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SLOVENIAN VETERINARY RESEARCH

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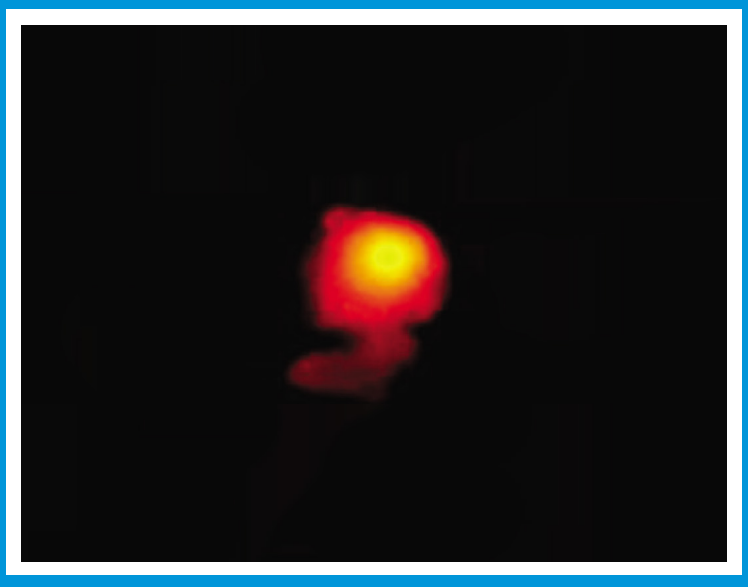

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Original Research Article

ACUTE AND SUB-ACUTE TOXICITY STUDIES ON *Combretum dolichopetalum* ENGL. & DIELS LEAVES

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Abstract: We studied the acute and sub-acute toxicity of *Combretum dolichopetalum* leaves in experimental mice and rats respectively using standard techniques. The LD_{50} of the methanol extract of *Combretum dolichopetalum* leaves as carried out in experimental mice was obtained as more than 5000 mg/kg body weight. Administration of graded doses (100, 200, 400 and 800 mg/kg) of the extract for 21 days resulted in increases in body weights, white blood cells (WBC), Neutrophils, red blood cells (RBC), packed cell volume (PCV), haemoglobin (HGB), mean corpuscular volume (MCV) and mean cell haemoglobin (MCH) of the rats; but did not affect ($P>0.05$) their monocytes, mean cell haemoglobin concentration (MCHC), platelet (PLT) levels. All doses of the extract did not affect ($P>0.05$) the sodium, potassium, chloride, bicarbonate, urea, creatinine, total and conjugated bilirubin, alanine and aspartate amino transaminase, aspartate amino transaminase, alkaline phosphatase activities; relative liver and kidney weights of the rats, a finding that was corroborated by histology of the liver and the kidney. The extract at 100 mg/kg had no effect on the PCV and HB of the rats. The study suggested the therapeutic potentials of *Combretum dolichopetalum* as a blood booster. Finally, the study revealed the safety in the usage of *Combretum dolichopetalum* leaves in Nigerian ethnomedicine.

Key words: ethnopharmacology; *Combretum dolichopetalum*; toxicology; herbal medicine; nutraceutical; pharmacotherapy

Introduction

For several years, traditional herbal medicines have been utilized all over the world to treat diseases and promote health (1,2). In view of this, the World Health Organization has come to recognize the importance of traditional/herbal medicines in the maintenance of human health. In addition, the World Health Organization estimated that nearly 80% of the human population worldwide, especially people residing in the developing countries rely on traditional herbal medicines for their health care needs (1).

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Irrespective of the growing popularity and perceived safety of herbal medicines, recent studies have shown that several medicinal plants that are being used as traditional medicines for the maintenance of human health and management of several diseases have adverse effects (3). This has therefore generated concerns about the potential toxicity that could arise from short and long-term exposure to such medicinal plants. To mitigate this, acute and sub-acute or sub-chronic toxicity studies on medicinal plants and their products are recommended as a way of assuring humans of the safety of exposure to these herbal medicines and to establish their safety margins (4).

The use of herbal medicines for the maintenance of health and treatment of diseases is a

common practice in Nigeria (5) and *Combretum dolichopetalum* Engl. & Diels (Combretaceae) leaf is one of such plants that are used in Nigerian and African traditional medicine for the maintenance of human health and treatment of a wide array of diseases.

The plant is commonly found in the Eastern part of Nigeria. It is known as "achichanza" (food of the sun bird) in Igbo land and "okoso" in Edo Nigeria (6). Some of the folkloric medicinal uses of the roots of this plant in Nigerian and African ethnomedicine include: relief of menstrual pain, enhancement of labour, facilitation of the removal of placenta after delivery, promotion of rich milk supply after delivery, treatment of burns and skin infections, whereas the decoction is taken as a purgative (7-9) while the folkloric and pharmacological proeprties of the leaves of this plant in Nigerian ethnomedicine include: Wound healing (10), antiulcer (11), antidiarrhea activity (12), relief of menstrual pain and enhancement of labour (Personal communication).

The antiulcer, anti-hepatotoxic, trypanocidal, anti-inflammatory, antidiabetic, gastric antisecretory, smooth muscle relaxant and antispasmolytic activities of this plant have also been reported (8-14).

Despite the wide spread usage of this plant in Nigerian and African ethnomedicine for the management of several health conditions, there is scarcity of information in literature on the toxicological implication of long term administration of this plant in humans or animals.

In the light of the above, the present study was designed to carry out acute and sub-acute toxicity studies on *C. dolichopetalum* leaves in experimental animals.

Materials and methods

Collection and Identification of Plant Materials

Fresh matured leaves of *C. dolichopetalum* were located and collected between February and March, 2017 from its natural habitat in Nsukka, Enugu State, Nigeria. The plant samples were identified by Mr. C.J. Onyeukwu, a taxonomist of the Plant Science & Biotechnology Department of the University and the voucher specimen (UNH No.49a) of the plant was deposited at the herbarium.

Preparation of extract

The leaves were washed and air dried at room temperature for 7 days after which they were pulverized using an electric blender (model ms-233, China). The flour (2 kg) was extracted with methanol for 48 h in a Soxhlet extractor using the method of Jensen (2007). At the end of the extraction period, the extract was collected and concentrated (40°C) to dryness after which it was weighed and thereafter constituted in the vehicle (distilled water) for acute and sub-chronic toxicity testing.

Animal Experiments

The animals that were used for this study were purchased from the Department of Veterinary Medicine, University of Nigeria, Nsukka.

Animal studies were done after ethical approval by the College of Medicine Research Ethics Committee, of the University of Nigeria, Enugu Campus, Enugu, Nigeria (protocol number: 026/02/2017) and which was in line with the ethical guidelines for the care and usage of laboratory animals as given by the National Institute of Health (15).

Acute Toxicity Study

The acute toxicity study was carried out following the OECD guideline 423 for testing of chemicals (16). Thirty healthy non preganant female Swiss albino mice were used for the acute toxicity study. Following acclimatization to their feeds and water, they were divided into 6 groups of 5 mice per group. The mice were administered graded oral doses of the extract (dissolved in distilled water) in the order: 500, 1000, 2000, 3000, 4000 and 5000 mg/kg body weight. Thereafter, they were kept in standard cages and allowed free access to feed and water *ad libitum*. Subsequently, they were observed for toxicity signs and the number of deaths in each group within 24 h for lethal dose (LD_{50}) calculation using the Karber's method, as reported by Enegide et al. (17) and Akomas et al. (18) respectively.

Sub-acute Toxicity Study

The sub-acute toxicity study on *C. dolichopetalum* was carried out following the OECD Guideline 407 (19) and it lasted for 21 days (20). Twenty five

(25) mature inbred healthy non-pregnant female albino rats (weighing between 87.66 to 95.82 g) of the Wistar strain were randomly grouped into five groups of five rats per group (one animal per cage) after acclimatization to their feeds and water. The rats in group I (Control) received distilled water while those in groups II, III, IV and V were administered 100, 200, 400 and 800 mg/kg respectively of *C. dolichopetalum* leaf extract (with distilled water as the vehicle) for 21 days using oral gavage. The rats were also kept in standard cages and they had access to their feeds and water *ad libitum.*

The changes in the weights of the rats were recorded on a daily basis. At the end of administration of the extract, the rats were fasted overnight (with access to only drinking water) and about 4 mL of blood samples were collected from the orbital route of each rat under mild ether anesthesia into anticoagulant tubes for the analysis of hematological parameters while the rest were poured into plain tubes for the assay of electrolytes, urea, creatinine, total and direct bilirubin, alkaline phosphatase (ALP), aspartate amino transaminase (AST) and alanine amino transaminase (ALT) activities respectively. The rats were later sacrificed by cervical dislocation and the liver and the kidneys were harvested and weighed (21). The body weights of the rats were recorded on a daily basis using an electronic weighing balance (Model Scout Pro, Ohaus Corporation, USA), and the changes in their weights were expressed as a percentage using the formula:

Percentage change in weight = [Final weight - Initial weight]/[Final weight] x100.

The relative organ weights were also expressed as a percentage using the formula:

Relative liver weight = [Liver weight]/[Final body weight] x100 and

Relative kidney weight = [Kidney weight]/[Final body weight] x100 (21).

Haematological parameters

The red blood cell count (RBC), packed cell volume (PCV), haemoglobin concentration (HGB), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), white blood cell count (WBC), and platelets count (PLT) were analyzed using Coulter® Ac-T 5Diff AL, Beckman Coulter, Inc. Port Matilda, Pennsylvania, USA.

Liver function assays

The total bilirubin, direct bilirubin, ALT, AST and ALP activities in the sera of the rats were determined with their respective kits (Biosystems kit) using A25 Biosystem Fully Automated Machines.

Kidney function assays

Sodium, potassium, chloride and bicarbonate were estimated with Easylyte® analyzer Medica Corporation, Bedford, USA while urea and creatinine were analyzed with their kits (Biosystems kit) using A25 Biosystem Fully Automated Machine.

Histology

The harvested organs were preserved in buffered 10% formalin saline solution for histopathological processing. They were washed in ascending grades of ethanol, cleared with xylene, embedded in paraffin wax. The tissues were finally sectioned using a rotary microtome (at 5µ thickness), stained with haematoxylin and eosin (H&E) and mounted on Canada balsam. All the sections were examined microscopically using standard techniques. The slides were examined under a light microscope using X 200 magnification. Photomicrographs of lesions were taken with an Olympus photo microscope for observations and documentation of histopathological lesions (22).

Statistical Analysis

Statistical analysis was carried out by the use of Microsoft Excel Statistical Packages (Microsoft Corporations, USA). All analyses were carried out in triplicates and the results were presented as means and standard deviation. One-way analysis of variance was used for comparison of the means. Differences between means were considered to be significant when $P<0.05$.

Results

General signs and mortality

There were neither behavioral changes nor signs of toxicity after administration of all the doses of the extract. The mice had normal disposition and were emotionally stable and all survived the 24

Table 1: Body weights of rats

Groups	Body weight before administration (g)	Body weight after administration (g)	Body weight gain (g)	% change in body weight
Control	91.38±3.40	153.10±0.59	61.72±1.64	40.31±1.15
II(100mg/kg)	92.18±2.10	137.10±1.82*	44.92±0.64*	32.76±0.06*
III(200mg/kg)	91.26±3.60	132.20±1.49*	40.94±1.01*	30.97±0.02*
IV(400mg/kg)	92.42±3.40	126.00±1.12*	34.19±0.61*	26.65±0.08*
V (800mg/kg)	91.61±2.10	123.12±0.93*	32.21±0.93*	25.59±1.06*

Values are reported as means ± SD, *= p< 0.05 versus control

Table 2: Effect of *C. dolichopetalum* leaf extract on haematological parameters in rats

Groups Parameters	Control	II(100mg/kg)	III(200mg/kg)	IV(400mg/kg)	V(800mg/kg)
WBC X 10^9/L	10.46±0.78	12.70±0.76*	14.12±0.42*	15.60±0.22*	18.78±0.66*
Neutrophils (%)	20.20±0.66	31.20±0.66*	36.60±0.51*	40.40±0.51*	44.00±1.18*
Lymphocytes (%)	78.40±0.85	66.60±0.85*	61.00±0.73*	57.00±0.94*	52.60±0.93*
Monocytes (%)	1.40±0.50	2.20±0.10	2.40±0.15	2.60±0.30	2.70±0.25
RBC X 1012/L	9.37±0.20	9.94±0.70*	10.28±0.72*	11.44±0.12*	12.56±1.15*
PCV (L/L)	42.02±0.30	43.00±0.33	43.80±0.15*	43.90±0.14*	44.32±0.33*
HGB (g/dL)	14.00±0.09	14.33±0.13	14.60±0.10*	14.62±0.05*	14.77±0.12*
MCV (fL)	49.12±0.24	50.12±0.06*	50.24±0.04*	50.30±0.09*	50.46±0.17*
MCH (pg)	18.10±0.08	18.36±0.02	18.70±0.05*	18.42±0.04*	18.45±0.15
MCHC (g/dl)	37.36±0.14	37.78±0.12	37.99±0.14	38.18±0.19	38.32±0.11
PLT X 10^9/L	684.20±5.94	684.60±6.12	685.60±5.33	686.10±6.22	687.40±6.54

Values are reported as means ± SD, *= p< 0.05 versus control

Table 3: Effect of *C. dolichopetalum* leaf extract on markers of renal function in rats

Groups Parameters	Control (distilled water)	II(100mg/kg)	III(200mg/kg)	IV(400mg/kg)	V(800mg/kg)
Sodium (mEq/L)	140.80±0.97	140.92±0.80	141.62±0.91	141.70±0.67	141.84±0.98
Potassium (mEq/L)	4.08±0.10	4.12±0.16	4.15±0.10	4.22±0.16	4.23±0.10
Chloride (mEq/L)	100.18±0.30	100.41±0.36	100.78±0.60	100.81±0.51	101.50±0.79
Bicarbonate (mEq/L)	15.58±1.81	17.52±2.05	17.74±2.02	18.92±2.46	18.46±2.30
Urea (mg/dL)	7.30±0.66	6.32±0.47	7.04±0.30	6.32±0.47	7.82±0.54
Creatinine (mg/dL)	1.46±0.23	1.42±0.08	1.47±0.22	1.54±0.05	1.71±0.18

Values are reported as means ± SD

Table 4: Effect of *C. dolichopetalum* leaf extract on markers of liver function in rats

Groups Parameters	Control (distilled water)	II(100mg/kg)	III(200mg/kg)	IV(400mg/kg)	V(800mg/kg)
Total bilirubin (mg/dL)	5.06±0.78	5.02±0.50	5.44±0.68	5.68±0.19	5.62±0.92
Conjugated bilirubin (mg/dL)	2.56±0.34	2.38±0.30	2.36±0.37	2.44±0.33	2.50±0.67
ALT (U/L)	18.02±2.61	15.62±2.37	16.96±2.49	15.78±0.33	15.06±3.78
AST (U/L)	26.02±4.20	25.72±4.35	22.44±4.76	22.64±4.07	22.28±4.19
ALP (U/L)	197.80±0.50	198.18±0.53	198.88±0.89	198.98±0.70	199.16±0.75

Values are reported as means ± SD

Table 5: Effect of *C. dolichopetalum* leaf extract on some relative organ weight in rats

Groups Parameters	Control	II(100mg/kg)	III(200mg/kg)	IV(400mg/kg)	V(800mg/kg)
Liver (%)	4.14±0.02	4.11±0.03	4.12±0.01	4.13±0.02	4.12±0.02
Kidney (%)	0.71±0.01	0.72±0.01	0.72±0.01	0.73±0.02	0.73±0.02

Values are reported as means ± SD

hours period of acute toxicity study. There were neither changes in the sensory nervous system responses nor adverse gastrointestinal effects. The oral administration of the extract up to the dose of 5000 mg/kg did not lead to 50% of mortality in mice. The LD_{50} of the extract was obtained as more than 5000 mg/kg body weight orally.

Body weights of rats

Table 1 shows the effect of administration of *C. dolichopetalum* leaf extract on the body weight of rats. Data presented in the Table revealed that there were no significant differences (P>0.05) in the body weights of groups II to V rats relative to the control before extract administration. However, after extract administration, the body weights of groups II, III, IV and V rats (that recorded 32.76, 30.97, 26.65 and 25.59% increases in weights) were significantly lower than that of the control group (that recorded 40.31% gain in weight).

Hematological parameters

The effect of administration of *C. dolichopetalum* leaf extract on the haematological parameters of rats is shown in Table 2. Data presented in the Table showed that there were significant increases (P<0.05) in the white blood cells of groups II to V rats relative to the control (Table 2).

Significant increase (P<0.05) was observed in the neutrophils levels of groups II to V rats compared with the control (Table 2).

There were decreases (P<0.05) in the lymphocyte levels of groups II to V rats compared with the control; but no significant differences (P>0.05) in the monocyte levels of groups II to V rats relative to the control (Table 2).

There were significant increases (P<0.05) in the red blood cells of groups II to V rats compared with the control; significant increases (P<0.05) in the PCV of groups III to V rats compared with the control but no difference (P>0.05) in the PCV of group II rats compared with the control (Table 2).

There were significant increases (P<0.05) in the hemoglobin levels of groups III to V compared with the control, but no difference (P>0.05) in the hemoglobin levels of group II rats compared with the control (Table 2).

There were significant increases (P<0.05) in the MCV of groups II to V compared with the control; significant increases (P<0.05) in the MCH of groups III to V compared with the control but no difference (P>0.05) in the MCH of group II rats compared with the control.

There were no differences (P>0.05) in the MCHC of groups II to V when compared with the control and no differences (P>0.05) in the PLT of groups II to V rats compared with the control.

Renal Function Parameters

Table 3 shows the effect of administration of *C. dolichopetalum* leaf extract on markers of renal function in rats. As shown in the Table, there were no significant differences (P>0.05) in the serum levels of Na^+, K^+, Cl^-, HCO_3^-, urea and creatinine in groups II to V rats when compared with the control.

Liver Function Parameters

The effect of administration of *C. dolichopetalum* leaf extract on markers of hepatic function in rats is shown in Table 4. As shown in the Table, there were no significant differences (P>0.05) in the serum levels of total and direct bilirubin and the activities of ALT, AST and ALP in the sera of groups II to V rats compared with the control.

Relative Organ Weights

The effect of administration of *C. dolichopetalum* leaf extract on some relative organ weight in rats is shown in Table 5. As shown in the Table, there were no significant differences (P>0.05) in the relative liver weights of groups II to V rats when compared with the control.

Similarly, there were no significant differences (P>0.05) in the relative kidney weights of groups II to V rats relative to the control.

Histology

The results of the histopathological assay of the liver and kidney of the rats that were investigated in this study are shown in Figures 1 and 2. A section of the liver from the control and treated groups showed normal arrangement of the hepatocytes (liver cells) in cords. The architecture of the portal triad comprising of the bile duct, hepatic portal vein and hepatic arteries were all

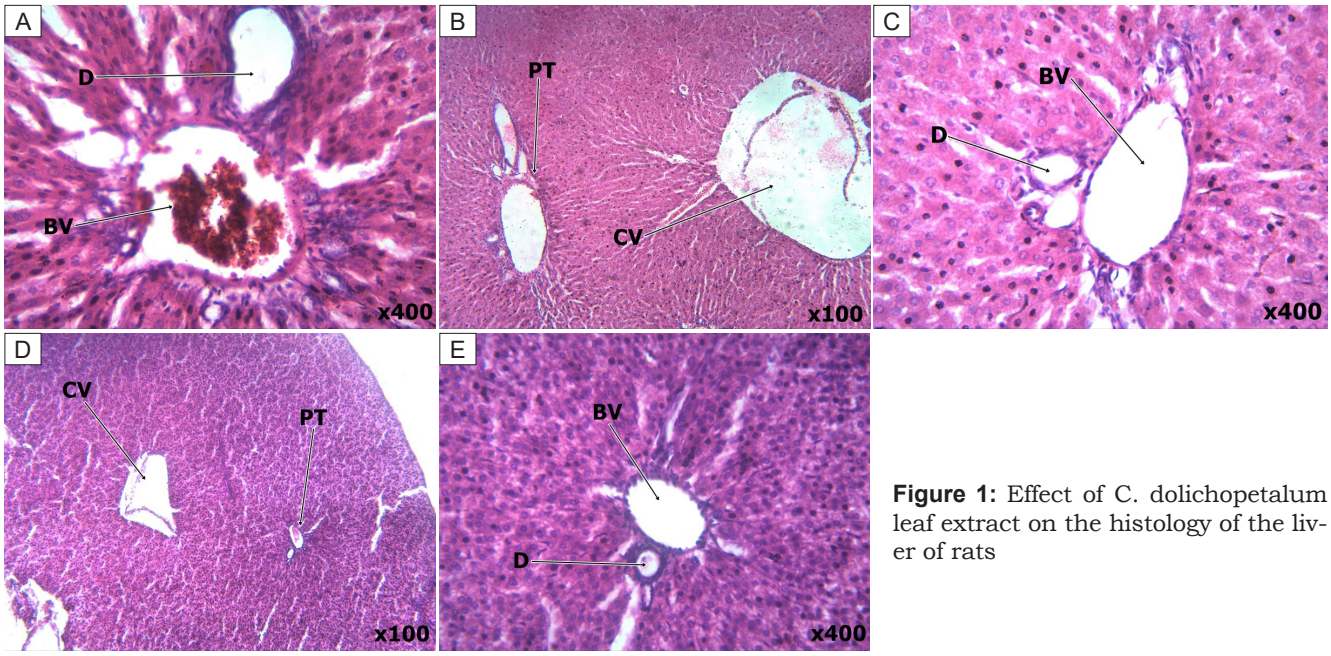

Figure 1: Effect of C. dolichopetalum leaf extract on the histology of the liver of rats

A: (Control group) Photomicrograph showing a well preserved liver architecture. The portal triads are evenly spaced around a central vein and there is no portal inflammation. (BV-blood vessel, D-ductule); **B:** (Group II) Photomicrograph showing a well preserved liver architecture. No inflammatory cells were seen. (PT – Portal triad, CV- Central vein); **C:** (Group III) Photomicrograph showing a well preserved liver architecture. No inflammatory cells were seen. No visible lesion seen. (BV-blood vessel, D-ductule); **D:** (Group IV) Photomicrograph showing a well preserved liver architecture. No inflammatory cells were seen. No visible lesion seen. (BV-blood vessel, D-ductule); **E:** (Group V) Photomicrograph showing a well preserved liver architecture. No inflammatory cells were seen. No visible lesion seen. (PT – Portal triad, CV- Central vein)

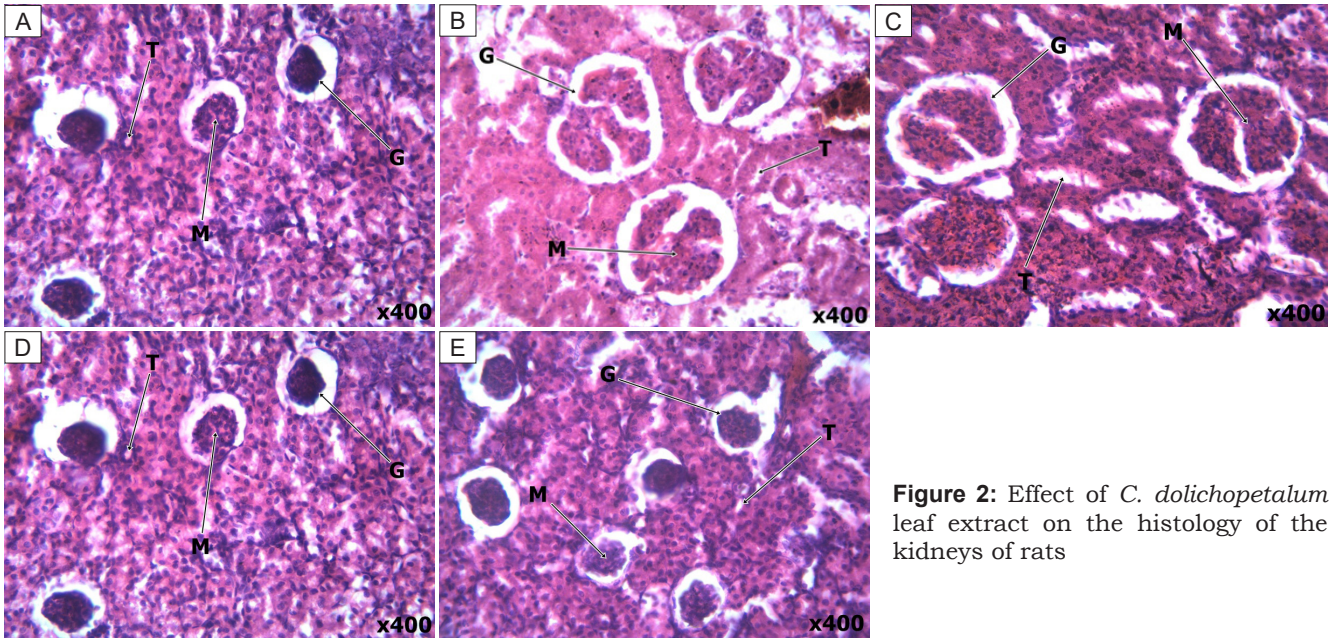

Figure 2: Effect of *C. dolichopetalum* leaf extract on the histology of the kidneys of rats

A: (Control group) Photomicrograph showing an evenly distributed glomeruli, of similar size, with normal mesangial cellularity. There are numerous open glomerular capillaries, and normal endothelium. The tubules are of normal density and tubular epithelium is viable. (M= mesangium, G=glomerulus, T=tubule)s; **B:** (Group II) Photomicrograph showing a well preserved kidney architecture. No visible lesion seen. . (M= mesangium, G=glomerulus, T=tubule); **C:** (Group III) Photomicrograph showing a well preserved kidney architecture. No visible lesion seen. (M= mesangium, G=glomerulus, T=tubule); **D:** (Group IV) Photomicrograph showing a well preserved kidney architecture. No visible lesion seen. (M= mesangium, G=glomerulus, T=tubule) **E:** (Group V) Photomicrograph showing a well preserved kidney architecture. No visible lesion seen. (M= mesangium, G=glomerulus, T=tubule)

normal. The central veins were also found to be normal. No inflammatory cells were seen in the connective tissue of the portal triad. No necrosis was also observed and the central vein showed no form of congestion and no infiltration with inflammatory cells.

The kidneys in both the control and all the treated groups were normal showing numerous renal tubules interstitials tissues and no signs of inflammations. The glomerulus and renal vein and artery also appeared normal.

Discussion

General behavior is amongst the parameters that are necessary for the assessment of first signs of toxicity (5). The acute toxicity study which revealed that the extract did not affect the general behaviours of the mice, suggests that the extract was well tolerated by the mice.

According to previous reports (22, 23), a substance with an LD_{50} of 1000 mg/kg body weight and above could be regarded to be safe for administration. The high LD_{50} that was obtained in this study for this plant therefore suggests its non-toxicity and the safety of its usage in herbal medicine. Going by the globally harmonized classification system for chemical substances and mixtures as given by the OECD (24), the LD_{50} that was obtained for the extract suggests the extract to fall under class 5 drug and as such, could be considered to be non toxic (1).

In addition to general behavior, changes in body weights are also considered as parameters that are of great importance for the evaluation of first signs of toxicity (6, 17, 22-23). Although the sub-acute toxicity study revealed that all the rats that were administered the extract had lower body weights compared with the control, when comparisons were made between before and after extract administration, it was observed that all the rats administered the extract recorded significant increases in body weights compared with their body weights before extract administration. This finding therefore suggests that the extract positively impacted on the normal growth of the rats. However, the decreased body weights of the rats administered the extracts when compared with the controls, may be attributed to improved feed consumption by the rats in the control group due perhaps to preference for the control diets over the extract.

Toxic compounds have the hematopoietic system as one of their targets which makes the hematopoietic system a crucial marker of physiological and pathological state in both humans and animals (5, 20, 25-31).

The increased WBC, Neutrophils, RBC, PCV, HGB, MCV and MCH of the rats administered the extract as observed in this study suggests that the extract is likely to stimulate hematopoiesis.

The non significant differences in the Monocytes, MCHC and PLT of the rats administered the extract at all doses when compared with the control suggests that administration of the extract at all the doses had no effect on the Monocytes, MCHC and PLT counts of the rats. Furthermore, findings of this study also showed that administration of the extract at 100 mg/kg had no effect on the packed cell volume and haemoglobin levels of the rats suggesting that the extract at this concentration was not able to initiate erythropoiesis in the rats. In all, the study revealed that administration of the extract had no toxicological effect on the haematopoietic system of the rats.

The kidneys receive about 25% of the cardiac blood flow and any substance that reaches the systemic circulation will reach the kidney. This therefore makes the kidneys quite vulnerable to to toxic compounds (1, 20, 32-34).

Renal function in this study was evaluated by serum levels of Na^+, K^+, Cl^-, HCO_3^-, urea and creatinine and by histological analysis.

Electrolytes play important roles in many body processes some of which include: control of fluid levels, acid-base balance, etc. In the event of renal impairment/disease, these functions of electrolytes could be grossly affected.

The major cation in the extracellular fluid is Na^+ and it plays an essential role in maintaining the water balance in the body and regulating the extracellular fluid volume (35-36). K^+ is the most abundant cation in the intracellular fluid and it is useful in the maintenance of osmotic pressure (35-36). Cl^- is the main anion that is found in the extracellular fluid and it aids the body to balance as well as maintain osmotic pressure and electrical neutrality. HCO_3^- is an important anion of the bicarbonate buffer system (35-36).

Urea which is formed in the liver as an end product of protein metabolism is thereafter eliminated by the kidneys (35). In the event of renal impairment or disease, the rate of elimination of urea by the kidneys will be affected leading to in-

creased blood levels of urea. Creatinine is a waste product that is derived from creatine phosphate in a non-enzymatic and spontaneous reaction. Creatinine is removed from the blood mainly by the kidneys by glomerular filtration and by proximal tubular secretion with little or no reabsorption. Hence, in the event of defective filtration by the kidneys due perhaps to renal impairment or disease, the tendency will be increased blood concentration of creatinine (35). Therefore, urea and creatinine are quite useful in evaluating kidney function.

In this study, the extract at all the doses administered had no effect on the concentrations of Na^+, K^+, Cl^-, HCO_3^-, urea and creatinine levels of the rats suggesting that the extract did not induce alteration in renal functions of the rats or induce kidney damage. These findings therefore indicate the non-toxicity of the extract to the kidney of the rats.

The liver is an important organ that plays a crucial role in the detoxification drugs and its normal function could be assessed by the concentrations or activities of various biomarker molecules/enzymes in the sera.

Bilirubin is a product of haemoglobin degradation, and increases in its serum levels are attributed to illnesses such as primary biliary cirrhosis, jaundice and hepatic cholestasis (1). ALT catalyzes the transfer of an α-amino group from alanine to α-KT to form glutamate and pyruvate respectively. The liver is the major source of this enzyme and its level in the sera increases during liver pathology. AST level, apart from being an indicator of liver dysfunction, is also used to assess muscle and heart diseases (35). ALP is mainly found in the cells lining the biliary duct of the liver and it is used in the diagnosis of bile duct pathologies (35). In addition, assay of the activities of aminotransferases and phosphatases is considered to be of clinical and toxicological importance as changes in their activities could indicate disease state or tissue damage by toxicants (37).

Findings of this study showed that the extract did not elicit significant changes in the levels of total and direct bilirubin as well as the activities of ALT, AST, and ALP in the rats, suggesting the non-hepatoxic action of the extract as well as its non-adverse effect on erythropoiesis.

Organ weight has been considered to be a very reliable and about the most sensitive indicator of the effect of drug toxicity (22, 38-39). This is because significant changes in organ weights between treated and control animals could occur in the absence of any morphological changes or may precede morphological changes (20, 39). In this study, the extract at all the doses administered had no effect on the relative weights of the liver and kidney of the rats, which further affirms the non-toxicity of the extract to the liver and kidney of the rats.

The results of the histology of the kidney and liver of the control rats and the rats administered the extract which results corroborate the results of the liver and kidney function assays and the relative liver and kidney weights of the rats, is a confirmation that administration of the extract to the rats did not induce any form of hepatic or renal toxicity.

Conclusion

The present study demonstrated the safety in the traditional usage of *combretum dolichopetalum* leaves in Nigerian ethnomedicine in the maintenance of health and treatment of various diseases.

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The authors declare that they have no conflict of interest.

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AKUTNA IN SUBAKUTNA ŠTUDIJA TOKSIČNOSTI LISTOV RASTLINE *Combretum dolichopetalum*

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Povzetek: S standardnimi metodami smo pri poskusnih miših in podganah proučevali akutno in subakutno toksičnost listov *Combretum dolichopetalum*. LD50 metanolnega izvlečka listov *Combretum dolichopetalum* je bil pri poskusnih miših nad 5000 mg/kg telesne teže. Enaindvajsetdnevno dodajanje naraščajočih odmerkov (100, 200, 400 in 800 mg/kg) izvlečka je pri poskusnih podganah povzročilo povečanje telesne mase, števila belih krvničk (WBC), nevtrofilcev, rdečih krvničk (RBC), volumna stisnjenih eritrocitov (PCV), hemoglobina (HGB), povprečnega volumna eritrocitov (MCV) in povprečno vsebino hemoglobina v eritrocitih (MCH), ni pa vplivalo ($p > 0,05$) na število monocitov, povprečno koncentracijo hemoglobina v volumnu eritrocitov (MCHC) ter na povprečno vrednost trombocitov (PLT). Nobeden od odmerkov izvlečka ni vplival na ($p > 0,05$) vrednosti natrija, kalija, klorida, bikarbonata, sečnine, kreatinina, skupnega bilirubina in vezanega bilirubina, alanina, aspartatne amino transaminaze, aspartatne amino transaminaze, alkalne fosfataze; relativno težo jeter in ledvic podgan, kar je bilo v skladu s histološko preiskavo jeter in ledvic. Izvleček v odmerku 100 mg/kg ni vplival na PCV in HB podgan. Študija tako kaže na možnost uporabe rastline *Combretum dolichopetalum* za izboljšanje krvne slike. Raziskava je dokazala varnost uporabe listov *Combretum dolichopetalum*, ki se tradicionalno uporabljajo v Nigeriji v etnomedicini.

Ključne besede: etnofarmakologija; *Combretum dolichopetalum*; toksikologija; zeliščna zdravila; hranila; farmakoterapija

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Original Research Article

ASSESSMENT OF MICROBIOLOGICAL LOAD OF SMALL RUMINANT CARCASSES, LIVERS, SOME LYMPH NODES, TOOLS AND KNIFE SAMPLES IN SLAUGHTERHOUSE

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Abstract: The aim of this study was to determine the microbiological loads of small animal carcasses, carcass lymph nodes, whole liver surface, liver lymph nodes and some tools contacting with carcass and offal. Total 630 samples taken from small animal carcasses, livers, hepatic lymph nodes, subiliac and prescapular lymph nodes, staff knives and slaughterhouse tools samples (stainless steel table, plastic crates, offal carts) were investigated for mesophilic aerobic bacteria, *Enterobacteriaceae*, *Escherichia coli* counts and *Salmonella* spp. The mean total aerobic mesophilic bacteria (TAMB), *Enterobacteriaceae* and *E. coli* numbers of the carcasses were 3.6, 0.6, and 0.1 $\log_{10}$ CFU/cm^2, respectively, and the most contaminated region among the carcass sampling points was flank. The mean TAMB, *Enterobacteriaceae* and *E. coli* counts of the liver surfaces were 6.0, 3.7, 2.9 $\log_{10}$ CFU/liver, respectively. The average TAMB, *Enterobacteriaceae* and *E. coli* numbers of the knives were found as 6.3, 2.9 and 2.1 $\log_{10}$ CFU/blade, and the average TAMB, *Enterobacteriaceae* and *E. coli* counts of the slaughterhouse surfaces were 5.1, 1.6, 0.5 $\log_{10}$ CFU/cm^2. *Salmonella* spp. was detected in 4% of the liver samples and 10% of the knives samples. Consequently, the presence of *Salmonella* on the surface of livers and blades, and high number of *E. coli* on the livers, blades and tools show that a public health risk may arise at any time, and staff should pay extra attention to the "Good Hygiene Practices" and Food Safety Management Systems (such as HACCP) applied in slaughterhouses.

Key words: carcass; liver; lymph node; microbiological quality; *Enterobacteriaceae*; *Escherichia coli*; *Salmonella* spp.

Introduction

Red meat is one of the important animal protein sources in human diet, and the meat under the skin of a healthy animal is considered sterile. However, carcass contamination is inevitable during the slaughtering process such as skinning and evisceration (1). Most of the microbial contamination on the carcass surface comes from different sources such as hide, intestinal contents, slaughterhouse equipment/tools and workers during the slaughtering.

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Salmonella is one of the pathogenic bacteria causing foodborne diseases (2). It is known that the animal skin, gastrointestinal tract and feces are the primary sources of *Salmonella* contamination for the carcass surface during the slaughter process (3). Besides, the studies conducted on carcass lymph nodes have shown that those nodes may harbor pathogenic microorganisms such as *Salmonella* spp. (4). It is known that completely removing lymph nodes from the carcass is impossible. The lymph nodes remaining in carcass meat become a part of the meat product after the mincing process of meat (5). Edible offal that is derived from carcass may be exposed to cross contamination with pathogen microorganisms in case of poor hygiene

in slaughterhouse (6). Most of the studies related to the *Salmonella* prevalence in lymph nodes, edible offal, carcass and on slaughterhouse tools have been conducted in beef carcasses and beef slaughterhouses (3-5, 7, 8), however, the studies conducted in small animal carcasses are very limited (9-11). Hence, this study was conducted to investigate (i) the microbiological condition of small animal carcasses and liver surfaces in slaughterhouse, (ii) *Salmonella* prevalence of carcass lymph nodes and hepatic lymph nodes in small animals, and (iii) the bacterial load of staff blade and some slaughterhouse tools contacting with offal.

Materials and methods

The study was conducted in a slaughterhouse that has beef and sheep/goat slaughter-lines with a line speed of approximately 150 small animals per hour, and there was no automatic hide puller for small animal carcasses. Samples of the study were composed of total 630 samples taken from small animal carcasses (200 samples taken from 50 carcasses; four sampling sites including rump, flank, brisket ad neck regions were sampled for each carcass), livers (50), hepatic lymph nodes (taken from 100 livers), subiliac (100 pairs, one pair per carcass) and prescapular lymph nodes (100 pairs, one pair per carcass), staff knives (blade) (40) and some tools (stainless steel tables (15), plastic crates (15), offal carts (10)) used in slaughterhouse. The animals sampled were not separated as goat and sheep; they all were defined as small animal because the slaughterhouse was cutting them as mixed, and the staff was using the same blade, same plastic crates, offal carts and tables for both goat and sheep. The lymph nodes samples were collected as a pair per carcass. The samples were collected between February and May 2018, and the slaughterhouse was visited once a week during this period.

Sampling procedures

Carcass samples were taken from four sampling sites including rump, flank, brisket and neck regions indicated in the ISO 17604 (12) at the stage after washing but before chilling. Briefly, samples were taken from carcass regions by swabbing an area of 100 cm^2 using sterilized stainless steel template (10 cm×10 cm) and sterile sponge (World bioproducts, EZ-ReachTM, US/Canada) which was premoistened with 25 ml sterile buffered peptone water (BPW) (Biokar, Beauvais/France). The entire liver surface was sampled by the sterile sponge premoistened with 25 ml sterile BPW. Samples from tools (stainless steel table, plastic crates, offal carts) were collected by swabbing an area of 100 cm^2 using the sterilized template (10 cm×10 cm) and the premoistened sterile sponge. Staff knives were sampled by swabbing the both surfaces of the blade with the premoistened sterile sponge. Hepatic, subiliac and prescapular lymph nodes were taken using sterile scalpel. All visible hepatic lymph nodes on the liver surface were collected.

Microbiological analysis

All the samples were transported to the laboratory in a thermo cool box containing pre-frozen ice bags within 2-3 h. The sponge samples taken from carcasses, livers, blades and tools were homogenized in a stomacher (Bag mixer 400, Interscience, France) for 2 min. Analysis of aerobic plate counts, *E. coli* and *Enterobacteriaceae* in all the samples were conducted according to the methods described in ISO 4833, ISO 16649-2 and ISO 21528-2, respectively (13-15). Briefly, Plate Count Agar (PCA), Tryptone Bile X-glucuronide (TBX) agar and Violet Red Bile Glucose (VRBG) Agar (Biokar, Beauvais/France) were used for the detection of aerobic plate count (APC), *E. coli* and *Enterobacteriaceae* counts. TBX and VRBG plates were incubated at 37°C for 24 h, and PCA plates were incubated at 30°C for 72 h. The adipose tissues surrounding the each lymph nodes were removed as much as possible, and each lymph nodes were dipped into 70% alcohol for 5 min in order to disinfect the outside of the node. After this procedure, the lymph nodes were kept in open air to remove the residual alcohol for 5 min. And then, hepatic, subiliac and prescapular lymph nodes were separately placed into sterile stomacher bags, and they were crushed with rubber mallet from outside of the stomacher bag. After this, 100 ml sterile BPW were added into stomacher bags and homogenized in a stomacher (BagMixer 400, Interscience, France) for 1 min, and the homogenized samples were incubated at 37°C for 24 h for *Salmonella* pre-enrichment procedure. Analysis of *Salmonella* spp., in all samples were conducted according to the methods described in ISO 6579 (16). Briefly; after pre-enrichment

procedure, 0.1 ml of the sample was added to 10 ml Rappaport-Vassiliadis broth (RVS; Oxoid, Hampshire/England) and 10 ml Muller-Kauffmann tetrathionate novobiocin broth (MKTTn; Oxoid, Hampshire/England). RVS and MKTTn were incubated at 41.5°C and 37°C for 24 h, respectively. RVS and MKTTn enrichments cultures were streaked to Xylose-Lysine-Deoxycholate agar (XLD; Lab M, Lancashire/United Kingdom) and Xylose-Lysine-Tergitol agar (XLT4; Lab M, Lancashire/United Kingdom), and the plates were incubated at 37°C for 24-48 h. At the end of the incubation, five suspected colonies with black center were transferred to tubes containing Triple Sugar Iron Agar and Lysine Iron Agar (Merck, Darmstadt/Germany) and incubated at 37°C for 24 h. After incubation, presumptive positive *Salmonella* colonies were confirmed with *Salmonella* latex test (Oxoid, Hampshire/United Kingdom) and Microgen GN-ID A (Microgen, Camberley/United Kingdom).

Statistical analysis

Statistical analysis were made using SPSS version 22 (IBM SPSS, IBM Corporation, USA). The microbiological data were converted to Log_{10} CFU. One way analysis of variance (ANOVA) was used to compare samples taken from different regions (rump, flank, brisket and neck) of the carcasses. Statistical significance level was accepted as $P<0.05$.

Results and discussion

In Turkey, decontamination of carcasses with any chemicals is not allowed, but washing with water. Carcass samples were taken after carcass washing stage before chilling. In the present study, the highest APC count in the carcass regions was found in the flank by average APC number of 3.6 $\log_{10}$ CFU/cm^2 (Table 1), and significant difference was observed between the flank and neck regions ($P<0.05$). Similarly, the highest *Enterobacteriaceae* and *E. coli* numbers were found in the flank while the lowest numbers were in the rump and neck regions ($P<0.05$). Since the microbiological results were expressed as $\log_{10}$ CFU/cm^2 in the study, statistical analysis for *E. coli* numbers of the rump, brisket and neck regions was not performed (because some samples had *E. coli* number of <25 CFU/100 cm^2 and negative logarithmic values). Gürbüz et al. (17) reported that the highest level of *Enterobacteriaceae* and APC were in rump and brisket regions after washing of sheep carcasses, respectively. However, unlike our study, they had chosen the three sampling points (rump, shoulder and brisket), not including flank region. In our study, the possible reason of the high microbial contamination of the flank compared to the other regions may be due to the dirty hands and blades of the staff. Staff hands and blades were frequently touching the flank region during the evisceration process. The average APC, *Enterobacteriaceae* and *E.coli* numbers of 50 carcasses were 3.6, 0.6 and 0.1 $\log_{10}$ CFU/cm^2, respectively. When the values obtained for *E. coli* in rump, brisket and neck regions of the carcasses were converted from CFU/100 cm^2 to CFU/cm^2, negative log values were obtained. Negative log value was used to imply that there was less than 1 bacterium in per cm^2 (Table 1). In Europe, the studies conducted in Italy, Spain, Finland and Poland (18-21) reported that APC and *Enterobacteriaceae* counts on sheep carcasses were between 2.27-3.88 $\log_{10}$ and between 0.27-1.03 $\log_{10}$ CFU/cm^2, respectively. Small differences among the bacterial load of carcasses should be taken as normal. It should kept in mind that some factors such as slaughter practices, the number of animals cutting per hours, sampling time (season), sampling methods (sponge, excision or swabbing) and hygienic conditions and procedures in slaughterhouses can affect the bacterial load on carcasses.

Salmonella spp. was not detected in 50 carcass samples collected in this study (Table 1). Similar to our result, some researchers reported no *Salmonella* spp. in sheep carcasses by excision and swabbing methods (17, 20-22). On the other hand, some researchers found that *Salmonella* spp. prevalence in small animal carcasses was between 0.62% and 14.1% (23-27). It is difficult to make comparison among the studies in point of the *Salmonella* prevalence. Since, some factors such as number of animals that harbor *Salmonella* spp. on their hide and in feces due to husbandry practices, sample numbers, sampling time (season) and frequency, sampling methods (sponge, excision or swabbing) and hygienic procedures in slaughterhouses should be taken into consideration. It should be noted that only 400 cm^2 surface area was sampled for each carcass in this study, hence, actual *Salmonella* prevalence may be higher than 0%.

Table 1: The mean Aerobic Plate Count (APC), *Enterobacteriaceae, Escherichia coli* counts and *Salmonella* spp. prevalence of the small animal carcasses and carcass regions (log_{10} CFU/cm^2±SD) (n=50)

	Carcass (general)	Carcass regions			
		Rump	Flank	Brisket	Neck
APC	3.6±0.8	3.1±1.2^{AB}	3.6±0.9^{B}	3.2±0.9^{AB}	3.0±1.1^{A}
Enterobacteriaceae	0.6±0.8	0.3±0.9^{A}	0.7±0.9^{B}	0.4±0.9^{AB}	0.1±0.8^{A}
Escherichia coli	0.1±0.9	-0.4±0.8	0.04±0.9	-0.2±0.9	-0.4±0.8
Salmonella spp.[a]	0/50	0/50	0/50	0/50	0/50

^{AB}: The values with different superscript within the same row are significantly different (P<0.05)
[a]: *Salmonella* positive sample/Total samples

Table 2: The average Aerobic Plate Count (APC), *Enterobacteriaceae and Escherichia coli* on the blade, liver and some slaughterhouse tools (plastic crates (15), stainless steel tables (15) and offal carts (10)) samples

	Blade[a] (n=40)	Liver[b] (n=50)	Slaughterhouse tools[c] (n=40)
APC	6.3±1.0	6.0±0.7	5.1±0.9
Enterobacteriaceae	2.9±1.3	3.7±0.9	1.6±0.8
Escherichia coli	2.1±1.1	2.9±0.9	0.5±0.9

[a]: Log_{10} CFU/blade [b]: Log_{10} CFU/liver [c]:Log_{10} CFU/cm^2

Table 3: *Salmonella* spp. prevalence on the knife blade, liver, some slaughterhouse tools [plastic crates (15), stainless steel tables (15) and offal carts (10)] and in lymph nodes.

Samples	Positive samples/Total samples
Knife	4/40
Liver	2/50
Slaughterhouse surfaces	0/40
Portal (hepatic) lymph nodes	0/100
Prescapular lymph nodes	0/100
Subiliac lymph nodes	0/100

Knife blades and other tools (plastic crates, offal carts, working tables) used by the staff at the slaughterhouse can be one of the contamination sources of the carcass (1, 28). In the present study, the average numbers of APC, *Enterobacteriaceae* and *E. coli* on the blades were detected as 6.3, 2.9 and 2.1 log_{10} CFU/blade, respectively (Table 2). *Salmonella* spp. was detected in 4 (10%) out of 40 blades (Table 3). Bakhtiary et al. (29) reported that *Salmonella enterica* was found on knives in a sheep and beef slaughterhouse in Iran. In the samples taken from the stainless steel table, plastic crates and offal carts, the mean numbers of APC, *Enterobacteriaceae* and *E. coli* were 5.1, 1.6 and 0.5 log_{10} CFU/cm^2, respectively (Table 2). *Salmonella* spp. was not found on the surface samples taken from stainless steel table, plastic crates and offal carts. To our knowledge, there are limited studies indicating bacterial load of staff knives in slaughterhouse. Hence, some references used in this study are old. Bell (30) detected APC number of 3.61 log_{10} CFU/cm^2 on the blade used for hide skinning. In another study, APC number on the blade was detected as 5.04 log_{10} CFU/cm^2 (31). Bell and Hathaway (31) and Bell (30) noted the bacterial load of the blade as per cm^2. If it is assumed that the entire surface area of a blade is more than 10 cm^2, then it can be said that the results of the studies are similar to each other. When the results obtained from tool and blades were evaluated, it was seen that the tool and blade samples had a high amount of microorganisms,

and they could pose an important role in the cross contamination of carcass and offal. In addition, the presence of *Salmonella* spp. in 10% of the blades showed that hygienic condition of blades should be taken care. The Regulation (EC) 853/2004 reported that "they (slaughterhouses) must have facilities for disinfecting tools with hot water supplied at not less than 82°C, or an alternative system having an equivalent effect" (32). The slaughterhouse where this study was conducted has equipment for disinfecting blades with hot water at 82 °C, however, it was observed that the equipment were not used by the staff. The results show that it is extremely important that staffs are to be informed and educated about the hygiene rules and public health. Moreover, staffs who do not comply with the hygiene rules should be warned during the slaughter process.

Liver surfaces can be contaminated with microorganisms during the production stage, and it can pose a risk for the public health. Woldemariam et al. (10) examined 107 small animals (sheep and goat) liver in Ethiopia and found that 5 (4.7%) of the livers were *Salmonella* spp. positive. In the present study, the average APC, *Enterobacteriaceae* and *E. coli* numbers of the 50 small animal livers collected from slaughterhouse were 6.0, 3.7 and 2.9 $\log_{10}$ CFU/liver, respectively (Table 2). *E. coli* was detected in 90% of the livers collected (detection limit was ≥1.4 $\log_{10}$ CFU/liver). *Salmonella* spp. was detected in 2 (4%) livers surface, while it was not found in any of the hepatic lymph nodes collected from 50 livers, (Table 3). This result indicates that *Salmonella* that were detected on the surface of the livers most likely originates from the hands or knife blades of the staff or the surfaces contacting with the livers such as offal carts, plastic crates. The presence of high amount of microorganisms on the surfaces of tools and blades may have contributed to the contamination of the liver surfaces. In addition, the presence of *Salmonella* spp. in 10% of the blades suggests that blades may also play an important role in the contamination of liver surfaces with *Salmonella* spp. However, it should not be forgotten that staff hands may also play an important role in the contamination of carcasses and offal.

There are limited studies on the *Salmonella* spp. prevalence of lymph nodes in small animal carcasses. El-Tom et al. (9) detected *Salmonella* spp. in 3 out of 78 mesenteric lymph nodes collected from goat carcasses. Hanlon et al. (11) collected mesenteric (223) and subiliac (223) lymph nodes from sheep and goat carcasses during 14 months, and they found *Salmonella* spp. prevalence as 5.8% in mesenteric and 7.6% in subiliac lymph nodes, respectively. It is almost impossible to use the mesenteric lymph nodes in the production of ground meat, however, removal of subiliac and prescapular lymph nodes from carcass can be forgotten; and they may probably be used in the production of ground meat. In the present study, *Salmonella* spp. was not found in any of the subiliac and prescapular lymph nodes collected from 100 carcasses (Table 3). The reason of these differences among the results may be depend on the condition of the farms where animals are come from and health/disease situations of the animals brought to the slaughterhouse. It has been also noted that the prevalence of *Salmonella* spp. in animals shows seasonal differences (5).

Conclusions

Consequently, the presence of high amount of *Enterobacteriaceae* and *E. coli* on the surfaces of blades and livers, and the detection of *Salmonella* spp. on the surfaces of the blades and livers show that staff should pay extra attention to the "Good Hygiene Practices" and Food Safety Management Systems (such as HACCP) applied in slaughterhouses. In this context, disinfection of blades with the hot water of ≥82°C, taking care of the sanitation of the surfaces that are contact with livers, and washing liver surfaces with the clean water in slaughterhouse can significantly reduce the microbial load on these surfaces. Although this study was conducted in one slaughterhouse, the samples were collected as many number as possible, and the study was continued for 4 months to reflect the general microbiological status of small animal carcasses and the surfaces contacting with offal in a slaughterhouse. The data of this study can be helpful in the microbiological risk assessment of small animal carcass and offal, and it may show the microbiological loads of the some contamination sources of the carcasses and offal.

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OCENA MIKROBIOLOŠKE OBREMENITVE TRUPOV MALIH PREŽVEKOVALCEV, JETER IN NEKATERIH BEZGAVK TER ORODIJ IN NOŽEV V KLAVNICI

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Povzetek: Namen študije je bil določiti mikrobiološko obremenitev trupov malih živali, bezgavk na trupih, celotne površine jeter, jetrnih bezgavk in nekaterih orodij, ki prihajajo v stik s trupom ter drobovjem. Pregledanih je bilo 630 vzorcev trupel malih živali, jeter, bezgavk, jetrnih bezgavk, nožev in orodij za klavnice (mize iz nerjavečega jekla, plastični zaboji, zaboji za drobovino). Ugotavljali smo prisotnost mezofilnih aerobnih bakterij, *Enterobacteriaceae* ter število bakterij *Escheria coli* in *Salmonella spp*. Povprečna skupna količina aerobnih mezofilnih bakterij (TAMB), *Enterobacteriaceae* in *E. coli* je bila 3,6, 0,6 in 0,1 log10 CFU/cm2. Najbolj onesnaženo področje pri vzorčenju trupov je bilo na boku trupov. Povprečno število TAMB, *Enterobacteriaceae* in *E. coli* na površinah jeter je bilo 6,0, 3,7 in 2,9 log10 CFU/jetra. Povprečno število TAMB, *Enterobacteriaceae* in *E. coli* na nožih je bilo 6,3, 2,9 in 2,1 log10 log10 CFU/rezilo, povprečno število TAMB, *Enterobacteriaceae* in *E. coli* na klavniških površinah pa 5,1 in 1,6, 0,5 log10 CFU/cm2. *Salmonello* spp. smo odkrili v 4 odstotkih vzorcev jeter in na 10 odstotkih nožev. Prisotnost salmonele na površini jeter in rezil ter veliko število bakterij *E. coli* na jetrih, rezilih in orodju kažejo na to, da te bakterije lahko predstavljajo tveganje za javno zdravje. Osebje bi moralo dodatno pozornost nameniti „dobri higienski praksi "in sistemom upravljanja varne hrane (na primer HACCP), ki se uporablja v klavnicah.

Ključne besede: trup zaklanih živali; jetra; limfni vozli; mikrobiološko onesnaženje; *Enterobacteriaceae*; *Escheria coli*; *Salmonella* spp.

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Original Research Article

AMELIORATIVE EFFECTS OF GRAPE SEED OIL ON CHROMIUM-INDUCED NEPHROTOXICITY AND OXIDATIVE STRESS IN RATS

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Abstract: The current study focused on investigating the renoprotective effects of grape seed oil (GSO) against hexavalent chromium (Cr (VI))-induced nephrotoxicity. A total of 40 male rats were randomly divided into four groups: group I served as the control group, group II received 1000 mg/L potassium dichromate (353.5 mg/L Cr(VI)) in drinking water for 12 weeks, group III received 3.7 g/kg body weight/day GSO orally for 12 weeks, and group IV received GSO together with potassium dichromate for 12 weeks. Cr(VI) significantly increased serum levels of urea, creatinine, potassium and glucose. In addition, Cr(VI) increased MDA levels and induced renal tissue damage and DNA damage. On the other hand, Cr(VI) decreased serum levels of sodium and antioxidant defence system [reduced glutathione (GSH) and catalase (CAT)]. However, treatment with GSO prevented elevation levels of serum urea, creatinine, potassium and glucose. In addition, GSO enhanced sodium level, renal tissue antioxidant defense system due to its curative effect ameliorated particularly oxidative stress, renal tissue and DNA damage. In conclusion, these results demonstrate that GSO is a promising nephroprotective agent against Cr(VI)-induced nephrotoxicity.

Key words: grape seed oil; hexavalent chromium; nephrotoxicity; DNA damage

Introduction

Grapes have high phenolic and essential fatty acid contents. Most phenolics are found in the seeds. Grape seed oil (GSO) contains large amounts of phenolic compounds such as epicatechin, gallic acid, catechin, procyanidins, and resveratrol and small amounts of hydroxytyrosol and melatonin (1-2).

GSO has high unsaturated fatty acids content that accounts for more than 89% of the total oil composition, containing 75% linoleic acid, 6% palmitic acid, 15% oleic acid, 1% linolenic acid and 3% stearic acid (3). Additionally, GSO has very high levels of antioxidants including vitamin E (120 mg/100 g), and phytosterols that can have an anti-atherosclerotic effect (4). Resveratrol (trans-resveratrol; trans-3,5,40-trihydroxystilbene) is a polyphenol in the stilbene family that is found at relatively high levels in grapes (5). The antioxidant property of GSO has been proposed to underlie its renoprotective activity (6-7).

GSO exerts important effects such as reducing platelet aggregation, normalizing lesions resulting from obesity and diabetes, and preventing hypertension caused by excess sodium (8).

GSO also exerts protective effects against acute liver injury induced by CCl_4 due to its powerful antioxidant, anti-inflammatory and antiapoptotic activities (9).

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Many forms of chromium (Cr) exist in nature; hexavalent Cr (Cr(VI)) is the main form of Cr emitted as an environmental pollutant and toxin in automobile exhaust and cigarette smoke (10). Additionally, Cr(VI)is widely used in chemical industrial processes such as wood preservation, dye production, alloy manufacturing, leather tanning and electroplating (11).

Long-term environmental Cr(VI) exposure from pollution may lead serious damage to human health (12); accumulation of Cr(VI) in the human body can cause dermatitis, chronic bronchitis, cancer, asthma, DNA mutation, hypertension (13) and testicular damage (14).

Cr(VI) is a powerful oxidizing agent. Upon chronic or acute exposure through inhalation, skin contact or consumption in drinking water, Cr(VI)exhibits carcinogenicity, cytotoxicity, mutagenicity and genotoxicity in very important organs such as liver, lungs and kidneys (15). The kidney is the main route of excretion of heavy metals such as Cr(VI); these metals are deposited in renal tissue, where they cause damage to the proximal tubule (16-17) and increase reactive oxygen species (ROS) production, promoting cellular and genomic damage and ultimately resulting in free radical-induced apoptosis in renal tissue (18-19).

There is much evidence to suggest that ROS over-production due to intracellular reduction of Cr(VI) leads to a high degree of instability and the presence of reactive Cr(III), Cr(IV) and Cr(V) species (10, 18, 20).

Therefore, this study was carried out to investigate the nephroprotective effects of GSO against nephrotoxicity induced by Cr.

Materials and methods

Chemicals

Cr(VI) was purchased from El Naser Pharmaceutical Chemicals Company, Cairo.

GSO was obtained from Haraz Egypt Company, Cairo, Egypt. Diagnostic kits for assaying serum urea, creatinine, and glucose were purchased from Biodiagnostic Company. Diagnostic kits used for determination of serum levels of sodium and potassium were purchased from Sensa Core Electrolyte, India. Diagnostic kits for assaying lipid peroxidation (as malondialdehyde, MDA) (Cat. No. MD 25 29), reduced glutathione (GSH) (Cat. No. GR 25 11) and catalase (CAT) (Cat. No. CA 25 17) activity in renal tissue were purchased from Biodiagnostic Company.

Animals

Forty male albino rats (140-160 g) were obtained from the Al-Zyade Experimental Animal Production Center, Giza, Egypt, assigned to 4 experimental groups of 10 rats each. The rats were housed in polypropylene cages at the animal facility of the Faculty of Veterinary Medicine, University of Sadat City, Egypt and were maintained under conditions of 22 °C and 55% humidity with a 12 h light/12 h dark cycle. They were supplied with a balanced diet and clean water ad libitum. Before the experiment began, the animals were placed under observation for a two-week acclimatization. All procedures were approved by the Animal Care Committee of University of Sadat City (Approval number: VUSC-022-5-19).

Experimental design

Forty male albino rats were assigned into 4 groups of 10 rats each. The experiment was conducted once.

Group I (control group). The rats were supplied with a balanced diet and clean water ad libitum

Group II *(Cr(VI))-intoxicated group).* The rats were given potassium dichromate in drinking water for 12 weeks at a concentration of 1000 mg/L (353.5 mg/L Cr(VI)) (21).

Group III (GSO-treated group). The rats were administered GSO at a dose of 3.7 g/kg body weight/day orally for 12 weeks (7).

Group IV (Cr(VI)) & GSO-treated group). The rats were administered GSO at a dose of 3.7 g/kg body weight/day orally together with potassium dichromate in drinking water at a concentration of 1000 mg/L (353.5 mg/L Cr(VI) for 12 weeks.

Sampling

At the end of the experimental period (12 weeks), the animals were subjected to 12 h of fasting. Then, after the animals were anaesthetized with diethyl ether (≥99.0%; Sigma Aldrich), blood samples were withdrawn from the medial canthus of the eyes with capillary tubes. The blood samples were collected

in glass tubes without anticoagulant, allowed to clot, and centrifuged for 10 min at 3000 xg. The collected serum samples were kept at -80 °C until they were used for biochemical assays. Then, the rats were sacrificed by cervical dislocation, and the kidneys were immediately excised and washed with 0.9% NaCl. Each kidney sample was divided into 3 parts. The first part was stored at -80 °C for lipid peroxidation (MDA) measurment and antioxidant defence system (GSH, CAT) assays. The second part was kept in PBS for genotoxicity investigation and was used to examine the rate of DNA damage (by comet assay). The third part of the kidney tissue was placed in 10% neutral buffered formalin for histopathological examination using haematoxylin and eosin (H&E) staining.

Biochemical analysis

Renal tissue homogenate was prepared according to the methods of Shawky et al. (22). Specific diagnostic kits were used to determine the serum levels of urea according to the methods of Fawcett and Scott (23). Specific diagnostic kits for determination of serum levels of creatinine were purchased from Diamond Diagnostics Inc. and were used according to the methods of Bartles et al. (24). Serum glucose was assayed using a Spinreact kit according to the methods of Trinder (25). Serum levels of sodium and potassium were determined according to the methods of El-Masry et al. (26).

Lipid peroxidation (MDA) was determined using a commercial kit from Biodiagnostic Company according to the procedure described by Ohkawa et al. (27). GSH content was determined in kidney homogenate according to the procedure described by Beutler et al. (28). CAT activity was determined in renal tissue homogenate according to the procedure described by Aebi (29).

Histopathological examination

Kidney tissue samples intended for histopathological investigation were fixed in 10% neutral formalin. The samples were prepared according to the methods of Bancroft et al. (30) and stained with H&E (31).

Genotoxicity assays (single-cell gel electrophoresis or comet assays)

Slides were prepared according to the methods described by Klaude et al. (32) and Orabi et al. (33). The fluorescent stain was visualized (at 400×magnification) using an automated fluorescence microscope, and images were captured on a computer equipped with Comet Score software (Komet IV). Three parameters were adopted as indicators of DNA damage: tail length (TL; length of DNA migration), comet tail DNA percentage (tail DNA%) and tail moment (TM) (34).

Statistical analysis

Statistical analysis of the obtained results was performed using analysis of variance (ANOVA) with SPSS software (SPSS version 13.0, IBM, Chicago, IL, USA).

Post-hoc Duncan Differences with values of $p < 0.05$ were regarded as statistically significant. The results are expressed as the mean ± standard error of the mean (SEM).

Results

Biochemical tests

The serum urea, creatinine, glucose and potassium levels were significantly elevated ($p<0.05$) in the oral Cr-treated animals compared to the control animals, while the serum sodium levels were decreased. GSO administration in combination with Cr promoted significant decreases ($p<0.05$) in the serum levels of urea, creatinine, potassium and glucose compared to Cr treatment alone (Table 1).

Levels of MDA, GSH and CAT in rat renal tissue

As shown in Table 2, the levels of MDA were significantly elevated in the Cr-intoxicated group compared to the control group, whereas the activity of the antioxidant enzymes GSH and CAT was significantly decreased ($p<0.05$). GSO treatment in combination with Cr significantly ameliorated ($p<0.05$) the changes in the levels of MDA and the activity of the antioxidant enzymes

Table 1: Effects of Cr(VI) and/or GSO on the levels of serum kidney function markers, electrolytes and glucose

	Control	Cr(VI)	GSO	Cr(VI)+ GSO
Urea (mmol/L)	13.66 ± 0.66^{c}	23.42 ± 1.07^{a}	13.48 ± 0.39^{c}	15.64± 0.64^{b}
Creatinine (µmol/L)	69.84±0.88^{bc}	87.52 ± 1.77^{a}	68.07 ± 1.77^{c}	72.49 ± 0.88^{b}
Na+ (mmol/L)	145.6 ± 0.86^{a}	138.8 ± 1.05^{b}	143.7 ± 0.49^{a}	143.0 ± 0.77^{a}
K^{+} (mmoI/L)	4.85 ± 0.089^{b}	5.55 ± 0.11^{a}	4.69 ± 0.12^{b}	4.88 ± 0.065^{b}
Glucose (mmol/L)	5.86 ± 0.23^{b}	6.89 ± 0.35^{a}	5.89 ± 0.18^{b}	5.96 ± .12^{b}

The values are expressed as the mean ± SEM; number of rats =10.
Values carrying different letters in the same row are significantly different. , p<0.05.

Table 2: Effects of Cr(VI) and/or GSO on the levels of MDA, GSH and CAT in renal tissue

	Control	Cr(VI)	GSO	Cr(VI)+ GSO
MDA (nmol/g)	168.9±4.62^{b}	222.2±18.7^{a}	167.2±6.54^{b}	157.8±3.93^{b}
GSH (mmol/g)	1.67±0.089^{a}	1.35±0.037^{b}	1.73±0.096^{a}	1.66±0.106^{a}
CAT (U/g)	4.19±0.06^{a}	2.30 ± 0.25^{b}	4.07 ± 0.01^{a}	4.32 ± 0.04^{a}

The values are expressed as the mean ± SE; number of rats =10.
Values carrying different letters in the same row are significantly different, p<0.05.

Table 3: Evaluation of DNA damage in the kidney tissue of the Cr(VI) and/or GSO-treated rats (Comet assay)

	Control	Cr(VI)	GSO	Cr(VI)+ GSO
Tail length(µm)	0.52±0.1^{b}	4.68± 1.35^{a}	0.54±0.31^{b}	1.80±0.31^{b}
Tail DNA%	1.97±0.75^{b}	12.19±1.27^{a}	1.81±0.51^{b}	6.19±1.7^{b}
Tail moment	0.01±0.001^{b}	0.57±0.02^{a}	0.01±0.001^{b}	0.12±0.005^{b}

The values are expressed as the mean ± SE; number of rats =10.
Values carrying different letters in the same row are significantly different, p<0.05.

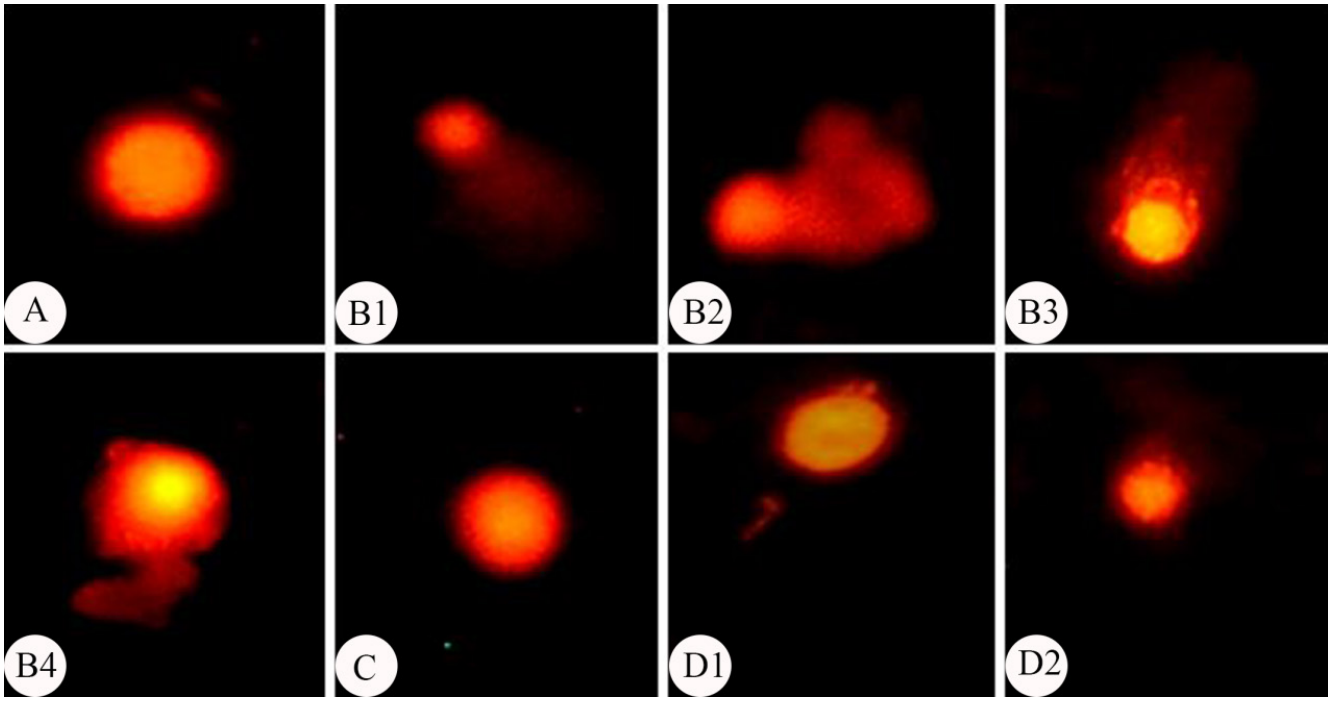

Figure 1: Comet assay for evaluation of renal tissues DNA damage: A, Control group; B (1-4), Cr VI intoxicated; C, GSO group; D, Cr VI + GSO group

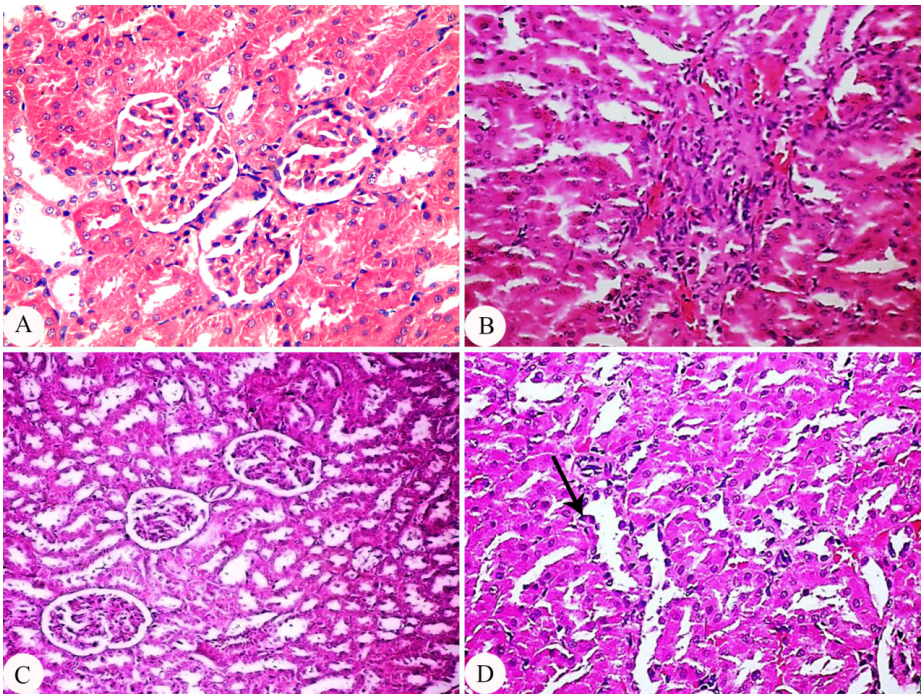

Figure 2: Photomicrographs of the kidney transverse sections stained with H&E in different groups. A&C kidney sections of control (GI) and GSO treated group (GIII) showing normal histological structure of renal corpuscles and renal tubules (H&E X 200). B: kidney sections of rat kidneys of Cr (VI) intoxication group (GII) showing focal area of fibrosis of cortical interstitial tissue. (H&E X 400). Kidney sections of GSO& Cr VI administrated group showing mild degeneration of lining epithelium with karyopyknosis of some renal tubules in cortical area. (H&E X400)

GSH and CAT caused by Cr alone.

DNA damage in rat renal tissue

A comet assay was performed to assess the protective effects of GSO against Cr-induced DNA damage in renal tissue of rats. Cr induced DNA damage in rats, as indicated by increases in TL, tail DNA% and TM in group II compared with the control group. Administration of GSO to Cr-intoxicated rats (in group IV) protected DNA from damage. On the other hand, administration of GSO alone (in group III) had no significant effect on renal tissue DNA as shown in Table 3 and Fig. 1.

Histological structure of rat renal tissue

Fig. 2 illustrates the histological changes in the renal tissue of the control and treated groups. Histopathological examination of the renal tissue of the control and GSO groups showed normal histological structures of the renal corpuscles and renal tubules (Fig.2A, 2C) while histopathological examination of the renal tissue of Cr intoxicated group showed marked necrosis of the renal tubular epithelium lining with fibrosis of interstitial tissue in the cortex and medulla (Fig. 2B). Administration of GSO to Cr-intoxicated rats (in group IV) protected the structure of renal tissues from damage and showed mild degenerative changes in the renal tubular epithelium lining (Fig.2D).

Discussion

Environmental exposure to Cr associated with stainless steel industrial processes, spray paints, drinking water, chrome plating, photography and metallurgy is well known to cause renal injury in animals and humans (15). Previous studies have suggested that Cr(VI) induces generation of ROS and thereby induces oxidative stress and apoptosis (35). Therefore, supplementation of Cr-intoxicated animals and humans with natural antioxidants may be healthful. GSO exhibits stronger antioxidant activity than vitamin E, vitamin C and β-carotene (36), enabling this substance to protect the kidney from contrast-induced nephrotoxicity (37). The present results revealed that intoxication with Cr altered kidney function, as indicated by the increased serum levels of urea, creatinine, and potassium and decreased serum levels of sodium in Cr-treated rats, which might have been due to renal tissue injury induced by Cr(VI). These findings are consistent with those of Abdel-Rahman et al. (38), who reported that administration of Cr(VI) significantly increased serum levels of urea and creatinine while simultaneously causing weight gain and pathological alterations in the kidney. The elevated levels of serum urea and creatinine

may have been due to toxic injury to the tubules induced by potassium dichromate. Additionally, Sahu et al. (39) demonstrated that a single injection of potassium dichromate resulted in significant increases in the serum levels of urea and creatinine that were linked to oxidative stress, inflammation and apoptosis accompanied by histopathological changes in renal tissues. Cotreatment with GSO significantly attenuated the elevations in serum urea and creatinine observed in Cr(VI)-administered rats. In the present study, the decreased levels of the antioxidants GSH and CAT and the increased levels of MDA in Cr(VI)-intoxicated rats indicated the presence of increased oxidative stress in the kidney. The decreased GSH levels may have been attributable to increased GSH consumption to neutralize Cr(VI)-induced free radicals or to binding of a –SH group to Cr(39). CAT is biologically necessary for the reduction of H_2O_2. In this study, the decline in the activity of CAT may have been due to intracellular accumulation of ROS, including H_2O_2 and superoxide anions, that overwhelmed the enzymatic activity (35). Cotreatment of Cr-intoxicated rats with GSO preserved the activity of CAT and GSH, indicating that GSO prevented the toxic effects of Cr(VI) through its antioxidant properties.

ROS-mediated oxidative stress is known to attack DNA and cause DNA lesions. In this study, Histopathological alterations, such as fibrosis of cortical interstitial tissue in Cr(VI)-administered rats were also considerably reduced in rats cotreated with GSO.These results were consistent with those of Song et al. (40) which recorded that exposure to Cr(VI), followed by a significant increase in tubular injury score in renal tissue .Furthermore, Mohamed et al. (41) reported that Cr (VI) induced various types of cell damage, inflammatory and vascular alterations in renal tissue

Renal tissue injury was related to renal tissue DNA damage, which was detected as increases in TL, tail DNA% and TM. These effects may have resulted from Cr(VI)-induced oxidative stress that subsequently damaged DNA. Sahu et al. (39) found that Cr induced DNA damage, renal oxidative stress, apoptosis, and inflammation in renal tissue. The renoprotective effect of GSO could be ascribed to its antioxidant effect. The antioxidant activity of the GSO is due to its high polyphenolic constituents such as resveratrol ,catechin, procyanidins, gallic acid, proanthocyanidins, and contents of vitamin E (42, 43, 44). Resveratrol has potant antioxidant, anti-carcinogenic, and anti-inflammatory activities that might be mediated by activation of silent information regulator protein 1 (SIRT1) gene expression (44) catechin, Procyanidins, and gallic acid were documented to be strong cellular preventive agents against oxidative DNA damage and apoptosis by induction of endogenous antioxidant enzymes (45), (46). Also GSO has played a key role in reducing oxidative stress and inhibiting the inflammatory responses. This could be due to the induction of antioxidant enzymes, the down-regulation of CYP2E1 and the expression of the iNOS gene, and the control of the inflammatory process through the down-regulation of NF-κB and activation of SIRT1 as well antiapototic effect mediated by down-regulation Caspase-3 gene expression, in addition to the regulation of the trace metals levels in tissues (47). In addition, grape seed proanthocyanidins extract (GSPE) can improve the nephrotoxicity and DNA damage caused by cisplatin in rats treated with grape seed extract and fish oil (48). Also, GSPE exhibits scavenging of peroxyl and superoxide radicals that can protect the renal tissue against oxidative stress that causes damage to the renal tissue, apoptosis, and fragmentation of DNA (49, 50). Furthermore, treatment of Ehrlich solid tumor (EST) induced renal injury in mice with GSPE improved renal tissue structure and reduced renal tissue DNA damage and P53, PCNA and ki67 proteins expression (51).

GSO, with its strong antioxidant properties, ameliorated the DNA damage caused by Cr(VI) intoxication

We concluded that GSO is a promising nephroprotective agent against Cr(VI)-induced nephrotoxicity

Acknowledgement

Authors declare that there is no conflict of interest.

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BLAŽILNI UČINKI OLJA GROZDNIH PEŠK PRI TOKSIČNI OBREMENITVI LEDVIC TER VPLIV NA OKSIDATIVNI STRES PODGAN, POVZROČEN S KROMOM

S. Hassan Orabi, S. Mohamed Shawky

Povzetek: Študija je bila osredotočena na proučevanje zaščitnih učinkov olja grozdnih pešk (GSO) pri toksični obremenitvi ledvic, povzročeni s heksavalentnim kromom (Cr (VI)). Štirideset samcev podgan je bilo naključno razdeljenih v štiri skupine: skupina I - kontrolna skupina, skupina II, ki je v pitni vodi 12 tednov prejemala 1000 mg/L kalijevega dikromata (353,5 mg/L Cr (VI)), skupina III, ki je peroralno 12 tednov prejemala 3,7 g/kg telesne mase/dan GSO ter skupina IV, ki je 12 tednov prejemala GSO skupaj s kalijevim dikromatom. Cr(VI) je znatno zvišal serumske ravni sečnine, kreatinina, kalija in glukoze v serumu. Poleg tega je Cr(VI) zvišal raven MDA in povzročil poškodbe ledvičnega tkiva in poškodbe DNK. Po drugi strani je Cr(VI) znižal serumsko raven natrija in antioksidativnega obrambnega sistema, zmanjšal raven glutationske peroksidaze in katalaze. Dodajanje GSO poskusnim živalim je preprečilo zvišanje ravni sečnine v serumu, kreatinina, kalija, natrija in glukoze. Poleg tega je GSO izboljšal obrambni sistem antioksidantov ledvičnega tkiva. Zaradi svojega zdravilnega učinka je izboljšal zlasti oksidativni stres, poškodbe ledvičnega tkiva in DNK. Rezultati kažejo, da je GSO obetavno zaščitno sredstvo za ledvica pri toksični obremenitvi, povzročeni s Cr(VI).

Ključne besede: olje grozdnih pešk; heksavalentni krom; nefrotoksičnost; poškodba DNK

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Original Research Article

WELFARE ASSESSMENT OF COMMERCIAL LAYERS IN SLOVENIA

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Abstract: Here we present the first welfare assessment of commercial layers conducted in Slovenia. Hens were assessed in four systems at the beginning of the laying period at 22 to 24 weeks and at 50 to 55 weeks of age. These systems were an enriched battery cage system, an aviary, and a litter system with or without outdoor access. Clinical inspections of flocks were performed, and animal-based welfare indicators were scored (e.g., keel bone damage, feather condition, foot pad lesions, beak deformities, and comb and skin wounds). Hens' fear level was scored using the novel object test and avoidance distance test. Among resource-based measures, selected micro-climate parameters were measured.

The results showed no obvious clinical signs related to infectious diseases and suggest that the selected climate conditions were satisfying in all systems. Among animal-based welfare indicators, keel bone damage was shown to be the most serious problem connected with hens' age and housing systems ($p < 0.05$). Enriched cages and aviary system were associated with significantly more keel deformities compared to the litter systems ($p < 0.05$). In addition, the least prevalence of foot pad dermatitis together with better feather condition was observed in the litter systems. In the family-owned aviary facility, hens were found to be the most motivated to approach a novel object or a human, and as such were recognized as the least fearful birds, with better human–animal interaction compared to other intensive housing systems.

Key words: laying hens; welfare; health; housing system

Introduction

The welfare of laying hens in modern intensive production units has been recognized as an important aspect of poultry management. Several factors can influence the welfare of laying hens, such as diseases, skeletal health, behavior, stress, nutrition, genetics, and management. Housing systems may play a critical role in the welfare of laying hens, and various systems have been implemented throughout the world. In the European Union, conventional battery cages were the dominant housing system for laying hens until their official ban on January 1st, 2012 (1). The ban on traditional cages was mainly adopted because of welfare concerns due to a lack of adequate space for performance of natural behaviors and an increased risk of bone deformities. According to an official report by the European Commission (2), enriched cages, aviaries, and floor housing are the most common housing systems used for commercial laying hens in the EU. In Slovenia, approximately 1.9 million hens are kept for egg production per year (3). Most hens (more than 40%) are kept in enriched cages, around 39% are kept in litter systems, including aviaries, and a smaller proportion of hens (16.3%) are in facilities under free-range conditions (2, 4).

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Enriched cages were introduced to allow birds more movement; they have extra facilities such as a nest box and at least 15 cm of perch per hen. Floor housing and aviaries are non-cage systems. These systems have the same facilities as enrichment cages (i.e., perches and nest boxes), but the group size and litter area are considerably larger. In a floor system, all hens are in a facility on one level, whereas in aviaries they have access to at least two levels and as such aviary systems allow higher stocking densities. The systems encourage birds to carry out natural behaviors such as nesting and perching, and they provide access to floor litter. The aviary and floor housing systems have the distinct disadvantage that the birds are exposed to litter and excreta, creating potential health and food safety concerns.

Finding a reliable method to assess bird welfare has been slow, and it was only in 2009 that the first protocol was published, presenting a gold standard. The Welfare Quality® assessment protocol for poultry (5) is based on the concept that welfare is multidimensional, addressing both physical and mental health (6). It focuses on animal-based measures (e.g., injuries and behavior) as well as on resource-based measures (i.e., design or management criteria), making possible welfare comparisons across farms and housing systems.

Until now no assessment of commercial layers has been conducted focusing on welfare indicators in Slovenia. This study included hens from four systems: an enriched battery cage system, an aviary, and a litter system with or without outdoor access. The selected animal-based welfare indicators were keel bone damage, feather condition, foot pad lesions, beak deformities, and comb and skin wounds. Hens' behavior was scored using the novel object and avoidance distance test. To investigate the possible development of selected welfare indicators connected with age, assessments were performed at two time points: at the beginning of the laying period at 22 to 24 weeks, and at 50 to 55 weeks of age. In addition, selected climate parameters were measured at each assessment.

Materials and methods

Animals and housing

Four commercial flocks of Lohmann Brown hens were assessed in the study. The birds were beak trimmed in the hatchery and reared in a floor system or in an aviary system. At approximately 16 weeks of age the pullets were moved to commercial egg facilities. Pullets reared in a floor housing system were housed in either the enriched cage system (ECS) or the litter system with (LOS) or without (LS) outdoor access, whereas pullets reared in the aviary system were moved to the facility with the aviary system (AS). The flocks had a similar vaccination program; they were vaccinated against Marek disease, infectious bronchitis virus, infectious bursal disease virus, Newcastle disease virus, and *Salmonella* Enteritidis. The vaccinations were performed before transfer to commercial egg facilities. The flocks had the same feed supplier during the rearing and the laying period and were managed according to the same standard practices, but the owners of the facilities and the birds' keepers were different. The ECS was populated by 37,860 hens. The facility had four double-sided rows with three tiers, and each cage housed 20 hens. The cages were furnished with perches, nest boxes, a scratching pad, and a water line (Big Dutchman). The AS (Big Dutchman) was populated with 13,800 laying hens. The hens had perches, a forage area, nest space, and a litter area accessible to the hens to perform foraging and dust-bathing behaviors. The LS was populated by 3,820 hens. The facility was equipped with a nipple water system, a feeder line, an automatic nest system, and wood shavings (Roxell). The LOS was populated by 4,420 hens. The hens had the opportunity to go outside for 4 to 6 hours a day. The equipment of the facility was the same in both litter systems.

Observations

Each flock was visited two separate times during the laying period, at 22 to 24 weeks and 50 to 55 weeks of age. The visits took place between November 2016 and October 2017 and were carried out by the same observers. Information on farm and flock management (e.g., hybrid, flock size, and age when birds were introduced to the farm) as well as cumulative flock mortality (e.g., percentage of dead or culled hens) was collected from the farm records. At each visit, the following climate parameters were checked: temperature, ammonia (NH_3), relative humidity, and air velocity. Each parameter was measured at three locations within the facility. Temperature, relative humidity, and airflow were measured with a Testo 543

instrument (Testo SE & Co. KGaA, Germany), and the level of ammonia was measured using Dräger Multiwarn II (Dräger, UK). All measurements were performed at animal level, and the average values of three measurements of each parameter were calculated. General clinical flock observation was performed by walking throughout the facility, and each flock was checked for the presence of clinical signs of respiratory disorders, diarrhea, enlarged crops, and leg problems. Clinical indicators of health problems were scored on a three-point scale (0 = none, 1 = fewer than three birds, 2 = three or more birds). Infestation with *Dermanyssus gallinae* was checked by looking for the presence of the parasite on hens and on the surface of equipment and eggs.

The following parameters were additionally scored for presence and severity on an individual hen: foot abnormalities, feather condition, comb wound, skin lesions, beak abnormalities, and keel bone damage. A description of the physical condition measurements and severity scoring system is presented in Table 1. Foot abnormalities were evaluated by examining both legs. Overall feather condition was evaluated by examination of the head, neck, back, and belly. For the assessment of skin lesions, the entire body of each hen was checked, including the region around the vent. Keel damage was determined by palpation to detect abnormal curvatures of the keel or bony callouses indicative of healed fractures. At each visit, 100 hens were examined in the AS and ECS, and 50 hens in the LOS and LS. Birds were caught individually from different locations in the facility and released after scoring.

Hens' behavior was scored using the novel object test (NOT) and the avoidance distance test (ADT) following the procedure described in the Welfare Quality® protocol. Both tests were performed after the clinical assessment of the flock to accustom the birds to human presence. The novel object (NO) used was a 45-cm stick with multicolored bands. In both litter facility systems and in the AS, the NO was placed in the litter area, and in the ECS the NO was placed in the feeder. At each visit, four locations in the facility were scored. The observer started to record the number of hens within one hen's distance of the NO (about 35 cm) every 10 s for a total period of 2 min. The average outcome of all four NOTs was calculated for each flock. The ADT was performed in 21 birds from different locations within each facility. In the AS, LS, and LOS the observer slowly approached the bird sitting on the edge of the slatted area.

Table 1: Description of physical condition measurements and severity scoring system

Condition	Score description
Foot pad lesions	0 = no lesion present
	1 = proliferation of epithelium
	2 = foot swelling dorsally visible
Feather condition	0 = completely or almost completely feathered, only single feathers damaged
	1 = damaged feathers (worn, deformed) or one or more featherless areas < 5 cm in diameter at largest extent
	2 = at least one featherless area ≥ 5 cm in diameter at largest extent
Comb wound	0 = no wounds present
	1 = < 3 fresh wounds present
	2 = > 3 fresh wounds present
Skin lesions	0 = no lesions present
	1 = < 3 lesions present
	2 = > 3 fresh wounds present
Beak abnormalities	0 = no trimming, no abnormalities
	1 = moderate to light trimming with moderate to no abnormalities
	2 = severe trimming with clear abnormalities
Keel bone damage	0 = no deformation
	1 = minor S-shape deviation
	2 = severe keel deformities

When the hen turned away, the distance from the hand of the assessor to the bird was measured. In the cage house the observer walked down the corridor and approached a hen with her head out of the cage. As soon as the hen pulled her head into the cage, the distance between the observer and the front of the cage was estimated. The mean avoiding distance was calculated for each flock.

Statistical analysis

The Statistical Package for the Social Sciences was used for the statistical analyses. Variables of individual welfare measurements (beak and foot abnormalities, comb wounds and skin lesions, feather condition, and keel deformities) were analyzed using the non-parametric Kruskal–Wallis test for comparison at each visit, followed by pair comparisons with the Wilcox Signed Rank test when significant ($p < 0.05$). The relationship between age and animal-based welfare indicators, and the association between housing system and animal-based welfare indicators were tested with Fisher's exact test ($p < 0.05$).

Results

General observation of health and welfare

The flocks examined were healthy at both visits. Signs of enteritis, eye abnormalities, respiratory disorders, leg problems, or pendulous crop were observed in fewer than three birds per flock at each visit. At the first visit no external parasites were detected, and good feather condition was noted in all the systems. At the second visit the AS hens showed greater feather loss compared to the other hens, but no signs of vent pecking were observed in any of the flocks. In the ECS birds, infestation with *Dermanyssus gallinae* was confirmed.

Cumulative flock mortality (percentage of dead or culled hens) is presented in Figure 1. At the first visit the lowest mortality was recorded in the ECS (0.21%), followed by the AS (0.56%) and both litter systems (0.9%). At the second visit the mortality ranged from 2.1% in the AS to 4.79% in the LS (Figure 1).

The selected climate conditions recorded at each visit are presented in Table 2. The average RH values within facilities ranged from 55.3 to 74.2%. The level of NH_3 exceeded the anticipated level of 20 ppm only at the first visit in the LOS and LS.

Specific observations

Beak abnormalities

Although the birds were beak trimmed at the hatchery, at the first visit four hens from the ECS had intact beaks. Significantly more abnormalities were observed in the LOS hens ($p < 0.05$) compared to the hens from other systems. At the second visit, 12% of the birds examined from the AS and ECS were scored 2, whereas all hens from the LOS and LS were scored 1 ($p < 0.05$) (Figure 2).

Comb wounds

Comb wounds were rare. At the first observation no comb wounds were observed in the ECS, AV, and LS, whereas in the LOS 18% of hens were scored 1. Significantly more wounds were confirmed in the LOS and ECS compared to the AS and LOS ($p < 0.05$). At an older age, no comb abnormalities were observed in the LOS, but they were noticed in individual birds from the other three systems. The highest score was obtained in the AS, but it did not significantly differ from the ECS and LS (Figure 3).

Skin lesions

At both visits, skin lesions were rarely detected, and no hen was scored 2. At the first visit, 1 to 4% of hens with a score of 1 were recorded in the ECS, LOS, and AS. Even better results were obtained at the second visit; mild skin lesions were detected in 2% of the AV and EC hens, but not in litter systems (data not shown). No significant differences were found between housing systems at any observation ($p > 0.05$).

Foot abnormalities

At the beginning of the laying period, no foot pad lesions were seen in the LOS and LS, whereas significantly more foot abnormalities ($p < 0.05$) were recorded in the ECS and AS; 6% of the ECS hens and 10% of the AS hens were scored 1, and one hen (2%) from the AS had severe inflammation (score 2). At the second visit, no score of 2 was recorded, although 16% of the ECS hens and 12% of the LOS hens had hyperkeratosis on one or both feet. In the AS or LS, only a few abnormalities were detected (Figure 4).

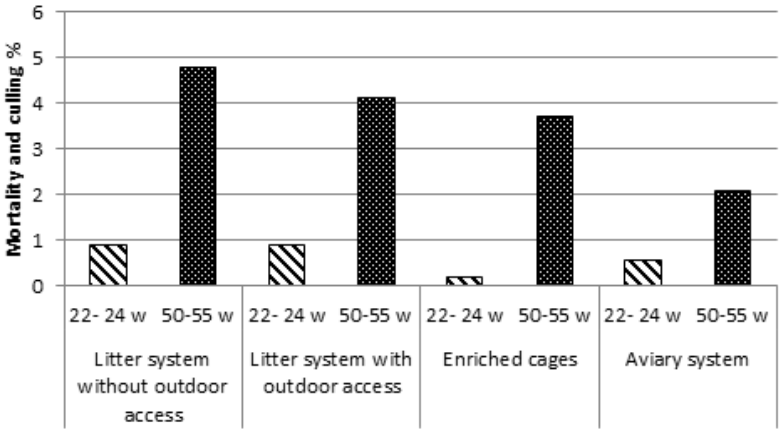

Figure 1: Cumulative mortality and culling per age and housing system

Table 2: Average values of selected climate parameters at both visits (V1, V2)

Parameter	Litter system, no outdoor access		Litter system, outdoor access		Enriched cages		Aviary system	
	V1	V2	V1	V2	V1	V2	V1	V2
Age (weeks)	22–24	50–55	22–24	50–55	22–24	50–55	22–24	50–55
Inside temp. (°C)	13.6	20.1	13.6	19.3	20.3	13.5	20.5	18.9
NH_3 (ppm)	24	4.3	24	4.3	nd	nd	4	3
Rel. humidity (%)	66.8	74.2	66.8	74.2	69.9	68.1	58.4	55.3
Airflow (m/s)	0.23	0.126	0.23	0.126	0.21	0.43	0.19	0.22

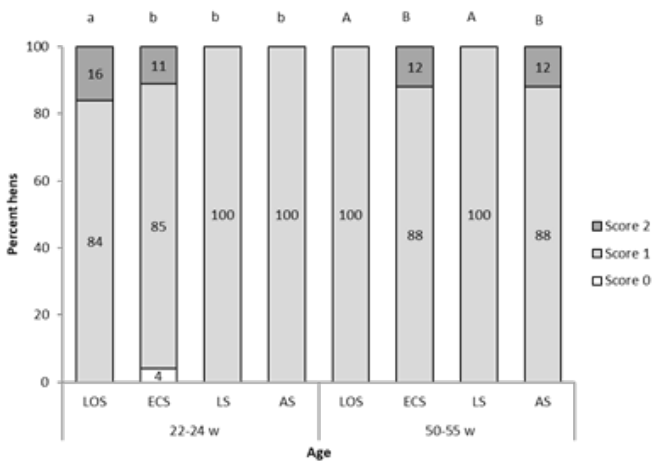

Figure 2: Percentage of hens with beak abnormalities at 22 to 24 and 50 to 55 weeks of age housed in a litter system with outdoor access (LOS), enriched cages (ECS), a litter system (LS), and an aviary system (AS). Beaks were scored 0 when no abnormalities were observed, 1 if abnormalities were mild, and 2 if severe. Bars with different letters (lower-case letters for the first assessment, upper-case letters for the second assessment) indicate significant differences ($p < 0.05$) between housing systems

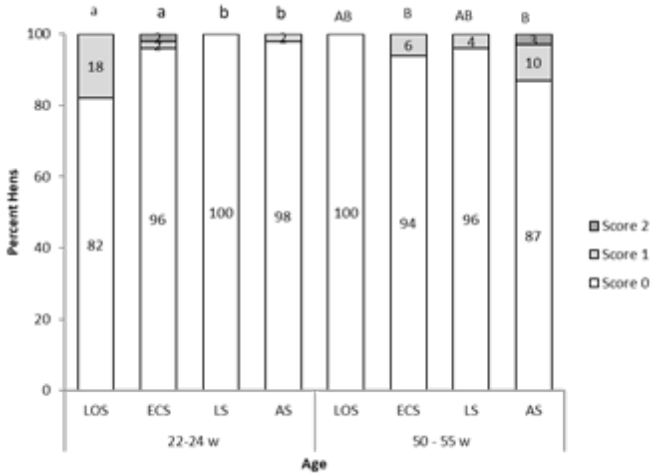

Figure 3: Percentage of hens with comb wounds at 22 to 24 and 50 to 55 weeks of age housed in a litter system with outdoor access (LOS), enriched cages (ECS), a litter system (LS), and an aviary system (AS). Combs were scored 0 when no wounds were observed, 1 if fewer than three fresh wounds were present, and 2 if three or more fresh wounds were present. Bars with different letters (lower-case letters = first assessment, upper-case letters = second assessment) indicate significant differences ($p < 0.05$) between housing systems

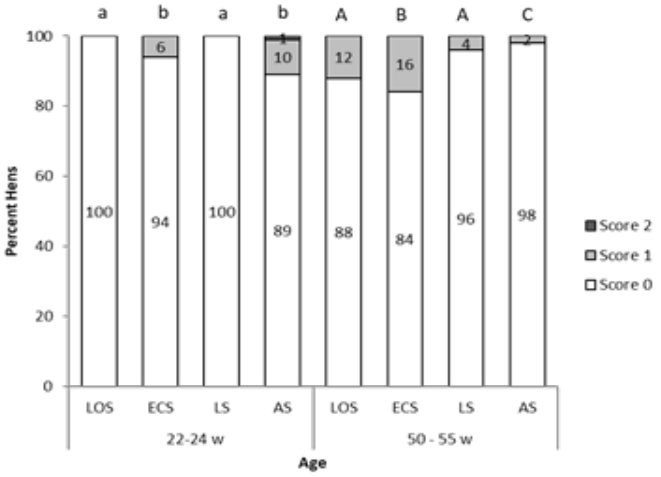

Figure 4: Percentage of hens with foot abnormalities at 22 to 24 and 50 to 55 weeks of age housed in a litter system with outdoor access (LOS), enriched cages (ECS), a litter system (LS), and an aviary system (AS). Foot lesions were scored 0 when no lesions were present, 1 if proliferation of epithelium was seen, and 2 if there was swelling on the dorsal surface of the foot. Bars with different letters (lower-case letters = first assessment, upper-case letters = second assessment) indicate significant differences ($p < 0.05$) between housing systems

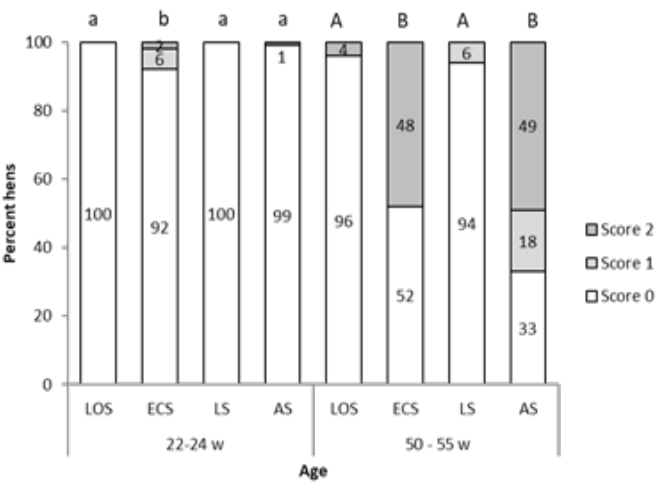

Figure 5: Percentage of hens with keel bone damage at 22 to 24 and 50 to 55 weeks of age housed in a litter system with outdoor access (LOS), enriched cages (ECS), a litter system (LS), and an aviary (AS). Keel bone damage was scored 0 when there was no deformation, 1 if minor S-shaped deviation was observed, and 2 if there were severe keel deformities. Bars with different letters (lower-case letters = first assessment, upper-case letters = second assessment) indicate significant differences ($p < 0.05$) between housing systems.

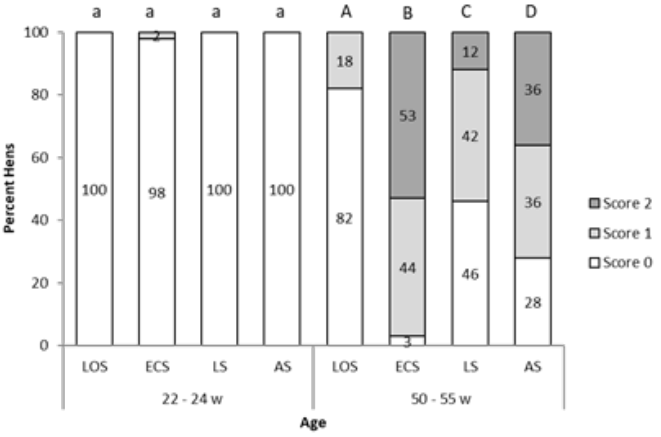

Figure 6: Percentage of hens with feather damage at 22 to 24 and 50 to 55 weeks of age housed in a litter system with outdoor access (LOS), enriched cages (ECS), a litter system (LS), and an aviary system (AS). Feather condition was scored 0 when a hen was completely or almost completely feathered, 1 if damaged feathers or one or more featherless areas < 5 cm in diameter were present, and 2 if there was at least one featherless area ≥ 5 cm in diameter present. Bars with different letters (lower-case letters = first assessment, upper-case letters = second assessment) indicate significant differences ($p < 0.05$) between housing systems.

Keel bone deformities

Keel bone damage was observed in 8% of the ECS hens at the first visit. Although one severe deformity was scored in the AS, there was no significant difference between the AS and both litter systems at this age ($p > 0.05$). At the second visit, the highest score of keel bone deviations was confirmed in the AS, followed by the ECS, but the difference was not significant ($p > 0.05$). In the litter system with or without outdoor access, significantly ($p < 0.05$) fewer hens with keel bone damage were identified (Figure 5).

Feather condition

A difference in feather condition between hens in different systems was found during the second visit ($p < 0.05$) but not the first ($p > 0.05$). The hens in the ECS had the highest score, followed by the AS and LS. The lowest score was recorded in the LOS, where 18% of hens scored 1 (Figure 6).

The similarity of proportions between housing-system and animal-based welfare indicators is presented in Table 3. The results show that except for comb wounds all other indicators differentiate between housing systems.

The relationship between animal-based welfare indicators and hens' age was tested using Fisher's exact test (Table 4).

Table 3: Occurrence of clinical welfare indicators in hens from different housing systems

Parameter	Score	Housing system				Fisher's exact test *p*-value
		LOS (*n* = 100)	ECS (*n* = 200)	LS (*n* = 100)	AS (*n* = 200)	
Feather condition	0	91	102	73	128	< 1e-07*
	1	9	46	21	36	
	2	0	53	6	36	
Keel bone damage	0	98	144	97	132	< 1e-07*
	1	0	6	3	18	
	2	2	50	0	50	
Comb wounds	0	91	190	98	185	0.2115
	1	9	8	2	12	
	2	0	2	0	3	
Skin lesions	0	98	197	100	194	0.0008*
	1	2	3	0	6	
	2	0	0	0	0	
Foot pad lesions	0	94	178	98	187	0.03492*
	1	0	22	2	12	
	2	0	0	0	1	
Beak deformities	0	0	4	0	0	0.00019*
	1	92	173	100	188	
	2	8	23	0	12	

*An asterisk indicates significant differences in the occurrence of clinical welfare indicators at different ages at the 0.05 level using Fisher's exact test

Table 4: Occurrence and scores of clinical welfare indicators at two ages

Parameter	Score	Observation period		Fisher's exact test *p*-value
		22–24 w (*n* = 300)	50–55 w (*n* = 300)	
Feather condition	0	298	95	< 2.2e-16*
	1	2	110	
	2	0	95	
Keel bone damage	0	291	180	< 2.2e-16*
	1	6	21	
	2	3	99	
Foot pad lesions	0	283	274	0.149
	1	16	26	
	2	1	0	
Skin wounds	0	283	296	0.566
	1	7	4	
	2	0	0	
Comb wounds	0	285	279	0.601
	1	13	13	
	2	2	3	
Beak deformities	0	4	0	0.1147
	1	277	276	
	2	19	24	

*An asterisk indicates significant differences in the occurrence of clinical welfare indicators among housing systems at the 0.05 level using Fisher's exact test

Table 5: Average results of the NOT and ADT obtained at different observation periods in four different housing systems

	Novel object test[1] (average hens close to the NO)		Avoidance distance test[2] (mean avoidance distance in cm and range)	
System	22–24 weeks	50–55 weeks	22–24 weeks	50–55 weeks
LS	0.68	0.75	33.10 (10–100)	30.70 (10–120)
LOS	0.68	0.74	32.99 (10–100)	28.09 (10–100)
ECS	0.21	0.39	19.20 (10–30)	20.90 (15–50)
AV	2.28	3.56	16.70 (10–50)	17.15 (10–60)

[1] Average of observations performed at four locations
[2] Average of 21 hens scored at three locations

The results showed that feather condition and keel bone damage were associated with hens' age.

Hens' behavior

Hens' behavior was scored on the NOT and the ADT, and the results are presented in Table 5. At both visits the highest average count of birds close to the NO was in the AS. In the ADT, a larger average distance indicative of increased fearfulness of humans was recorded in the LS hens, followed by the LOS and ECS hens. The overall results show that the AS hens expressed less general fearfulness as well as a better human–animal relationship compared to hens kept in the other three systems.

Discussion

This study evaluated some aspects of welfare in the four most common rearing systems for layers used in Slovenia; enrichment battery cages, aviaries, and litter systems with or without outdoor access. To investigate the possible development of selected welfare indicators with age, assessments were performed at two time points; at the beginning of the laying period at 22–24 weeks and at 50–55 weeks of age. The results suggest that the intensive housing conditions for laying hens in Slovenia are satisfactory from the health and welfare point of view. Finding a better psychological profile for birds in the aviary system on a small family farm may emphasize the importance of human contact for birds' welfare.

Records of the clinical condition of farm animals such as mortality or diseases are among the earliest welfare indicators used (7). The four flocks included in our study were in good health, and no obvious clinical signs related to infection diseases were seen. In the ECS hens, infestation with *Dermanyssus gallinae* was confirmed at the second assessment. The flock was treated with Byemite® and no obvious effect on mortality was noted. The mortality from placement to 50 to 55 weeks of age did not exceed the expected rate for each farm. The lowest cumulative mortality was recorded in the AS hens (2.1%), followed by the ECS (3.7%) and both litter systems (4.2 and 4.8%). Mortality rates in layer flocks have previously been reported between 2.9 to 15.5% in enriched cages (8), and 5 to 20% for non-cage systems (6). Weeks (9) conducted a quantitative analysis on the mortality of 3,851 commercial flocks recorded across Europe. The results showed that mortality rates in layer flocks tend to be higher in non-cage systems compared to enriched cage systems. In addition, a review by Nicol (10) noted that mortality in free-range and aviary systems is highly variable.

Air quality in the housing systems could have an important effect on health. High concentrations of ammonia can have adverse health effects and, when very high, can even influence production performance. The most profound effects seen are lesions in the respiratory tract and keratoconjunctivitis (11). According to European regulations (12, 13), occupational exposure limit values are set at 20 ppm. Slightly exceeded levels of ammonia were recorded at the first assessment in the facilities with litter systems. It is known that concentrations of ammonia could be high in floor housing systems in which manure is not regularly removed. Both observations were made in the winter, and the levels of ammonia might be due to reduction of ventilation to maintain a suitable indoor temperature. Overall, it can be concluded that the indoor climate was satisfactory in all systems.

This study showed that the condition of the keel bone and feathers in layers housed in various housing systems in Slovenian commercial farms were affected by hens' age, and that the ECS and AS were associated with significantly more keel deformities and poorer feather condition compared to the litter systems.

The keel bone is known to be a site of frequent fractures during the production life of laying hens, with incidence rates ranging from 5% to over 85%, and it is known that hens in all types of housing systems are susceptible to keel fractures (14). Several studies have also shown that the prevalence of keel bone fractures increases throughout the laying period (15–18). In our study, keel bone deformities were already detected at the beginning of the laying period in the ECS and AS hens. These early deformities could be caused by the keels being damaged during handling or transport from rearing to the production facility. Although keel bone damage was observed at the second assessment in all systems compared, the differences between systems were significant. Moderate to severe deformities were found in 67% of the AS hens and 48% of the ECS hens, whereas in the LOS and LS hens only 4 to 6% had keel bone deformities. Bone fragility in laying hens is related to high egg production, musculoskeletal health, and restricted movements (19). However, bone fractures are a risk when hens fall during flight on objects such as perches, feeders, and drinkers within the facility, which might be the main reason for the high occurrence of keel bone damage found in the AS. On the other hand, hens housed in the ECS are less active than in non-cage systems, which increases the susceptibility to weak bones and osteoporosis (17).

Previous studies comparing feather damage in different housing systems have reported diverging results; some studies showed a lower prevalence in cage system compared to non-cage system (20, 21). On the contrary, other studies showed a higher prevalence in hens housed in enrichment cages compared to hens housed in litter systems (22). The poor feather condition found in almost all the ECS hens in our study might also be linked to the red mite infestation that was present in the facility. The presence of mites in a production house induces a high level of stress from pain and skin irritation associated with repeated mite bites. In addition, mite infestations induce increased self-grooming and aggressive feather-pecking behavior (23).

Foot lesions are present in laying hens housed in all systems, although some studies have indicated that layers in enriched cages have better foot health than those in other production systems (19). Wet litter, a high level of ammonia, and poorly designed and maintained perches have been associated with foot injuries in litter systems (19, 24, 25). In cage systems, hens are kept on wire mesh, which may cause superficial epithelial lesions and hyperkeratosis (26, 27). In this study the occurrence of foot injuries was low and almost no severe inflammation (i.e., bumblefoot) was seen in any system. At both observations, hens kept in systems using wire mesh as flooring material (the AS and ECS at the first assessment and the ES at the second assessment) had more foot pad dermatitis compared to both litter systems. The prevalence of foot pad lesions has previously been found to be higher in younger hens compared to older ones (17). In our study no association between hens' age and foot lesions was confirmed.

The assessment of mental wellbeing of hens was performed by using two tests included in the Welfare Quality® protocol. The NOT measures the conflicting motivation to approach and avoid a novel object (28). The time spent near a novel object can therefore be used to quantify an animal's fearfulness. The results of our study showed that the AS hens were the most motivated to approach a NO at both observation periods. The lowest numbers were obtained in the ECS hens, possibly because of the lack of free space and because the object was not visible for all hens. In the ADT, which reflects animals' fear of humans (29), hens in both litter systems showed more fear compared to the AS hens. However, it should be mentioned that the results of both tests in the ECS might be difficult to compare with non-cage systems because there is a wire door between the observer and hens. It was shown that hens in large commercial facilities tend to be fearful of human contact (30) and that fearfulness can be reduced through appropriate familiarization (31). Three flocks (ECS, LOS, and LS) in our study were housed on large commercial farms, whereas the hens from the AS were kept on a small family farm. At both assessments these hens expressed less general fearfulness and a better human–animal relationship compared to hens from other systems. A review by Nicol noted that fearfulness might be better correlated with the quality of human contact that hens are exposed to more

than with rearing systems (10), which may also be the case in our study.

This study is the first to provide results of welfare indicators of commercial layers in Slovenia. We cannot assume that our restricted sample of flocks is representative for the commercial hen industry in Slovenia, but it offers some estimation of the problems that may exist, such as keel bone damage.

Acknowledgements

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OCENA DOBROBITI V INTENZIVNIH REJAH KOKOŠI NESNIC V SLOVENIJI

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Povzetek: Opravili smo prvo celovito oceno dobrobiti kokoši nesnic v Sloveniji. V raziskavo smo vključili nesnice iz štirih različnih sistemov reje in raven dobrobiti ocenili v dveh starostnih obdobjih; na začetku nesnosti, v starosti od 22 do 24 tednov in pri 50 do 55 tednih. Nesnice so bile rejene v obogatenih kletkah, v voljerah, v talni reji brez možnosti izpusta in v talni reji z možnostjo izpusta. Ob vsakem ocenjevanju smo jate klinično pregledali in s pregledom posameznih živali ocenili specifične indikatorje dobrega počutja (poškodbe prsnice, operjenost, poškodbe podplatnih blazinic, deformacije kljuna in poškodbe grebena ter kože). Plašnost kot indikator socialnega obnašanja smo ocenili s testom novega predmeta in s testom odmika od človeka. Spremljali smo tudi mikro-klimatske pogoje reje.

Ves čas spremljanja nismo ugotovili vidnih kliničnih znakov kužnih obolenj. Rezultati meritev mikro-klimatskih parametrov nakazujejo, da so bili pogoji v rejah dobri. Poškodba prsnice se je izmed specifičnih kazalnikov izkazala za najresnejši problem, na katerega vplivata tako starost kot sistem reje ($p < 0,05$). Poškodbe prsnice so bile značilno bolj izražene pri kokoših iz obogatenih kletk in voljer ($p < 0,05$) v primerjavi z nesnicami iz talnih rej. Kokoši iz talnih sistemov so bile tudi boljše operjene in so imele nižjo prevalenco poškodb na podplatnih blazinicah. Nesnice, ki so bile rejene v voljerah na družinski kmetiji, so kazale največ zanimanja za nove predmete in človeka. Te kokoši so bile ocenjene kot najmanj plašne in so izražale boljšo interakcijo človek–žival kot kokoši iz drugih primerjanih sistemov.

Ključne besede: kokoši nesnice; dobrobit; zdravje; sistem reje

SLOVENIAN VETERINARY RESEARCH
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