by the farmer at the time of the study or in the past. However, stress could be one of the reasons for the development of GUs and vice versa (31). It is hypothetical, if GUs can increase the risk of ear biting. According to empirical reports, it is assumed that increased chewing leads to more production of saliva with its buffering components and can be triggered by gastrointestinal disorders (32,33). In a previous study a relation between abnormal oral behavior, as increased chewing, and gastric ulceration was found (34).

For pigs, various dietary fibre sources such as straw, hay or sunflower hulls are used as feed and as suitable manipulable material for rooting and chewing. Dietary fibres contain NSP (non-starch polysaccharides) as pectins, cellulose, hemicelluloses, ß-glucans and fructans (35). Dietary fibres have generally low energetic value, could stimulate satiation and reduce feed intake (36). Different fibre sources vary in their water-holding capacity, which is generally high. In the case of stem-rich material, as hay or straw, the material can be bulky due to their coarse structure (37). All fibre sources differ in their digestibility and fermentability based on content of NDF (neutral detergent fibre) and ADF (acid detergent fibre). The digestibility of dietary fibre increases with age of swine. Hay is more digestible than straw because of its less NDF content (38) and may lead to a better feeling of satiety. For the case reported here, other options for provision of manipulable material as well as alternative sources can be taken into account as a supplementation to diet.

Conclusion

Gastric health examination during routine carcass monitoring at the abattoir can support decision-making for analysis of GU predisposing factors on farm and for rapid preventive interventions. In this study no difference in the GU prevalence was found between pigs provided with straw and more space and those kept under conventional conditions. As a main predisposing factor, a high fraction of small feed particles in diet was identified. Gastric scores were higher in pigs provided with straw and more space. A positive correlation between ear tip lesions and gastric scores indicate that either both diseases influence each other or were triggered by the same factors on this farm.

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OCENJEVANJE GASTRIČNIH ULKUSOV PRI PRAŠIČIH PITANCIH, NASTANJENIH Z NASTILJEM ALI BREZ NASTILJA IN DODATNEGA PROSTORA – MAKROSKOPSKA IN MIKROSKOPSKA ŠTUDIJA NA KONVENCIONALNIH AVSTRIJSKIH KMETIJAH

I. Krauss, L. Schwarz, K. Schodl, C. Knecht, R. Brunthaler, B. Metzler-Zebeli, C. Leeb, I. Hennig-Pauka

Povzetek: Gastrične ulceracije pri prašičih pitancih v zaključni fazi lahko povzročijo omejevanje rasti, nenadno smrt in kontaminacijo trupa z invazivnimi mikroorganizmi. Cilj raziskave je bil primerjava makroskopskih in histoloških ugotovitev v sluznici želodca pri pitovnih prašičih, ki so nastanjeni na 1 m²/prašiča in imajo nastilj sestavljen iz dolge slame (10 skupin, 113 prašičev) v primerjavi s kontrolno skupino, ki je bila nastanjena na 0,7 m²/prašiča in z nastiljem brez slame (11 skupin, 120 prašičev). Pri zakolu je bilo želodčno zdravje prašičev ocenjeno z makroskopskim in histološkim točkovanjem, opravljenem na 233 želodcih, z ocenami, ki se gibljejo od 0 (brez spremembe sluznice) do 3 (ulceracija). Rezultate opazovanj sluznice želodcev smo nato povezali s spremembami organa, poškodbami trupa in krvnimi parametri.

Medtem ko se obseg sprememb sluznice lahko oceni z makroskopskim merjenjem pri zakolu, histološki pregled razkrije globino sprememb. Mediana prevalenca želodčnih ulkusov, diagnosticiranih z makroskopskim pregledom, je bila 5 % v kontrolni skupini (razpon od 0 do 40 %) in 18 % v skupini z nastiljem iz slame (razpon od 0 do 50 %), pri čemer ni bilo opaziti značilne razlike med skupinama. Makroskopski rezultati so bili precej višji v skupini z nastiljem iz slame. Razširjenost poškodb ušes je bila pozitivno povezana z zdravjem želodca ($p < 0,05$). Analiza porazdelitve velikosti delcev v krmi je pokazala, da več kot 50 % krme sestavljajo delci s premerom manj kot 0,5 mm. Prehrana z drobno zmleto krmo je bila v čredi opredeljena kot pomemben dejavnik tveganja za razvoj razjed želodca.

Znani faktor tveganja z velikim deležem majhnih delcev v prehrani ni bil kompenziran z morebitnimi pozitivnimi učinki slame in več prostora, zato ga je treba iz reje čim prej odpraviti.

Ključne besede: poškodbe na vrhu ušesa; histologija; spremembe želodčne sluznice; želodec; slama; prašiči

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Original Research Article

RESIDUAL LEVELS OF ORGANOCHLORINE PESTICIDES AND HEAVY METALS IN SHELLFISH FROM EGYPT WITH ASSESSMENT OF HEALTH RISKS

Taghred M. Saber[1]*, Mariam H. E. Khedr[2], Wageh Sobhy Darwish[3]

[1]Forensic Medicine and Toxicology Department, [2]Department of Veterinary Public Health, [3]Food Control Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44519, Egypt

*Corresponding author, Email: taghred_saber@yahoo.com

Abstract: This study investigated the levels of organochlorine pesticides (OCPs) and heavy metal residues in shellfish (shrimp, oyster and crab) collected from three Egyptian governorates (Ismailia, Damietta and Alexandria). Levels of 12 OCPs such as hexachlorocyclohexanes (HCHs), aldrin, endrin and dichlorodiphenyltrichloroethanes (DDTs) residues were determined. The dominant detected OCPs were ß-HCH, p,p-DDE and endrin. The contamination pattern of OCPs was in the order of other OCPs (HCB, heptachlor, heptachlor-epoxide, aldrin, endrin and γ chlordane) > HCHs > DDTs. Residual levels of some heavy metals and trace elements were also estimated. The highest residual levels of OCPs and heavy metals were found in oysters collected from Damietta. The health risk assessment was determined by calculating hazard ratio and hazard index. Concentrations of OCPs and heavy metals in examined shellfish were below the maximum residual level set by United States Food and Drug Administration and FAO. Therefore, shellfish collected from these studied sites could be considered safe for human consumption.

Key words: organochlorine pesticides; heavy metals; shellfish; health risks; Egypt

Introduction

Organochlorine pesticides (OCPs) are considered as persistent pollutants all over the world due to their persistence in the environment; bioaccumulation; their magnification ability in food chain and induction of toxicity for human and wildlife (1). These pollutants enter the aquatic environment and could be transported into food chains, then accumulated in the aquatic organisms. Lastly, OCPs might reach human via consumption of fish and fish products, drinking water and agriculture foods (2). In Egypt since 1980s, several OCPs and their metabolites were found with different levels in fish where the OCPs use has been banned (3, 4).

Heavy metals, as major environmental contaminants, has detrimental effects on the aquatic organisms and human (5).Consequently, these metals reach the aquatic ecosystem through the natural and anthropogenic sources and causing serious threats due to their toxicity, bioaccumulations in food chain and non-biodegradable nature in the aquatic environments (6). The levels of heavy metals were elevated in natural water because of increasing the industrial and agricultural activities (7). Metals such as copper, chromium, zinc and nickel and iron are

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essential trace elements playing a vital role in the biological systems, whereas non-essential metals including lead, cadmium, arsenic and mercury are bioaccumlated in tissues leading to intoxication, damage of cells and tissues, reduced fertility, cell death and organ dysfunctions (8, 9).

Fish and shellfish are delicious food that support humans with high quality protein, various minerals, polyunsaturated fatty acids and vitamins. In addition, fish and shellfish are considered as one of the valuable bioindicators for pollution in the aquatic habitats as a result of their lower detoxification enzymes (e.g. mono-oxygenases) than those in mammals and thereby allowing a higher bioaccumulation for toxicants (10, 11). Besides, these metals will provide more reliable information on the impact on public health arising from seafood consumption (12). Information available about OCPs and heavy metal residues in shellfish in Egypt is very limited. This study aimed to investigate the levels of OCPs and heavy metal residues in three shellfish species (shrimp, oyster and crab) collected from Ismailia, Damietta and Alexandria Governorates, Egypt, and to assess the potential risks on the public health arising from shellfish consumption.

Materials and methods

Study area and sample collection

Ismailia is a province situated near the Delta with low industrial and agricultural activities, while Damietta is close to Nile Delta, where agricultural activities and industries are predominant. Alexandria is located in the North West of Delta extending for about 75 km along the Egyptian Mediterranean coast with medium industrial and agricultural activities (Fig. 1). Sixty-three samples of three shellfish species (shrimp, crab and oyster) were collected from fish markets at Ismailia, Damietta and Alexandria Governorates at Egypt during March to June 2015. The collection scheme was twenty-one samples per each governorate (as divided seven shellfish samples per each species).

The collected shellfish species; including white shrimp (*Penaeus setiferus*), blue crab (*Callinectes sapidus*) and oyster (*Crassostrea gigas*); were stored at -20°C until analysis. The residues of OCPs and heavy metals were analyzed in the edible portions of shellfish samples.

Figure 1: Location of sampling sites; Ismailia, Damietta and Alexandria

Analysis of OCPs residues

The processing and analysis of shellfish samples were carried out according to the previously described method by Yohannes et al. (13). In brief, approximately 10 gm of each edible shellfish sample was homogenized with anhydrous sodium sulfate, and then extracted with 150 mL hexane: acetone (3:1, v/v) for 6 h in a Soxhlet S306AK Automatic Extractor System (Gerhardt, Germany). Firstly, the extract was concentrated with a rotary evaporator to about 2 mL, and then secondly was diluted with hexane to 10 mL. An aliquot from the extract was cleaned-up after the evaporation of solvent on the glass column that is packed with 6 gm of activated florisil, then eluted with 80 mL hexane containing a diethyl ether of 25%. The rotary evaporator concentrated the elute, then was dried using gentle nitrogen flow. The extract was redissolved in n-decane (100 µL), then transferred to gas chromatography (GC) vials for the analysis process.

The analysis of twelve OCPs including hexachlorocyclohexanes (HCHs; α-, β- and γ-HCH), hexachlorobenzene (HCB), heptachlor, heptachlor-epoxide, aldrin, endrin, γ chlordane, and dichlorodiphenyltrichloroethanes (DDTs; p,p-DDE, p, p-DDD and p, p-DDT) was achieved with a gas-chromatography equipped with a detector

of Ni electron capture (GCECD: Shimadzu GC-2014,Kyoto, Japan). An ENV8MS capillary column with the splitless injection was utilized to separate OCPs. One µl of each sample was injected. The temperature of GC oven was initially set at 100 °C for 1 min, then raised up to 180 °C at 20 °C/min, and then to 260 °C at 4 °C/min, finally was held for 5 min. The temperature of injector was 250 °C, while that of detector was 310°C. Helium was used as a carrier gas at a flow rate (1.0 mL/min), while nitrogen (as a make –up gas) was at a flow rate of 45 ml/min.

Heavy metal analysis

Fish samples were digested according to the method of Finerty et al. (14). In brief, one gm of each sample was mixed with 10 mL 3:2 nitric acid (65% v/v): Perchloric acid (70% v/v). The mixture was allowed to digest overnight at room temperature, then it was heated for three h in a water bath at 70 °C with whirling at intervals of 30 min for the accuracy of complete digestion. After cooling, the digested shellfish samples were diluted with 20 mL de-ionized water then filtered through *a Whatman* filter paper (*No. 42).* Similar procedure was applied for the blank. For determination of Pb, Cd, As, Cu, Cr, Zn, Ni and Fe residue levels in shellfish samples, the analysis of filtrate was performed using Buck V210GP atomic absorption spectrophotometer (Buck Scientific Instrument Manufacturing Co., Norwalk, CT, USA) using lambs of hollow cathode, equipped with air-acetylene flame. While, Hg was measured a cold vapor atomic absorption spectrophotometer (Varian VGA-77; Agilent Technologies, Santa Clara, CA, USA).

Quality assurance and quality control

The OCPs were identified by comparing their retention time with reference to the corresponding standard. The quality control was conducted by analysis of procedural blanks and spiked blanks for each 7 samples. The detection limits based on 3:1 signal to noise ratio (S/N) were between 0.05 and 0.1 ng/g for all analyzed OCPs. The recovery rate of OCPs was ranged from 80-102 % and the results have not been corrected for recoveries.

For testing the accuracy and validity of analytical procedures of heavy metals, the reference material; DORM-3 (Fish protein, the National Research Council, Canada) was used. Replicate analysis of this reference material demonstrated good accuracy with recovery rates ranged from 80% to 115%. The detection limits for heavy metals were 0.1 µg/g for lead (Pb), 0.005 µg/g for cadmium (Cd), 0.02 µg/g for arsenic (As), 0.2 µg/g for mercury (Hg), 0.02µg/g for copper (Cu), 0.05 µg/g for chromium (Cr), 0,005 µg/g for zinc (Zn), 0.01µg/g for nickel (Ni) and 0.005 µg/g for iron (Fe).

Estimated daily intake (EDI)

The EDI was calculated on the basis of incorporation of data from heavy metals analysis, rates of fish consumption, and body weight of Egyptian adults. EDI (µg/kg/day) for heavy metals was calculated by using the following equation which is explained by the Human Health Evaluation Manual (US Environmental Protection Agency, EPA) (15):

$$EDI = \frac{C_m * F_{IR}}{BW}$$

Where C_m is the metal concentration in the sample (mg/kg wet weight); F_{IR} is the food (fish) ingestion rate in Egypt, which was determined at 48.57 g/day (16); BW is the body weight of Egyptian adults, which was determined at 70 kg.

Health risk assessment

The US EPA (15) quantitatively evaluates the health risks for humans in terms of non-cancer and cancer risks. This study was designed to quantify the non-cancer risks imposed on the three locations under the study at Egypt, by consumption of metal contained fish. The assessment of risks followed the guidelines adopted by the US EPA (2007)(15). For the non-cancer risks, EDI was compared with the recommended reference doses (RfD) (4E03, 1E03, 3E04, 5E04, 0.3, 3E03, 0.3, 2E02 mg/kg/d for Pb, Cd, As, Hg, Cu, Cr, Zn, Ni; respectively) (15), as was illustrated in the following equation:

$$\text{Hazard Ratio (HR)} = \frac{EDI}{RfD}$$

The hazard ratios (HRs) could be added together to estimate a hazard index (HI) to evaluate the risk of mixed contaminants. HI was measured using the following equation:

$$HI = \sum HRi$$

Where *i* represent each metal. HR and/or HI of >1 demonstrates that there is a potential risk for human health, while a result of ≤ 1 shows no risk for detrimental health effects.

Statistical analysis

The obtained data were expressed as the mean ± standard error (SE). The statistical analysis was performed using two-way analysis of variance (ANOVA) to evaluate the statistical differences in the concentrations of heavy metals between different shellfish species and localities followed by the post-hoc Duncan's test. This was carried out using IBM SPSS Statistics computer software (version 21). All the statistical analyses were done at the significance level of 0.05 ($P<0.05$).

Results and discussion

Concentration of OCPs

In this study, the residual levels of OCPs in shellfish on wet weight basis (ng/g ww) were dominated by other OCPs (HCB, heptachlor, heptachlor-epoxide, aldrin, endrin and γ chlordane) followed by HCHs (α-, β- and γ-HCH) then DDTs (p,p-DDE, p, p-DDD and p, p-DDT). OCPs concentrations were in the range of ND-63.53 ng/g ww (Table 1). The maximum concentration of OCPs was found in oysters collected from Damietta. This highest OCPs residual level in oysters could be due to their feeding habits, where these molluscan shellfish are filter feeders, and thus, they could concentrate these pollutants at higher levels than others found in the water environment (17). In addition, the highest residual levels of OCPs in shellfish from Damietta may be related to the increase in the agricultural and industrial activities.

HCHs

Shellfish revealed total HCHs within the range of ND-12.27 ng/g ww (Table 1). This concentration level was nearly similar to that reported in shellfish from Qiantang River, China (18). However, higher HCHs levels (16.20- 183.40 ng/g ww) were detected in mussels from the Red Sea (19).The highest concentration of total HCHs was recovered in oysters at Damietta. The β-HCH was the predominant isomer in HCHs for all shellfish followed by γ-HCH then α-HCH. Similar finding was cited in shellfish from Qiantang River, China (18). This result might be attributed to the stability, environmental existence, resistance for microbial degradation, long half-life with vapor pressure and low solubility of β-HCH (20). However, some studies reported that α-HCH was the predominant HCHs isomer in fish (10, 21).

DDTs

Total DDTs concentrations in examined shellfish had a range from ND to 8.64 ng/g ww (Table 1). Although, higher concentrations of total DDTs were recorded in shellfish from Qiantang River, China (18) and in mussels from the Red Sea (19). The highest level for total DDTs was also detected in oysters from Damietta. Moreover, these results may be accounted for the high chemical stability, hydrophobicity of p,p- DDE, its long half-life and persistence in the biotic and abiotic components of aquatic ecosystem (22).The high levels of DDE and low DDT concentrations in shellfish could indicate that the DDT had not been recently used in the agricultural activities after its ban (23).

Other OCPs

Total other OCPs concentrations in shellfish ranged from ND to 42.62 ng/g ww. Endrin was predominant among other OCPs with the range of ND–29.00 ng/g ww. Oysters collected from Damietta showed the highest residual levels of total other OCPs (Table 1). The residual levels of OCPs detected in fish samples were below the maximum residual limit (MRL) set by United States Food and Drug Administration (US FDA) (24) and Food and Agriculture Organization (FAO)(25) (Table 1).

Thus, their human health risk assessment was not assessed in this study. Concerning the Commission Regulation of European community (EC) No 396(26) and amendments (27, 28, 29), we found that MRLs of pesticide residues in fish not applicable until the individual products are identified and listed. OCPs residues in studied shellfish may be attributed to unauthorized use of pesticides in the agriculture or as a result of marine water contamination

Heavy metal concentrations

The concentration of heavy metals was expressed as µg/g ww in shellfish samples (Tables 2 and 3). There was no a significant difference in the toxic metal levels between different species from the same locality. On the other hand, the mean concentrations of trace elements were significantly ($P<0.05$) different among different species. Moreover, edible portions of oysters harboured the highest residual levels of Cu and Ni. This was supported by Rainbow (17), who declared that the highest trace elements levels in oysters might be attributed to their feeding habits. Furthermore, there was asignificant difference in heavy metal concentrations among different localities. Our data depicted that the highest heavy metal concentrations were noticed in Damietta due to an increase in industrial and agricultural activities.

Lead (Pb) is a toxic heavy metal causing retardation in growth, anemia and neuronal defects in children. In addition, Pb chronic posioning could induce toxicity in different organs such as liver, kidney and brain (30).The present study showed a range for pb concentrations in shellfish (0.84 to 1.63µg/g ww) with a mean value of 1.19 µg/g ww (Table 2). The highest Pb level was observed in oysters from Damietta, while the lowest concentration was found in crabs from Ismailia. Pb levels in this study were within the range (0.67-0.99 µg/g ww) recorded in mussels from Alexandria, Egypt (31). Conversely, it was higher than that reported (ND-0.55 µg/g ww) in fish from Palestine (32). It is surprising, Pb levels in this study exceeded the maximum permissible limit (MPL) (0.5 µg/g ww) recommended by FAO (25) in fish and Commission Regulation (EC) No 1881(33) in crustacean fish. Although, these Pb levels were lower than MPL proposed by US FDA (24) in shellfish and Commission Regulation (EC) No 1881(33) in oysters .

Acute cadmium (Cd) intoxication in humans is manifested by nausea, vomiting, diarrhea, pain in abdomen and shock. While, the Cd chronic toxicity causes dysfunction of renal tubules and appearance of Itai–itai disease (34). The Cd residual concentrations in shellfish ranged from 0.21-0.55 µg/g ww with a mean value of 0.38 µg/g ww (Table 2). The highest concentration was detected in oysters from Damietta; whereas the lowest level was found in crabs from Ismailia. In the present study, Cd concentrations were nearly close to levels (0.16-0.65 µg/g ww) that reported in fish, Giza, Egypt (35). However, lower levels (ND-0.09 µg/g ww) were detected in fish from Palestine (32). Cd levels in the present study were below the MPL adopted by FAO (25) (0.5 µg/g ww) in fish, US FDA (24) and Commission Regulation (EC) No 1881(33) in shellfish.

Arsenic (As) is a toxic element that has carcinogenic effects and non-carcinogenic effects including genotoxicity and immunotoxicity (36, 37). The range of As levels in shellfish was 0.81 to 1.45µg/g ww with an average concentration of 1.13 µg/g ww (Table 2). It was clear that shellfish collected from Damietta showed the highest As residual levels. However, lower mean concentrations of As were recovered in shellfish from Ismailia. On the contrary, the residual As levels in this study were lower than those reported in fish from New Jersey (38). However, it was higher than those detected in seafood from Mumbai, India (39). In addition, As levels in this study were lower than the MPL for shellfish set by US FDA (24).

Mercury (Hg) is a highly toxic element causing different adverse health effects that include neurologigal, immune, renal and developmental disorders (40). The concentrations of Hg in shellfish were varied from 0.53 to 1.16 µg/g ww with a mean value of 0.83 µg/g ww (Table 2). The highest Hg levels were found in shellfish from Damietta, while the lowest concentrations were detected in the collected samples from Ismailia. The observed values of Hg in shellfish were higher than those found in fish from Mumbai Harbor, India (0.01-0.23 µg/g ww) (41).The levels of Hg in the present study were below MPL of 0.5-1 µg/g ww (33, 42) except Hg concentrations in oysters from Damietta and Alexandria.

The levels of Cu in shellfish was ranged between 1.13 and 5.72 µg/g ww with an average value of 3.33µg/g ww (Table 3).The maximum

Table 1: Concentrations (range) of OCPs residues (ng/g ww) in the examined fish species from different localities

Loactions	Shellfish	α-HCH	β-HCH	γ-HCH	HCB	Hepta-chlor	Heptachlor-epoxide	Aldrin	Endrin	γ Chlordane	p,p-DDE	p, p-DDD	p, p-DDT	ΣHCHs	ΣDDTs	Σother OCPs	Σ OCPs
Ismailia	Shrimp	ND - 0.30	ND - 2.32	ND - 2.15	ND - 0.25	ND - 0.33	ND - 4.80	ND - 0.70	ND - 9.44	ND - 0.56	ND - 1.50	ND - 0.22	ND - 0.48	ND - 4.77	ND - 2.20	ND - 16.08	ND - 23.05
	Oyster	ND - 0.39	ND - 4.29	ND - 2.21	ND - 0.35	ND - 0.46	ND - 5	ND - 1.05	ND - 10.18	ND - 0.62	ND - 2.44	ND - 0.34	ND - 0.54	ND - 6.89	ND - 3.32	ND - 17.66	ND - 27.87
	Crab	ND - 0.31	ND - 2.45	ND - 2.10	ND - 0.29	ND - 0.36	ND - 4.20	ND - 0.65	ND - 9.00	ND - 0.20	ND - 1.90	ND - 0.20	ND - 0.40	ND - 4.86	ND - 2.50	ND - 14.70	ND - 22.06
Damietta	Shrimp	ND - 1.12	ND - 4.15	ND - 2.75	ND - 0.36	ND - 4.00	ND - 6.00	ND - 0.85	ND -28.00	ND - 0.78	ND - 2.70	ND - 0.53	ND - 0.60	ND - 8.02	ND - 3.83	ND - 12.27	ND - 24.12
	Oyster	ND - 1.25	ND - 8.22	ND - 2.80	ND - 0.54	ND - 4.20	ND - 6.60	ND - 1.40	ND - 29.00	ND - 0.88	ND - 5.40	ND - 2.44	ND - 0.80	ND - 12.27	ND - 8.64	ND - 42.62	ND - 63.53
	Crab	ND - 1.15	ND - 5.21	ND -2.70	ND - 0.34	ND - 3.96	ND - 6.30	ND - 0.81	ND - 27.32	ND - 0.72	ND -2.30	ND - 0.49	ND - 0.65	ND - 9.06	ND - 3.44	ND - 39.45	ND - 51.95
Alexandria	Shrimp	ND - 0.89	ND -3.76	ND - 2.43	ND - 0.28	ND - 0.71	ND - 5.34	ND - 0.77	ND - 14.87	ND - 0.72	ND - 2.26	ND - 0.46	ND - 0.54	ND - 7.08	ND - 3.17	ND - 22.69	ND - 32.94
	Oyster	ND - 1.07	ND - 6.01	ND - 2.53	ND - 0.46	ND - 0.97	ND - 5.60	ND - 1.23	ND - 15.02	ND - 1.23	ND - 2.70	ND -0.56	ND - 0.75	ND - 9.61	ND - 4.01	ND - 24.51	ND -38.13
	Crab	ND - 0.96	ND - 4.02	ND - 2.38	ND - 0.31	ND - 0.76	ND - 5.30	ND - 0.79	ND - 14.80	ND - 0.78	ND - 2.00	ND - 0.40	ND - 0.61	ND - 7.36	ND - 3.01	ND - 22.74	ND - 33.11
MRL			300[1,2]	300[1,2]		300[1,2]	300 [1]	300[1,2]	300[1,2]	300 [1]					5000[1] 300[2]		

ND; Non detetected (1): US FDA (24) (2):FAO (25)

Table 2: Toxic metal concentrations (µg/g ww) in the examined fish species from different localities

	Sampling sites	Shrimp	Crab	MPL		
		Mean ± SE	Mean ± SE	Regulation(EC) No 1881(33)	US FDA(24)	FAO (25)
Pb	Ismailia	0.85 ± 0.007^{aB}	0.84 ± 0.003^{aB}	Crustacean 0.5	Crustacean 1.5	
	Damietta	1.47 ± 0.002^{aA}	1.49 ± 0.007^{aA}	Oysters 1.5	Oysters 1.7	0.5
	Alexandria	1.23 ± 0.004^{aAB}	1.24 ± 0.002^{aAB}			
Cd	Ismailia	0.25 ± 0.011^{abB}	0.21 ± 0.019^{bB}	Crustacean 0.5	Crustacean 3	
	Damietta	0.44 ± 0.011^{aA}	0.37 ± 0.011^{aA}	Oysters 1	Oysters 4	0.5
	Alexandria	0.42 ± 0.008^{aA}	0.36 ± 0.016^{aA}			
As	Ismailia	0.81 ± 0.016^{aB}	0.82 ± 0.013^{aB}		Crustacean 76 Oysters 86	-
	Damietta	1.32 ± 0.009^{aA}	1.30 ± 0.013^{aA}	-		
	Alexandria	1. 14 ± 0.011^{aAB}	1.14 ± 0.014^{aAB}			
Hg	Ismailia	0.53 ± 0.007^{aB}	0.55 ± 0.011^{aB}		-	-
	Damietta	0.96 ± 0.010^{aA}	0.96 ± 0.012^{aA}	0.5-1		
	Alexandria	0.82 ± 0.009^{aA}	0.82 ± 0.009^{aA}			

Table 3: Trace element concentrations (µg/g ww) in the examined fish species from different localities.

	Sampling sites	Shrimp	Oyster	Crab	MPL	
		Mean ± SE	Mean ± SE	Mean ± SE	US FDA (24)	FAO (25)
Cu	Ismailia	1.13 ± 0.02^{cC}	2.44 ± 0.06^{aC}	1.86 ± 0.02^{bB}	-	30
	Damietta	4.75 ± 0.10^{aA}	5.72 ± 0.08^{aA}	4.56 ± 0.06^{aA}		
	Alexandria	3.14 ± 0.02^{bB}	4.41 ± 0.06^{aB}	1.97 ± 0.03^{cB}		
Cr	Ismailia	0.84±0.01^{aA}	1.02±0.01^{aA}	0.86 ± 0.01^{aA}	crustacean 12 oysters 13	-
	Damietta	1.18±0.01^{aA}	1.37±0.02^{aA}	1.17 ± 0.01^{aA}		
	Alexandria	0.96±0.10^{aA}	1.18± 0.01^{aA}	1.06 ± 0.01^{aA}		
Zn	Ismailia	11.25 ± 0.01^{aB}	13.33 ± 0.01^{aC}	11.71±0.16^{aB}	-	40
	Damietta	19.19 ± 0.04^{abA}	22.19± 0.08^{aA}	15.94±2.27^{bAB}		
	Alexandria	17.06 ± 0.03^{aAB}	18.17± 0.05^{aB}	17.18 ± 0.03^{aA}		
Ni	Ismailia	0.15±0.01^{bC}	0.25±0.02^{aC}	0. 17±0.01^{bC}	crustacean 70 oysters 80	-
	Damietta	0.52±0.01^{cA}	1.30 ±0.01^{aA}	0.72 ± 0.01^{bA}		
	Alexandria	0.34±0.01^{bB}	0.55±0.03^{aB}	0.44 ± 0.01^{abB}		
Fe	Ismailia	34.49± 1.01^{bB}	46.17 ± 0.94^{aB}	34.89 ± 0.86^{bB}	-	-
	Damietta	173.95 ± 0.90^{aA}	189.00 ± 0.85^{aA}	173.64 ± 0.76^{aA}		
	Alexandria	154.51 ± 0.79^{aA}	165.75 ± 0.82^{aA}	153.38 ± 0.80^{aA}		

Table 4: Estimated daily intakes (EDI) and hazard ratio (HR) of toxic metals through consumption of shellfish from different studied areas

	Sampling sites	Shrimp		Oyster		Crab	
		EDI	HR	EDI	HR	EDI	HR
Pb	Ismailia	0.58	0.15	0.68	0.17	0.58	0.14
	Damietta	1.03	0.26	1.13	0.28	1.03	0.26
	Alexandria	0.85	0.21	0.93	0.23	0.86	0.22
Cd	Ismailia	0.17	0.17	0.23	0.23	0.15	0.15
	Damietta	0.30	0.30	0.38	0.38	0.25	0.25
	Alexandria	0.30	0.30	0.37	0.37	0.25	0.25
As	Ismailia	0.56	1.87	0.68	2.27	0.56	1.86
	Damietta	0.92	3.05	1.01	3.36	0.90	3.00
	Alexandria	0.79	2.64	0.86	2.87	0.79	2.64
Hg	Ismailia	0.37	0.73	0.45	0.89	0.36	0.73
	Damietta	0.66	1.33	0.80	1.60	0.66	1.33
	Alexandria	0.57	1.14	0.71	1.41	0.57	1.14

Table 5: Estimated daily intakes (EDI) and hazard ratio (HR) of trace elements through consumption of shellfish from different studied areas

	Sampling sites	Shrimp		Oyster		Crab	
		EDI	HR	EDI	HR	EDI	HR
Cu	Ismailia	0.78	0.003	1.69	0.01	1.29	0.004
	Damietta	3.29	0.01	3.97	0.01	3.16	0.01
	Alexandria	2.18	0.01	3.06	0.01	1.37	0.005
Cr	Ismailia	0.58	0.19	0.70	0.23	0.59	0.20
	Damietta	0.82	0.27	0.95	0.32	0.81	0.27
	Alexandria	0.67	0.22	0.82	0.27	0.73	0.24
Zn	Ismailia	7.80	0.03	9.25	0.03	8.13	0.03
	Damietta	13.32	0.04	15.40	0.05	11.06	0.04
	Alexandria	11.84	0.04	12.61	0.04	11.92	0.04
Ni	Ismailia	0.10	0.01	0.17	0.01	0.10	0.01
	Damietta	0.36	0.02	0.50	0.02	0.90	0.04
	Alexandria	0.30	0.01	0.38	0.02	0.31	0.02
Fe	Ismailia	23.93	ND	32.03	ND	24.21	ND
	Damietta	120.70	ND	131.14	ND	120.48	ND
	Alexandria	107.21	ND	115.00	ND	106.43	ND

Table 6: Hazard index (HI) due to consumption of shellfish in different localities in Egypt

	Shrimp	Oyster	Crab
Ismailia	3.15	3.84	3.12
Damietta	5.28	6.02	5.2
Alexandria	4.57	5.22	4.55

concentration of Cu was found in oysters from Damietta; while the minimum value was found in shrimp from Ismailia. Cu concentrations in this study were comparable with those reported in fish from Alexandria, Egypt (31). On the other hand, the obtained results were higher than those detected in fish collected from Galas River and Beranang mining pool, Selangor (0.01-0.05 µg/g ww) (43) and Palestine (0.25-0.91 µg/g ww) (42). The Cu values observed in this study were lower than MPL (30 µg/g ww) in fish as permitted by FAO (25).

The concentrations of Cr in shellfish were in the range of 0.84-1.37 µg/g ww with an an average concentration of 1.07 µg/g ww (Table 3).The highest value of Cr was detected in oysters from Damietta while the lowest value was observed in shrimp from Ismailia. The Cr levels in this study were nearly similar with those detected (0.1-1.10 µg/g ww) in fish and shellfish from Calicut region, India (11). Although, lower concentrations were reported in (0.03-0.34 µg/g ww) in fish from New Jersey (38).The observed values of Cr were below MPL for shellfish recommended by US FDA (24).

The residual levels of Zn in shellfish were ranged from 11.25-22.19 µg/g ww with a mean concentration of 16.22 µg/g ww (Table 3). The highest level of Zn was found in oysters from Damietta; meanwhile, the lowest level was detected in shrimp from Ismailia. The observed values of Zn in shellfish were nearly similar with those detected (16.53-22.12 µg/g ww) in mussels from Alexandria, Egypt (31) and in investigated fish from Palestine (3.71-20.54 µg/g ww) (32). However, higher levels (3.35- 41.87 µg/g ww) were reported in fish collected from El Menofiya Governorate, Egypt (44). Zn concentrations in this study were below MPL (40 µg/g ww) in fish set by FAO (25).

The concentrations of Ni in shellfish was ranged between 0.15 and 1.30 µg/g ww with a mean concentration of 0.49 µg/g ww (Table 3). The highest levels were observed in shellfish from Damietta; whereas the lowest levels were detected in collected samples from Ismailia. Ni values observed in this study were comparable with those reported in blue crab from Mediterranean Lagoons (0.24-1.96 µg/g ww) (45). However, Baharom and Ishak (43) cited that concentrations of Ni in fish from Galas River and Beranang mining pool, Selangor were lower (0.06-0.07 µg/g ww) than those detected in this study. The levels of Ni detected in the present study were below MPL for shellfish adopted by US FDA (24).

Iron (Fe) was the most abundant trace element in shellfish in this study. The maximum concentration of Fe was found in oysters from Damietta (189.00 µg/g ww) and the minimum Fe concentration was detected in shrimp from Ismailia (34.49 µg/g ww). The average value of Fe reported in this study was 125.14 µg/g ww (Table 3). The observed Fe values in shellfish were nearly similar with those detected in fish from El Menofiya Governorate, Egypt (34.97-165.30 µg/g ww) (44) and in blue crab from Mediterranean Lagoons (25.50-170.00 µg/g ww) (45). On the other hand, higher levels were reported in mussels from Alexandria, Egypt (261.16-332.15 µg/g ww) (31). Fe levels in this study were higher than MPL in fish set by WHO/ FAO (46) (43 µg/g ww) except Fe values in shrimp and crab from Ismailia.

Daily intake and human risk assessment

EDI of different heavy metals owing to consumption of shellfish in the three examined localities was evaluated in the present study as was found in Tables (4 and 5). It was clear that the highest EDI of the investigated heavy metals was found at Damietta especially due to oyster consumption. EDI values of Pb, Cd, As and Hg were 1.13, 0.38, 3.36 and 1.60 µg/Kg/day, respectively; due to consumption of oyster at Damietta (Table 4). The highest recorded EDI values of different trace elements were similarly reported at Damietta due to oyster consumption (Table 5). The recorded EDI values in this study were strongly higher than that recorded at Catalonia, Spain (47). For instance, they reported that EDI values of Pb, Cd, As and Hg due to consumption of shellfish are 0.06, 0.04, 2.52 and 0.11 µg/Kg/day; respectively. Therefore, further investigation for the hazard ratio (HR) and hazard index (HI) is necessary due to shellfish consumption (Tables 4, 5 and 6). Values of HR and/or HI increasing than 1 indicate that there is a potential risk to human health.

HR exceeded 1 for both As and Hg in all examined shellfish species (Table 4). However, HI of different heavy metals exceeded 1 in all localities and in different shellfish (Table 6). In spite of having heavy metal load within the permissible limits, consumption of shellfish in these geographic areas may constitute a public health hazard. Thereby, some adverse effects such as hepatic and renal dysfunctions may be expected. Future approaches necessitate finding solutions to reduce the concentrations of heavy metals in the edible shellfish. Continuous monitoring studies are highly warranted to screen heavy metal load in fish and shellfish.

In Conclusion, this study confirmed that the residual levels of OCPs (HCB, heptachlor, heptachlor-epoxide, aldrin, endrin and γ chlordane) were the predominant then followed by HCHs then DDTs. The highest concentrations of β-HCH HCH and p,p-DDE in shellfish were attributed to their resistance nature for the microbial degradation, and thus the long half life. Of interest, oysters from Damietta showed the highest residual levels of OCPs and heavy metals among the analyzed shellfish species. The levels of OCPs and heavy metal residues were within the recommended level adopted by US FDA, FAO and EU except in few instances. From the analysis of EDI, HR and HI values for the metals in examined shellfish, it was declared that heavy metal load in shellfish must be reduced to decrease the

possible toxicological complications arises from shellfish consumption.

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RAVNI PREOSTANKOV ORGANOKLORINSKIH PESTICIDOV IN TEŽKIH KOVIN V LUPINARJIH IZ EGIPTA IN OCENA ZDRAVSTVENIH TVEGANJ

T.M. Saber, M.H.E. Khedr, W.S. Darwish

Povzetek: V tej raziskavi smo proučevali ravni organokloriranih pesticidov (OCP) in ostankov težkih kovin v lupinarjih (kozice, ostrige in rakovice), zbranih iz treh egiptovskih provinc (Ismajlija, Damietta in Aleksandrija). Določene so bile vrednosti 12 OCP-jev, kot so heksaklorocikloheksani (HCH), aldrin, endrin in diklorodifeniltrikloroetani (DDT). Prevladujoči odkriti OCP-ji so bili ß-HCH, p,p-DDE in endrin. Vzorec kontaminacije OCP je bil v vrstnem redu drugih OCP-jev (HCB, heptaklor, heptaklor-epoksid, aldrin, endrin in γ klordan) > HCHs > DDT. Ocenjeni so bili tudi ostanki nekaterih težkih kovin in elementov v sledovih. Največje preostale količine OCP-jev in težkih kovin so bile najdene v ostrigah, zbranih v provinci Damietta. Ocena tveganja za zdravje je bila določena z izračunom razmerja nevarnosti in indeksa nevarnosti. Koncentracije OCP-jev in težkih kovin v pregledanih školjkah so bile pod najvišjo stopnjo preostankov, ki so jo določile Združene države Amerike za prehrano in zdravila ter FAO. Zato bi bili lupinarji, proučeni v tej raziskavi, varni za prehrano ljudi.

Ključne besede: organoklorirani pesticidi; težke kovine; lupinarji; tveganje za zdravje; Egipt

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