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INTUSUSSCEPTION SECONDARY TO GASTROINTESTINAL FOREIGN BODY IN AN ELEVEN MONTH OLD JUVENILE AFRICAN LION (*PANTHERA LEO*) CASE REPORT

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ABSTRACT

An eleven month old male, juvenile lion brought for rehabilitation at the Olusegun Obasanjo Presidential Library (OOPL) Wildlife Park was diagnosed with gastrointestinal foreign body and intussusception based on the history of persistent vomiting following ingestion of a cotton towel, and radiographic findings of a radiopaque gastric foreign body extending from the fundus to the pylorus and a cylindrical soft tissue mid-abdominal mass. The lion was premedicated with an intramuscular injections of ketamine (5 mg.kg^{-1}) and midazolam (0.25 mg.kg^{-1}), while anaesthesia was induced with an intravenous injection of propofol (2 mg.kg^{-1}). A celiotomy followed by gastrotomy and subsequent intestinal resection and anastomosis was performed to remove the gastrointestinal foreign bodies and correct the intussusception. The lion recovered well without any complication and was gradually introduced back into the group housing three weeks later following successful alimentation process. This report is probably the first case of intussusception in a lion that was associated with a gastrointestinal foreign body.

Key words: foreign-body; intestine; lion; intussusception; stomach

INTRODUCTION

An intussusception is an invagination of one segment of the intestine into another and is due to changes in peristalsis in the intestinal segments [12]. It results in either partial or complete intestinal obstruction with associated clinical

signs depending on the chronicity, size or location of the intussusception [9]. The majority of intussusceptions are idiopathic [16], however, causes such as: gastrointestinal foreign body, intestinal parasitism, neoplasms and enteritis have been documented [2, 9, 12, 16]. Intussusceptions have been reported in various animal species such as: dogs and domestic cats [6, 9], cattle [8], Maine coons [16], and a Red corn snake [2]. However, there is no record of an intestinal intussusception in a lion in the literature.

A gastrointestinal foreign body is not common in lions. There is only one report of a gastrointestinal foreign bodies in a lion and that was associated with the ingestion of a blanket used to provide warmth for the cubs, resulting in gastric outflow obstruction, perforation and subsequent toxaemia [15]. There has been no report of a gastrointestinal foreign body resulting in an intussusception in lions either. This report presents the diagnosis and successful management of a gastrointestinal foreign body with an intussusception in a lion.

CASE PRESENTATION

An eleven month old juvenile male lion weighing 22 kg was referred to the Veterinary Teaching Hospital, Federal University of Agriculture, Abeokuta, Ogun State, from the Olusegun Obasanjo Presidential Library (OOPL) Wildlife Park, Abeokuta, Ogun State, where the lion was undergoing rehabilitation. The Zoo keeper complained that the lion developed a sudden onset of vomiting two days after being suspected to have eaten the cotton towel kept in the house. The lion was weak and lethargic, and appeared severely dehydrated. The examination following anaesthesia with an intramuscular injections of 5 $mg.kg^{-1}$ ketamine (Ketamine®, Kepro, Holland) and 0.25 $mg.kg^{-1}$ of midazolam (Dormicum®, Claris life, India) revealed that ocular and oral mucous membranes were dry and moderately congested, while the rectal temperature was normal (37.2 °C). Also, the heart rate was rapid (165 $beats.min^{-1}$), while the femoral pulse was weak and rapid (172 $beats.min^{-1}$). A survey abdominal radiograph obtained with a mobile digital X-ray machine (Siemen, Germany) revealed a radio-opaque gastric foreign body (Fig. 1) with a cylindrical soft tissue opaque mid-abdominal mass. In addition, blood obtained from the cephalic vein for a complete blood cell count and determination of plasma concentrations of urea, creatinine and electrolytes revealed normal packed cell volume (38.0 %) with neutrophilia (neutrophils: $18.4 \times 10^3.l^{-1}$) and leukocytosis (WBC: $24.9 \times 10^3.l^{-1}$). There was mild hypernatremia (sodium: 158 $mmol.l^{-1}$), hyperchloridaemia (chloride: 125 $mmol.l^{-1}$) and metabolic acidosis (HCO_3: 35 $mmol.l^{-1}$). The values for the level of creatinine (1.7 $mg.dl^{-1}$), urea (18 $mg.dl^{-1}$) and potassium (4.0 $mmol.l^{-1}$) were normal. Based on the findings, a tentative diagnosis of a gastrointestinal foreign body was made. The lion was therefore scheduled for celiotomy and gastrotomy.

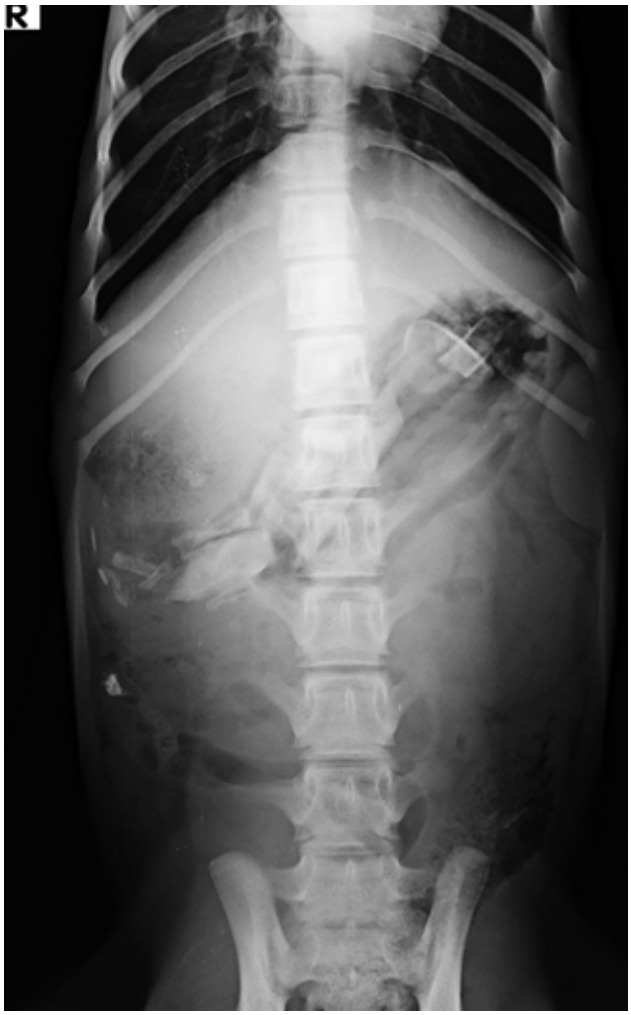

Fig. 1. Ventro-dorsal abdominal radiograph of an eleven month old lion showing the gastric foreign body

Management and Outcome

The lion was premedicated with intramuscular injections of 5 $mg.kg^{-1}$ ketamine and 0.25 $mg.kg^{-1}$ of midazolam and the ventral abdomen was prepared aseptically for surgery. Thereafter, venous access was secured using

Fig. 2. Intraoperative picture of an eleven months old lion showing removal of the ingested cotton towel (Red arrow)

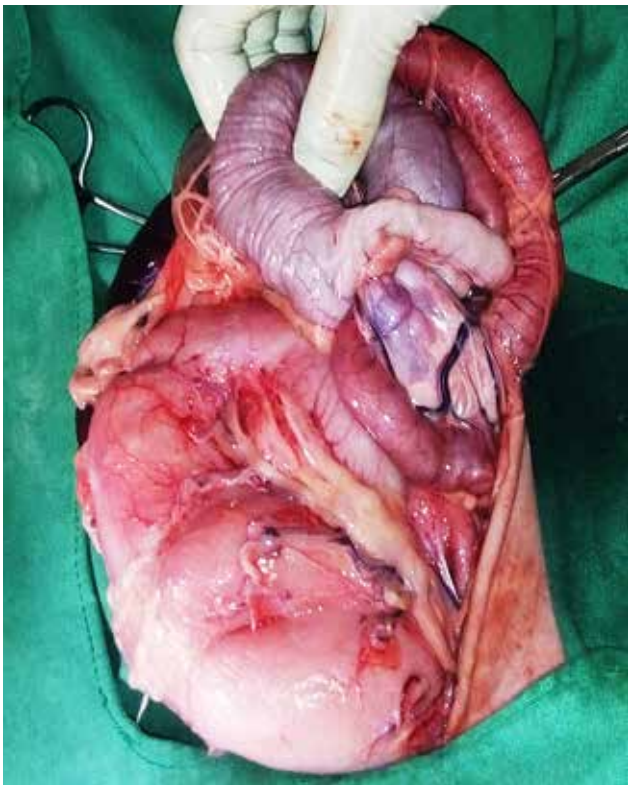

Fig. 3. Intraoperative picture of an eleven months old lion showing the intestinal intussusception

23 gauge intravenous cannula and a lactated ringers solution was administered at the rate of 5 $ml.kg^{-1}.hr^{-1}$. Anaesthesia was induced with 2 $mg.kg^{-1}$ of 1 % Propofol (Diprivan, ICI — Zeneca Pharmaceuticals) and maintained with continuous propofol infusion at the rate of 0.2 $mg.kg^{-1}.hr^{-1}$. The lion was then placed on dorsal recumbency and the limbs secured to the table. A standard celiotomy incision was made extending from the xyphoid cartilage to the prepubic tendon. The stomach was exteriorized and an incision was made at the less vascularized area to access the gastric foreign body (Fig. 2). A partially digested cotton towel measuring about 30 centimetre long was removed from the fundus of the stomach. Following the removal of the gastric foreign body, the gastric incision was closed with a double row of a Lembert suture pattern using size 2-0 Polyglactin 910 (Vicryl, Anhui Kangning Ltd, China). Thereafter, the intestine was explored and a portion of the jejuno-ileum with the intussusception (Fig. 3) was exteriorized and freed. The devitalized portion was removed, and an end to end anastomosis was done with a Lembert suture pattern using size 2-0 braided Polyglactin 910 (Vicryl, Anhui Kangning Ltd, China). Before closure of the laparotomy incision, the intestinal anastomosis was tested for leakage and obstruction by injecting saline into the anastomosed site. The laparotomy incision was then closed in three layers. The linea alba was closed with simple continuous suture pattern using size 0 braided Polyglactin 910 (Vicryl, Anhui Kangning Ltd, China). The subcutaneous layer was closed with a subcurticular pattern using size 0 Polyglactin 910 (Vicryl, Anhui Kangning Ltd, China), while the skin was closed with a horizontal mattress suture pattern using size 1 nylon monofilament (Agary Ltd, China). Following recovery, 2 $mg.kg^{-1}$ of tramadol injection (TramadolR, Gland Pharma, India) was administered intramuscularly. The lion was then returned to the transport cage until full recovery. Postoperatively, the lion was treated twice daily with 500 mg of ciprofloxacin (R. K. Laboratories, India) dissolved in drinking water for seven days. Thereafter, meat was gradually introduced to the lion until full alimentation was restored. The lion was introduced back into its housing three weeks after surgery

DISCUSSION

Although gastrointestinal foreign bodies are commonly encountered in domestic cats, they are not common in wild felidae. They may present with a variety of clinical signs depending upon the location, the degree and the duration of the obstruction resulting from them [1, 11]. This is probably the first report of an intussusception in a lion resulting from foreign body ingestion. Intestinal obstructions have been reported to result in disturbances of fluid balance, acid-base status and serum electrolyte concentrations due to hypersecretion and sequestration within the gastrointestinal tract [4]. In this report, the lion was presented with vomiting, dehydration and metabolic alkalosis resulting either from gastric outflow obstruction or the intussusception.

Chemical immobilization and anaesthesia is an integral component in conservation, diagnostic and surgical procedures in wild animal species. The risks involved in chemical immobilization and anaesthesia in lions are: loss of thermoregulation, rigidity, depressed respiration, shock, unpredictable recovery, delayed recovery and convulsions [3]. Although inhalational anaesthesia appears to be the safest technique for anaesthesia of the lion, this might not be feasible in field settings, coupled with the difficulty of endotracheal intubation when compared with domestic cats. Ketamine and propofol have been reported to be suitable for the induction of anaesthesia in lions [3]. In this report, anaesthesia was successfully induced with propofol and maintained also with propofol using a constant rate infusion technique. Many patients with gastrointestinal disorders are dehydrated. The hypotension associated with anaesthesia as well as the distension of the gut occasioned by redistribution of fluid will compound the fluid deficit. This explains why the lactated Ringers solution was administered at 5 $ml.kg^{-1}.hr^{-1}$ throughout the intra-operative period.

Majority of obstructive non-linear intestinal foreign bodies compromise the blood supply to the intestinal segment leading to intestinal wall oedema and progressive necrosis. These factors contribute to ileus and to an increase in the number of pathogenic intraluminal bacteria resulting in the breakdown of the mucosal barrier and systemic endotoxemia [5]. This may be responsible for the neutrophilic leukocytosis observed in the lion. The packed cell volume of the lion was normal probably due to the early detection of the intussusception or may be as a result of the haemo-concentration resulting from the dehydration.

The major challenges with the management of intestinal foreign bodies are early diagnosis of the condition, and risk associated with anaesthesia of patient with compromised electrolyte and acid base status [14]. Early presentation and recognition of the condition in this lion might have been responsible for the favorable outcome recorded compared to the previous record of gastrointestinal foreign body in lion cubs in which the animals were dead prior to intervention. Surgical management and wound healing are compromised by intestinal wall viability, intraluminal bacterial overgrowth, ileus and hypoproteinaemia [13]. There were no complications resulting from wound dehiscence or anaesthesia in this lion. Enteric wound breakdown and leakage are the most serious and catastrophic complications of surgery on the gastrointestinal tract [7].

A technique of single enterotomy removal of linear foreign bodies has been reported and used with good success [7, 16]. However, many chronic foreign objects may not be safely manipulated due to the severe compromise of the local gastrointestinal segment. This explains the choice of resection and anastomosis technique over enterotomy. The choice of resection and anastomosis also allows for the removal of devitalized intestinal segments occasioned by the intussusception. However, the technique is more time consuming and with higher risk of leakage. In addition, several techniques have been reported for the anastomosis of the intestinal segments following resection. These include end to end, end to side and side to side [10]. The technique of choice depends on the length of the intestine that is resected and the diameter of the two ends. An end to end anastomosis was performed using a Lembert suture pattern because the resected ends were of the same diameter

In conclusion, captive lions are prone to consume indigestible materials such as blankets out of curiosity, playfulness, or even nutritional deficiencies resulting in gastrointestinal complications; thus the use of such materials to provide warmth for young or sick lions should be discouraged. Prompt diagnosis of gastro-intestinal foreign body in lions followed by appropriate selection of anaesthetic and surgical technique with adequate intensive post-operative follow-up is essential for successful management.

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EFFECTS OF CELL SEEDING METHODS ON CHONDROGENIC DIFFERENTIATION OF RAT MESENCHYMAL STEM CELLS IN POLYHYDROXYBUTYRATE/CHITOSAN SCAFFOLDS

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ABSTRACT

The aim of our study was to examine the effects of passive and active cell seeding techniques on *in vitro* chondrogenic differentiation of mesenchymal stem cells (MSC) isolated from rat bone marrow and seeded on porous biopolymer scaffolds based on polyhydroxybutyrate/chitosan (PCH) blends. This paper is focused on the distribution of the cells on and in the scaffolds, since it influences the uniformity of the created extracellular matrix (ECM), as well as the homogenity of the distribution of chondrogenic markers *in vitro* which ultimately affects the quality of the newly created tissue after *in vivo* implantation. The three types of cell-scaffold constructs were examined by: fluorescence microscopy, SEM, histology and quantitative analysis of the glycosaminoglycans after chondrogenic cultivation. The results demonstrated that the active cells seeded via the centrifugation of the cell suspension onto the scaffold guaranteed an even distribution of cells on the bulk of the scaffold and the uniform secretion of the ECM products by the differentiated cells.

Key words: bone marrow; glycosaminoglycans; ECM; mesenchymal stem cells; polyhydroxybutyrate/chitosan scaffold

INTRODUCTION

The healing process of injured cartilage is insufficient due to the fact, that cartilage is an avascular and aneural tissue with a low number of chondrocytes which results in osteoarthritic changes and the production of inferior fibrocartilage. Several techniques designed to restore injured articular cartilage like: e. g. autologous chondrocyte implantation, microfracture, mosaikoplasty, are known in medicine [26]. At the present time, much attention is paid to the field of regenerative medicine and cartilage tissue engineering (TE), where with the appropriate cell type, suitable scaffold for cell seeding and biological factors or substances, which control the cell differentiation into desired lineages are studied. The key role of the scaffold is to support cell colonization, migration, growth, differentiation, and the development and integration of formed tis-

sue [27]. Scaffolds for *in vitro* chondrogenesis has been characterized as biomaterials based on biopolymers; e.g. collagens, polyhydroxyalkanoate, hyaluronate alginate, and polyurethanes [3, 4, 10, 14]. Chitosan is a natural aminopolysaccharide consisting of sugars close to natural glycosaminoglycans (GAGs) characteristic for cartilage tissue. Chitosan is formed by the alkaline deacetylation of chitin—the second most abundant natural polysaccharide in the world. Another benefit for the utilization of chitosan includes its antimicrobial properties [11]. M a d i h a l l y and M a t t h e w studied animal tissue tolerance to chitosan based implants with the conclusion, that this type of material causes a minimal body response and is considered as biocompatible [19]. These implants were degraded hydrolytically with lysozymes and the rate of degradation was inversely proportional to the degree of crystallinity. Y a m a n e et al. compared the *in vitro* properties of a hybrid composite consisting of hyaluronic acid (HA) coated chitosan fibers and the properties of pure chitosan [34]. Cell adhesion, proliferation and aggrecan synthesis were significantly higher in the hybrid composite with HA than in chitosan. SEM observations showed a typical chondrogenic phenotype of cells with a lot of extracellular matrix. Immunohistochemical staining has demonstrated the rich production of collagen type II by chondyrocytes.

C h o et al. demonstrated the ability to differentiate MSC into chondrocytes using an injectable gel based on chitosan-Poly-N-isopropyl acrylamide [15]. MSCs were cultured *in vitro* and after the injection of the cell-gel complex into the animal organism the cartilage tissue formation was revealed.

T a n et al. studied hydrogels consisting of N-succinyl chitosan and aldehyde hyaluronate with encapsulated bovine chondrocytes [29]. The hydrogel allowed the survival of chondrocytes and the maintenance of their typical phenotype. The authors concluded that the composite system has the potential for tissue engineering applications.

C h e n et al. manufactured three-dimensional substrates consisting of chondroitin sulphate (CS), dermatan sulfate (DS) and chitosan in various formulations with potential use in cartilage tissue engineering [13]. The addition of CS and DS positively affected the cell morphology, glycosaminoglycan and collagen production as well as expression of the corresponding genes. W a n g et al. implanted a 3D substrate consisting of poly (3-hydroxybutyrate (PHB) and 3-hydroxyhexanoate) seeded with rabbit chondrocytes into rabbits after 10 days of *in vitro* culture [33]. The treated defects in rabbits were filled with cartilage tissue with good connection with the subchondral bone. The scaffolds showed higher accumulation of ECM with Type II collagen and GAGs.

Mesenchymal stem cells (MSCs) are used as a cell source for TE and specifically in cartilage regeneration due to their relatively simple availability from multiple tissues (bone marrow, hair follicles, dental pulp, adipose tissue), high proliferation capacity in laboratory conditions and the ability to differentiate among other cell types (osteocyte, adipocyte) including chondrocytes [1, 6, 31]. Their main tasks in chondrogenic differentiation of MSC are affecting and control of the differentiation process from the point of view of enhancing the synthesis of collagen II, aggrecan and GAGs by differentiated cartilage cells. The effective biological active molecules responsible for the *in vitro* differentiation of MSC into the chondrogenic lineage are dexamethasone and transforming growth factor as supplements in chondrogenic differentiation culture media [8, 28].

For successful tissue regeneration using cartilage tissue engineering, it is recommended that the optimal pore size of scaffolds be between 100—300 μm. A critical step involves the cell seeding on to the porous scaffold [22]. The passive seeding technique is based on dropping cells onto the scaffold surface followed by the cell infiltration through the scaffold microstructure. On the other hand, the active cell seeding utilises a certain external factor, which improves the penetration of cells into the interior of the substrate (rotation, centrifugation, magnetic field) and the results have demonstrated improved cell distribution and ECM formation by differentiated cells by this method [2, 9, 30].

The aim of this study was to examine the effects of passive and active cell seeding techniques on the *in vitro* chondrogenic differentiation of mesenchymal stem cells isolated from rat bone marrow and seeded on porous biopolymer scaffolds based on polyhydroxybutyrate/chitosan (PCH) blends.

MATERIALS AND METHODS

Scaffold preparation

Porous biopolymer polyhydroxybutyrate/chitosan (PCH) scaffolds were prepared according to M e d v e c k y

et al. [21]. The PCH scaffolds with the PHB:Chit ratio equal to 1 : 1 were prepared by the precipitation of PHB (PHB, GoodFellow, dissolved in propylene carbonate) and chitosan (Chit, SigmaAldrich, dissolved in 1 % acetic acid) mixture. After stirring for 10 minutes, acetone was added to the slurry until complete precipitation of the biopolymers occurred. The resulting polymer blends were washed with distilled water, filtered, molded in molds (scaffold type A, B—discs) or microcentrifuge tubes (scaffold type C—cone) and frozen at –20 °C. Finally samples were lyophilized (Ilshin) for 6 hours and sterilized in an autoclave. The microstructure of the scaffold was modified by changing the water content in the suspension.

The microstructure of the scaffolds was observed by the scanning electron microscopy (FE SEM JEOL7000) after the deposition of the conductive carbon layer on the scaffold surface. The distribution of the molecular weights of PHB and chitosan in the mixtures was determined by gel permeation chromatography (GPC, Watrex, RI detector). Due to the high porosity of the scaffolds, they were free of closed pores and the true density of the PCH scaffolds was determined by Helium Pycnometer (AccuPyc II, Micrometics). The porosity of the scaffolds (%) was calculated from the true density of the blend, mass and dimensions of the individual scaffold.

Isolation and culture of rat MSCs

The bone marrow was isolated from the long bones (femur, tibia) of an adult male Wistar rats (300 g) cadavers (for up to 3 hours at 4 °C). The full bone marrow was flushed with ice-cold DMEM LG (Dullbecco's Modified Essential Medium; low glucose, Sigma-Aldrich, UK) + 10 % FBS (fetal bovine serum, Biowest, France) culture medium, homogenized, and centrifuged at 200 g for 10 min. The cell pellet was plated on a 75 cm^2 culture flask (SPLLife Sciences, Korea) and cultured in 15 ml of DMEM LG with 10 % FBS, and 1 % penicillin-streptomycin-amphotericine (ATB-ATM solution, Sigma-Aldrich, UK); and incubated at 37 °C in a humidified atmosphere with 5 % CO_2. Non-adherent cells were removed by changing the medium after 48 h. The cells were passaged upon reaching 90 % of confluence. The subconfluent cell layer from passage 2 were released and cells were used for the determination of the MSC multidifferentiation capacity, confirmation of MSC surface markers (CD29, CD90, CD45) by flow cytometry and *in vitro* chondrogenesis experiments. The cells were seeded in biopolymeric scaffolds by simple dropping of the cell suspension on to the surface of the scaffold and infiltrated the cells to the porous structure of the scaffold by gentle centrifugation.

Phenotypic characterization of MSC by flow cytometry

For flow cytometric analysis of the cells, direct immunofluorescence staining was used, with a combination of conjugated monoclonal antibodies: CD45/CD29/CD90.1 (eBioscience, USA). The specification of the antibodies used is presented in Table 1.

The flow cytometric analysis was performed on a six color BD FACSCantoTM flow cytometer equipped with blue (488 nm) and red (633 nm) lasers (Becton Dickinson Biosciences, USA). The data were analyzed using the BD FACS DivaTM software. The proportions of cells expressing analyzed CD markers were expressed in percentages.

Multidifferentiation capacity of MSC

The multidifferentiation ability of isolated adherent cells was confirmed by a commercially purchased kit StemPro Chondrogenesis, Adipogenesis and Osteogenesis Differentiation Kit (Gibco) according to the manufacturer instructions. The cells were cultured in the differentiation media for up to 21 days; the medium was changed three times a week. The differentiated cells were fixed with 4 % formaldehyde and stained—fat vacuoles of adipocytes with Oil Red (Sigma), calcium deposits produced by osteoblasts with Alizarin Red S (Sigma) and GAGs in micromasses of chondrocytic cells with Alcian Blue (Sigma).

Table 1. Specification of the anti-mouse monoclonal antibodies used

Type	Fluorochrome	Clone	Isotype	Concentration	Volume/10^5cells
anti-CD45	APC	OX1	IgG1, κ	0.2 $mg.ml^{-1}$	2.5 µl
anti-CD29	R-PE	HMb1-1	IgG	0.2 $mg.ml^{-1}$	5 µl
anti-CD90.1	FITC	DX5	IgG2, κ	0.5 $mg.ml^{-1}$	0.25 µl

Cell seeding into the biopolymeric scaffolds

The MSC were enzymatically released from the culture flasks, counted and the concentration of the cells in suspension was adjusted. The final scaffold characteristics (size and volume), cell concentration as well as method of the cell seeding are described in Table 2. Scaffolds were after seeding transferred separately into wells of 48 nonadherent culture plate (Greiner Bio-One) and incubated (37 °C, 5 % CO_2, 95 % humidity) for 2 h. Following the 0.5 ml of complete chondrogenic medium (DMEM HG—high glucose 4.5 $g.l^{-1}$), 1 % ITS + 3.50 $\mu g.ml^{-1}$ ascorbic acid, 40 $\mu g.ml^{-1}$ proline, 10 $ng.ml^{-1}$ TGF b1, 1 % HEPES, 1 % NEAA, 1 % ATB-ATM solution (all from Sigma) was added to each well containing the cell-scaffold construct. The medium was changed three times a week.

Morphology and topography of MSC seeded in scaffolds by fluorescence staining

Live/dead staining (fluorescein diacetate/ propidium iodide), acridine orange and DAPI(4',6-diamidino-2-phenylindole) staining were used for the visualisation of the cells focused on their morphology, density and topography on the scaffold surfaces so as to evaluate the cross-sections after 2 and 4 weeks of chondrogenic cultivation.

Fluorescein diacetate is metabolised by live cells to a fluorescent product and stains the living cells green. Propidium iodide is permeable by damaged cell membranes and stains the dead cells red (live/dead staining). DAPI stains the cell nuclei blue. Acridin orange stains the cell nuclei yellow-green to orange-red. After washing with PBS, the stained cell-scaffold constructs were observed by a fluorescence optical microscope Leica DM IL LED, blue filter.

For obtaining quantitative data, which could more precisely characterize the distribution of cells across the substrate, the the conical scaffold C was sectioned into 3 parts: surface, middle and bottom part. The thickness of each part was about 2 milimeters and the cell nuclei on captured images stained with DAPI were counted on areas of 1 mm2.

Histological staining of cell-scaffold constructs

After 4 weeks of cultivation in complete chondrogenic media, the constructs were removed, washed and fixed in 4 % paraformaldehyde. The specimens were sectioned (Leica RM 2255) in 5—10 μm slides and stained with alcian blue for 30 minutes (GAGs staining); the cell nuclei were stained with nuclear fast red. Scaffolds C failed to prepare for histological staining due to their disruption in paraffin.

Scanning electron microscopy staining of cells—scaffold constructs

After 2 and 4 weeks of cultivation of the cell-scaffold constructs in chondrogenic media, scaffolds were removed, washed with PBS and fixed in 2.5 % glutaraldehyde in PBS for 24 hours at 4 °C. After dehydratation in an ethanol gradient from 30 to 100 % and freeze drying (Illshin), the specimens were sputter-coated with carbon and observed (JEOL FM SEM JSM-7000F).

Determination of DNA and GAG-s content in cell-scaffold constructs

After 4 weeks of cultivation in chondrogenic medium, scaffolds were removed and washed with PBS and lysed in papain buffer after homogenization (Tissuerupter, Quagen) at 60 °C for 24 hours. The aliquots of supernatants were used for GAGs determination by the DMMB methodology and DNA estimation by Hoechst 33258. To determine the GAGs content, the 250 μl solution of the dimethylmethylene blue (DMMB) was added to 50 μl of the supernatant and absorbance at 450 and 525 nm was measured by UV VIS spectrophotometer (Shimadzu, UV-1800). The

Table 2. Scaffold characteristics, cell density on scaffolds and methods of cell seeding

Type	Composition	Cell seeding method	Average scaffold porosity*	Scaffold volume (μl)*	Cells/scaffold	Cells.cm-3 scaffold
A	PCH (1 : 1)	dropping	85 ± 4.1	50 ± 5	3.5×10^5	7.0×10^6
B	chitosan (100 %)	dropping	92 ± 4.8	50 ± 5	3.5×10^5	7.0×10^6
C	PCH (1 : 1)	centrifugation (1400 rpm/5 min)	94 ± 3.8	100 ± 15	3.5×10^5	3.5×10^6

* — mean ± standard deviation

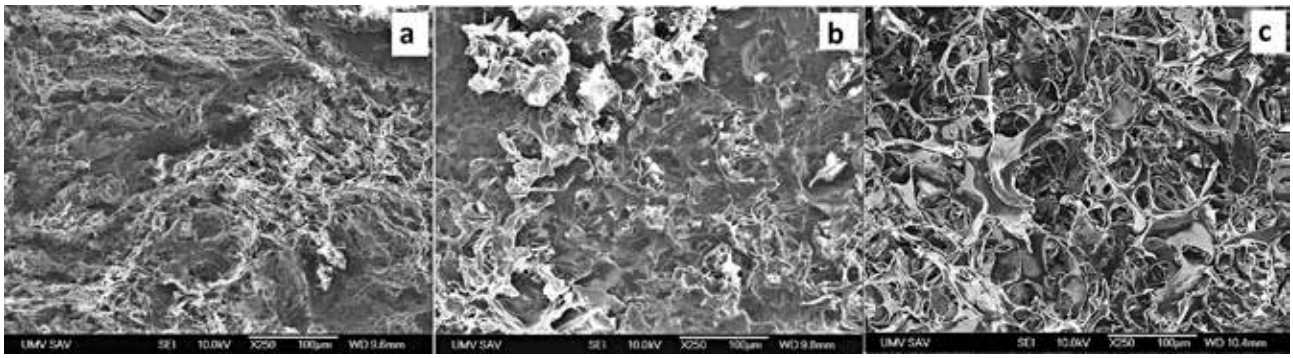

Fig. 1. SEM images of scaffold microstructures: a) A; b) B; c) C-type

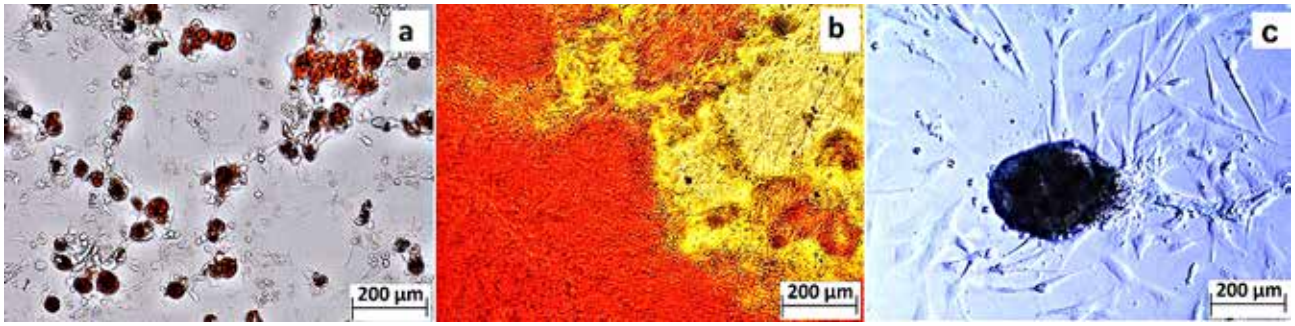

Fig. 2. Multidifferentiation ability of MSC:
b) adipogenic differentiation, oil red staining of fat vacuoles; c) osteogenic differentiation, alizarin red staining of calcium deposits;d) chondrogenic differentiation, alcian blue staining of GAGs

chondroitin sulfate from shark cartilage (Sigma) was used as a standard for GAG calibration.

The DNA content in cell lysate from cell constructs was determined using Hoechst 33258. The 20 µl of supernatant was added to Hoechst 33258 buffer solution (180 µl) and the DNA content was determined from the calibration curve by fluorimetry (Picofluor, Turner biosystems). The DNA from a calf thymus (Sigma) was used as a standard.

All of the quantitative measurements were performed on cell-scaffold constructs (n = 3) and then statistically evaluated by ANOVA (Statmost32 statistical programme). The statistical significance of results was determined by one- and two-way ANOVA ($P < 0.05$).

RESULTS

Scaffold characterization

The highly porous spongy-like microstructure of the scaffolds were obtained after lyophilization (Fig. 1). The images document a heterogeneous open microstructure with a high proportion of more regular macropores up to 100 µm size with mutual interconnection via smaller spherical pores of < 40 µm. This microstructure allows a faster diffusion of media into the interior of the scaffolds after cell seeding. Also a dense network of fine spherical micropores (diameter < 10 µm) were observable in the pore walls of the scaffolds (Fig. 1b, c). The calculated porosities of the scaffolds are listed in Table 2 and all of the scaffolds achieved the ≥ 85 % level. The gel permeation chromatography (GPC) analysis showed that the average molecular mass (Mw) of Chit and PHB in the mixtures were 41 kDa and 80 kDa respectively.

Multidifferentiation capacity and phenotype characterization of MSC

The flow cytometric analysis confirmed that > 95 % of the cells expressed CD90, CD29 and around 1.1 % of the cell population expressed CD45. The isolated cells were able to differentiate to adipocytes: red fat vacuoles in adipocytes stained with oil red (Fig. 2a); osteoblasts: red colored calcium deposits stained with alizarin red (Fig. 2b); and chondrocytes: blue stained GAGs in micromasses stained

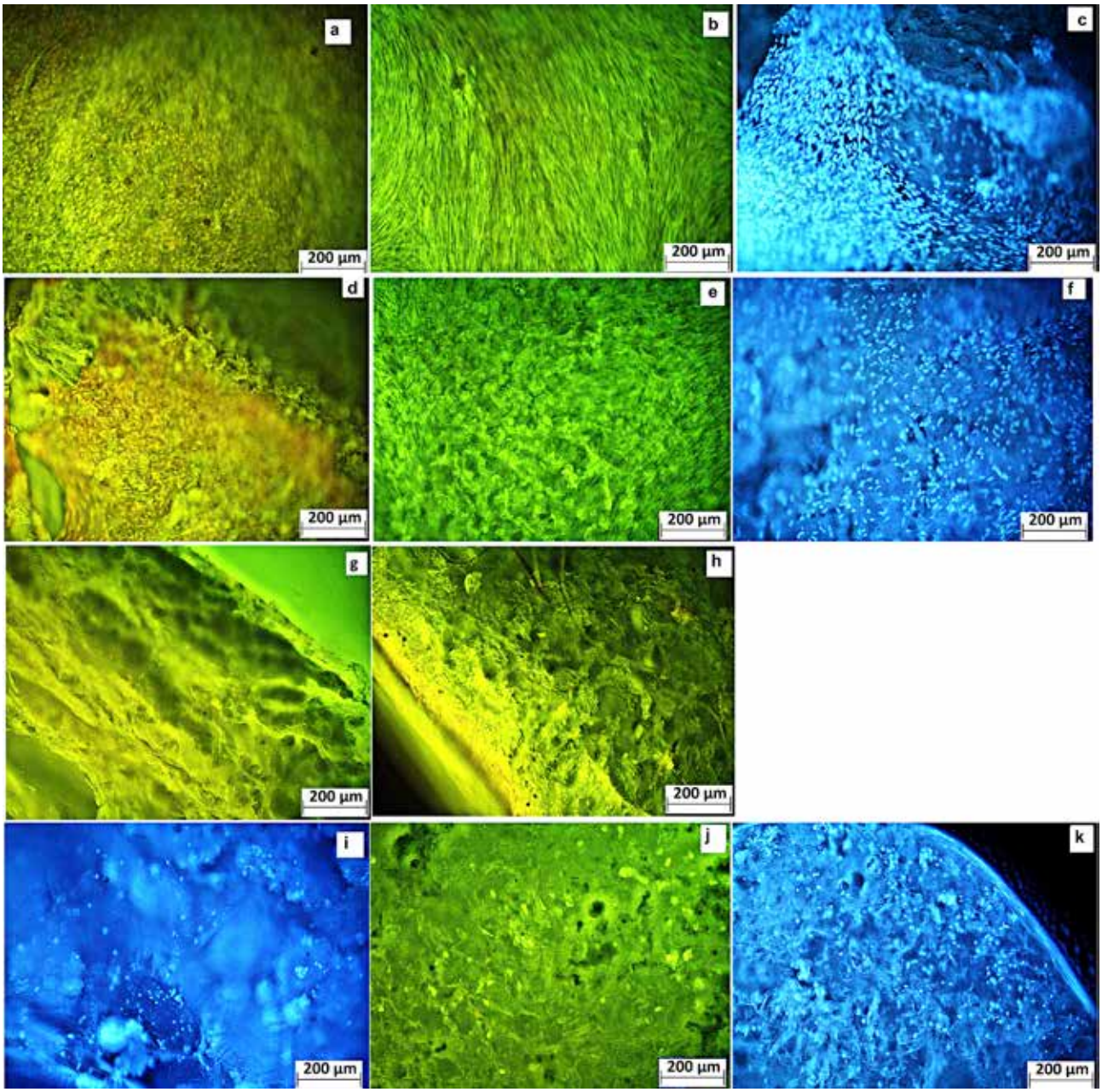

Fig. 3. Distribution of cells on scaffolds characterized by fluorescence microscopy:
A-type—surface after 2 (a-acridine orange) and 4 weeks (b-live/dead; c- DAPI) of chondrogenic cultivation; cross-sections after 4 weeks of culture (g-acridine orange); B-type—surface after 2 (d-acridine orange) and 4 weeks (e-live/dead; f- DAPI) of chondrogenic cultivation; cross-sections after 4 weeks of culture (h-live/dead); C-type- cross-sections after 2 (i-DAPI) and 4 weeks (j- live/dead; k- DAPI) of chondrogenic cultivation

with alcian blue (Fig. 2c). These facts confirmed that cells isolated from rat bone marrow were MSC's.

Morphology and topography of MSC seeded in scaffolds by fluorescence staining

Figs. 3a, b, c, d, e, and f showed cell distributions on A and B scaffolds after 2 and 4 weeks of MSC cultivation in the chondrogenic media. An enormously dense cell population, adhered to the surface was revealed on the surface of the scaffolds. Multiple cell layers with extracellular matrix production were identified using the fluorescence staining techniques. The cross-section of the scaffolds confirmed the negligible penetration of cells into the inner porous structure, as shown in Fig. 3g and h. A layer of living cells is clearly visible on the scaffold surfaces. Also the live/dead staining (Figs. 3b, e) did not show the presence of dead cells on the scaffolds, which confirms the low cytotoxicity of the biocomposites.

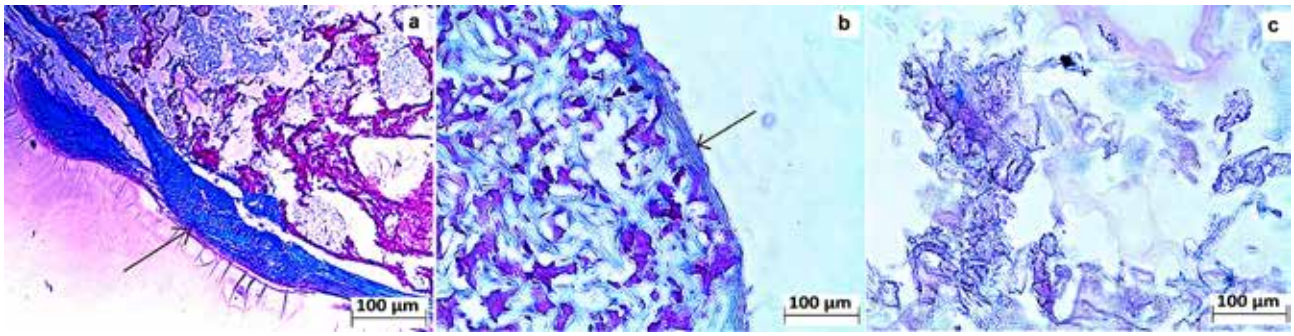

Fig. 4. Histological staining with alcian blue of scaffold A (a) and B (b) after 4 weeks of chondrogenic cultivation and scaffold A without cells (c). Arrows show layer of cells producing cartilage like tissue (positive GAG-s)

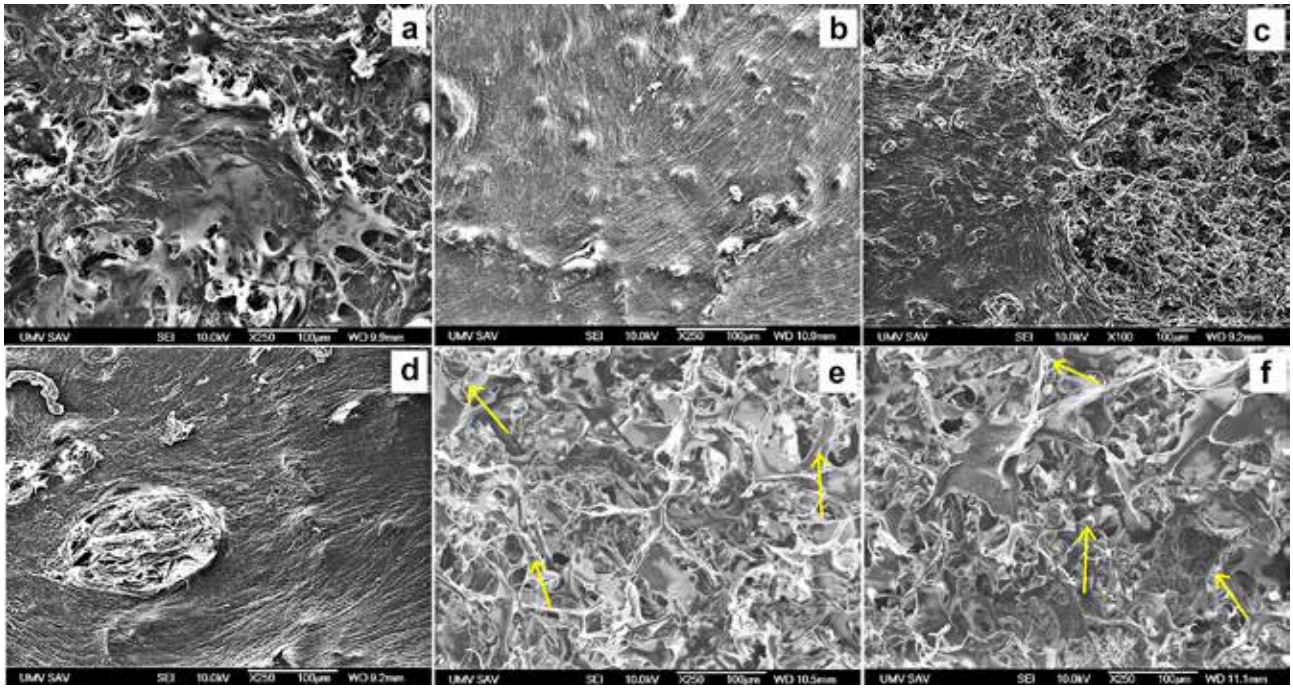

Fig. 5. Surface microstructure of scaffolds A (a, b), B (c, d) and cross-section of scaffold C (e, f). Arrows indicate adhered cells) after 2 weeks and 4 weeks of chondrogenic cultivation

In Figs. 3i, j, and k; a uniform distribution of cells is observable on the surfaces and on the cross-sections of scaffolds C, but the density of cells was lower than on the A or B samples. The acridine orange staining of scaffolds C was qualitatively unsatisfactory.

The average amount of cells on each assessed part of the scaffold C was 1470 ± 180 cells.mm^{-2}. On the surface part, there were 1320 ± 200 cells.mm^{-2}; on the middle part and bottom parts there were 1180 ± 270 cells.mm^{-2}. Based on these findings it can be stated that cells were randomly distributed and the number of cells in each part of the scaffold C was not statistically significantly different ($P < 0.05$).

Histological staining of cell-scaffold constructs

Figs. 4a and b revealed the histological staining of A and B type scaffolds by Alcian Blue, which demonstrated the presence of GAGs. The arrows indicate cell layers on the surface producing a cartilage-like tissue positively staining dark blue by Alcian blue. The scaffold cross section without seeded cells characterized by porous structure with polymer fibers created pore walls is demonstrated in Fig. 4c. Note, the partial staining of biopolymer blends (chitosan) can be visible in Fig. 4, but GAGs were stained more intensely blue in the multilayers of cells.

Table 3. Measured content of GAGs and DNA in cell-scaffold constructs

Scaffold	Seeding of MSC	GAG-s content (ng/scaffold) *	DNA content (μg/scaffold)*
A	dropping	130 ± 30	0.903 ± 0.1
B	dropping	220 ± 30	0.609 ± 0.09
C	centrifugation	75 ± 8	0.944 ± 0.1

* — mean ± standard deviation

SEM evaluation of cells—scaffold constructs

The SEM images of A and B scaffold surfaces after 2 and 4 weeks of MSC cultivation are shown in Figs. 5a, b, c, and d. The images are consistent with the observations from the fluorescence microscopy and histological staining. The scaffold surfaces are almost completely covered with cell multilayers and clearly indicate the excellent adherence of the cells and layers to the scaffold surface. These facts verify a low cytotoxicity of PCH scaffolds.

In the case of the scaffold type C, the SEM showed the microstructure with a cell population adhered and distributed on individual pore walls with partial filling of the scaffold pores. The individual cells are relatively difficult to distinguish from the highly porous, plate-like interconnected microstructure of the biopolymers (Figs. 5e and f).

Determination of DNA and GAG-s content in cell-scaffold constructs

The average GAG contents determined by DMMB after 4 weeks of culture of each cell-scaffold type construct are shown in Table 3. We observed statistically significant differences in GAG contents ($P < 0.05$) between the individual sample types A, B and C with the higher GAG content in the B substrate (220 ± 30 ng) and much lower in the C type substrate (75 ± 10 ng).

Table 3 shows also the amount of DNA in cell-scaffold constructs after chondrogenic cultivation using Hoechst 33258. Similar amounts of DNA were found in the A and C scaffolds, whereas a statistically significant decrease was revealed in scaffold B ($P < 0.05$).

DISCUSSION

The isolated MSC's from rat bone marrow represent an adherent population of spindle-shaped fibroblast-like cells, which were able to differentiate into bone, cartilage and fat cell lineages during cultivation under defined conditions. During the *in vitro* chondrogenic differentiation of MSCs seeded on scaffolds, the supplementation of the culture media with growth factors and other active biological substances is strongly recommended. Certain biologically active molecules which bind to the MSC receptors affects both the entire differentiation process and the synthesis of extracellular matrix components by the cells. In the chondrogenic differentiation of MSC, glucocorticoid—dexamethasone and growth factor TGFb1 played crucial roles [28]. The chondrogenic differentiation was mostly carried out in a culture medium without FBS (FBS is a mixture of biological agents like growth factors, hormones, etc. which can adversely affect the behavior of the cells in the differentiation process) and FBS was replaced by the supplement containing insulin, transferrin, selenite, linoleic acid, oily acid and bovine serum albumin (commercial product ITS + 3), which is used as a serum replacement in serum-free cell cultivation during the chondrogenic differentiation of the stem cells. Also, one of the most used culture media is DMEM HG. DMEM HG contains up to 4.5 g of glucose per liter of medium and enhanced glucose amount is very important in the cultivation of an enormously high number of MSCs because glucose serves as a source of readily available energy for the cells [16, 28, 32]. In our experimental work, the isolation and expansion of MSC took place in DMEM LG medium with the addition of 10 % FBS. However, MSC differentiation to chondrocytes (4 weeks) was performed in a culture medium of DMEM HG with chondrogenic supplements and successful cell differentiation was experimentally demonstrated.

The 3D-porous scaffolds must meet the requirements of biocompatibility, bioresorbability or biodegradability, good mechanical strength, shape, interconnected pores of appro-

priate size distribution to allow the cells to penetration into the scaffolds as well as allow the flow of nutrients and metabolite between the culture medium and the cells [10, 18, 24,]. The above conditions fulfill biomaterials of polymer origin, typically collagen, hyaluronic acid, and chondroitin sulphate in the case of cartilage regeneration [7, 12, 14, 25]. A number of authors have developed chitosan-based scaffolds and their composites with other polymers or inorganic compounds (e. g. calcium phosphates). As is well known, chitosan is characterized by favorable properties which predispose its use as a cell scaffold, especially in the regeneration of articular cartilage. Glucosamine groups of chitosan are structurally similar to the GAGs of the cartilage extracellular matrix and positively affect chondrocyte differentiation. In the living organism, it can be partially enzymatically degraded with lysozymes [5, 20, 23, 35]. The cost of chitosan is several times lower than collagen and hyaluronate which is a prerequisite to its wider availability for patients with traumatic cartilage damage. The porous interconnected structure of the polymer scaffolds provides a large area for the proper distribution, adhesion and cell proliferations as for neovascularization of the scaffold [17, 22]. The pore size of the polymer scaffolds macropores prepared in our work was in the range of 50—100 μm with interconnection via micropores of < 30 μm diameter. The open pore spongy-like microstructure significantly improves the nutrients inflow to the deeper parts of the scaffold. A critical step in the cartilage tissue engineering is the cell seeding on the porous scaffold and our results are comparable with results of other research groups [2, 30]. The most appropriate method ensuring the uniform distribution of cells to the inner scaffold pores was active seeding via introducing the cell suspension to the scaffold by centrifugation. In the second method based on dropping the cell suspension on to the scaffold surface, the cells didn't penetrate into the pores and extracellular matrix formation was formed only on the scaffold surface. The cell seeding with very high concentrations for the *in vitro* chondrogenesis is parallel to cell condensation during embryonic development of cartilage in a living organism. For induction of MSC into a chondrogenic lineage, tight contacts between cells are desirable and the recommended cell concentrations applied on the scaffold is between 5 and 10 million cells per cm3 of porous scaffold. The much higher cell concentrations as described above were even applied for successful chondrogenesis [2]. In the case of PCH scaffolds, the applied concentrations of MSC varied depending on the method of cell seeding. The cell concentrations used in dropping method met the requirement for cell seeding density on the porous cell scaffold contrary to this one in the centrifugation method with the lower cell density. The PCH scaffolds showed the suitable properties for chondrogenic differentiation of MSC´s with production typical of the extracellular matrix component of hyaline-like cartilage—GAGs.

For comparison in Table 3, the amount of DNA in the cell-scaffold constructs of A and C was approximately the same (0.9 μg DNA/scaffold) contrary to the B type samples where about a 30 % lower amount was found. The dense multilayers of cells were formed after culture on scaffolds A and B (Figs. 3a–f; 4a, b; 5a—d) and such a cell arrangement does not allow for further cell proliferation on scaffolds because it is not of sufficient size for another adhesion and spreading of cells. Simply said, the confluence was achieved. On the other hand, a 3D macroporous microstructure with large areas of pore walls in the scaffold C firstly makes it possible to obtain more homogeneous and uniform distribution of cells in the scaffold volume (Figs. 3i, j, and k; and 5e, and f), but the density of the adhered cells in the scaffold was not high enough for sufficient mutual cell interconnections during the short-time period after seeding which resulted in lower production of GAGs by the cells on these samples. In the case of scaffolds type A and B, the amount of produced GAGs was around 3 times higher than in the case of substrate C. We concluded, that the abundant production of GAGs by cells in the A and B scaffolds was related to the fact, that the cells formed a multilayer on the scaffold surface and the contact between the cells with each other was very tight (parallel to the culture of the chondrogenic pellets or micromasses formed by the very concentrated population of MSC), and this fact supported the differentiation of the cells in multilayers with good GAGs production. It is possible to assume that the prolonged cultivation of MSC's can enhance the cell population with significant effects on the production of cartilage markers. Based on recent papers, it can be assumed that the integrity of the constructs with active cells evenly distributed therein will be more satisfactory compared to scaffolds with active cells adhered to outer scaffold surface only and following overgrown with a differentiated cell layer on its surface and partially ingrowth tissue after implantation. However, it is necessary to consider the cur-

rent health condition of the patient with injured cartilage, the cartilage lesion position relative to the anatomy of the entire joint, lesion size, its thickness, and the surgery technique used.

In our future research work, we will focus on increasing the initial cell seeding concentration and improving the active cell seeding techniques into porous scaffolds of polymeric origin.

CONCLUSIONS

Two different methods of seeding cells into biopolymer scaffolds were compared. The synthesized biopolymer scaffolds were not cytotoxic; the cells grew and differentiated on scaffold surfaces and in scaffold pores. The cells seeded by dropping on scaffolds A and B produced higher amounts of GAGs in comparison to cells seeded by centrifugation into scaffold C. For chondrogenic cultivation and GAGs production by differentiated cells seeded in suitable porous scaffolds, there is a key prerequisite that a high enough cell seeding concentration and selecting the right cell seeding techniques be utilized. The developed porous PCH scaffold could be utilized in cartilage tissue engineering in veterinary and human medicine due to its satisfactory properties and low cost of input materials for scaffold synthesis.

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PREVALENCE OF PORCINE ROTAVIRUS ANTIGEN AND ASSOCIATED RISK FACTORS IN PIG-RAISING COMMUNITIES AND INSTITUTIONAL PIGGERIES IN ZARIA, KADUNA STATE, NIGERIA

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ABSTRACT

Porcine rotaviruses are potential reservoirs for genetic exchange with human rotaviruses. A cross-sectional study was carried out to determine the prevalence of porcine Rotavirus antigen and associated risk factors in pig-raising communities and institutional piggeries in Zaria, Kaduna State, Nigeria. A total of 376 faecal samples from pigs of all ages were collected from backyard and institutional piggeries by convenience sampling. The faecal samples collected were analysed using commercially available ELISA kit: BioK 343/2, for the antigenic diagnosis of rotavirus in porcine faeces. The overall prevalence of rotavirus antigen in pigs was 9.8 % (37/376). Piglets (10.4 %) had a higher prevalence than adults (9.1 %), while males (10.1 %) were more infected than females (9.6 %). Breed-specific prevalences revealed 5.9 %, 12 % and 15.5 % for local, exotic and cross-breeds, respectively. There was a significant association between breed ($P < 0.05$) (Odds Ratio OR = 2.927; 95 % Confidence Interval CI on OR = 1.288—6.653) and rotavirus infection. Management system revealed 14 % and 8.2 % prevalence for intensive and semi-intensive systems, respectively. There is evidence of Rotavirus infection (9.8 %) in pigs in Zaria, and the breed is a risk factor. This study provides the first data on the prevalence of rotavirus and risk factors of rotavirus infection among pigs in Zaria and environs, Kaduna state, Nigeria. There is a need to enlighten the public on the zoonotic implication and economic impacts of rotavirus infections.

Key words: backyard piggeries; ELISA; institutional piggeries; rotavirus

INTRODUCTION

Rotaviruses are important causal agents of diarrhoea in animals, belonging to the family *Reoviridae* with an eleven-segment double-stranded RNA genome [13, 21]. Presently, based on the VP6 structural protein of the genus *Rotavirus*, ten species designated *Rotavirus* A–J have been antigenically identified [4, 14, 17, 21]. Rotaviruses are generally species-specific, but cross-species transmission is possible. Several case studies have indicated the infection of humans by animal rotaviruses [20, 27]. Porcine rotavirus is one of the three major causative agents of viral diarrhoea in swine herds worldwide [6], the others being porcine epidemic diarrhoea virus (PEDV) and transmissible gastroenteritis virus (TGEV). Porcine rotavirus infects neonates between the first and the second weeks of life, affecting the productivity of the herd as reflected in the economic losses associated with growth retardation, the cost of veterinary treatment and, in some cases, the death of the animal [26]. These factors make it necessary to develop a diagnostic system that permits the specific detection of porcine rotavirus strains in the affected populations [12]. Rotaviruses can also infect and cause gastroenteritis in a broad range of animal species resulting in significant economic losses in livestock animals, including young hogs and cattle [13, 20].

Rotavirus A causes acute enteritis in young piglets and a fairly large proportion of neonatal diarrhoea [18, 19]. They have been shown to infect mammals (for example apes), cattle, pigs, sheep, rats, cats, dogs, mice, horses, rabbits and birds (chickens and turkeys). These are potential reservoirs for genetic exchange with human rotaviruses [10]. There is evidence for interspecies transmission between humans and other animals as well as between various animal species [15, 20]. There are two major routes to cross the host barriers: direct interspecies transmission and transmission coupled with reassortment [19]. Management failure in pig herds might be pivotal in the development of the disease, as well as infection by bacteria, protozoa and other viruses [11, 19, 35].

Rotavirus infection is endemic in pig herds worldwide [28]. Antigen detection in diarrhoeic pigs have demonstrated rotavirus infection in two-thirds of herds; and seroprevalence studies in pigs have demonstrated that almost all animals in a herd could be exposed [7, 28]. It therefore becomes important, to screen for the presence of rotavirus in asymptomatic infections [29]. Rotavirus infection in animals can cause great economic loss to the farmer due to the cost of treatment associated with increased morbidity and mortality rates. There is a paucity of information on the prevalence of rotavirus in pigs in Zaria, where very few pigs are mainly raised in traditionally restricted pens. These animals live in close proximity to humans, a situation which may result in significant zoonotic implications with rotavirus infections.

The aim of this study was to investigate the prevalence of rotavirus antigen in the faeces and the risk factors associated with rotavirus infection in pigs in Zaria.

MATERIALS AND METHODS

Study area

This study was carried out in Zaria and environ, comprising of Zaria, Sabon Gari and Giwa Local Government Areas (LGAs) in Kaduna State in North-Western Nigeria. Zaria is about 2,800 feet above sea level. It lies on latitude 110 North and 70 42" East [23]. It is characterized by a tropical climate with two main seasons; a rainy season (May to October) and a dry/harmattan season (November to April). The monthly mean temperature records show a range from 13.8 to 36.7 °C and a mean annual rainfall of 1092.8 mm [1]. It has an estimated human population of 547,000 and a growth rate of 3.5 % per annum [23]. Hausa and Fulani are the main ethnic groups and their major occupation is agriculture. Approximately 40—70 % of the population derive their livelihood from agriculture [1, 24]. Animals reared include: cattle, sheep, goats, poultry and pigs.

Study design and sample collection

A cross-sectional study design was used for the study and convenience sampling method was carried out during sample collection due to the availability of the pigs and consent of the farmers. A sample size of 376 was determined for the study using the formula described by Thrusfield [31]. The study subjects were selected regardless of age, sex or breed. For each study subjects, data was generated on the age, sex, breed and other possible risk factors associated with the subject. Fresh faecal samples (n = 376) were collected directly from the rectum of well-restrained pigs. The samples collected were transported to the Viral Zoonoses Laboratory in the Department of Veterinary Public Health

and Preventive Medicine, Ahmadu Bello University Zaria, Nigeria, in separate plastic containers placed on ice-packs and stored at 4 °C until analysed. The samples were analysed for the presence of rotavirus antigen using enzyme-linked immunosorbent assay (ELISA) test kits: ELISA kit, BioK 343/2 (Bio-X Diagnostics, Belgium).

Questionnaire survey

A total of three hundred and seventy-six (376) structured questionnaires were administered to the farm owners to obtain information on: age, sex, breed, management system, the presence of other animals on the farm, and other biosecurity measures carried out on the farm. For the purpose of this study, some variables are defined as follows: Intensive; animals that are confined at all times and not allowed to scavenge, Semi-intensive; animals that are allowed to scavenge but come back to their pens to be fed. Adult — pig ≥ 4 months old; piglet — < 4 months old pig.

Data analysis

Data obtained from the study as well as the questionnaire survey were subjected to statistical tests using GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla California, USA) and Microsoft Excel. The frequency of occurrence of rotavirus in pigs was determined. Chi-square, Fisher's exact test and odds ratios were appropriately calculated where applicable and values of $P < 0.05$ were considered statistically significant.

RESULTS

This study recorded a 9.8 % prevalence of rotavirus antigen in swine faecal samples obtained from pigs of all ages and from both backyard and institutional piggeries in Zaria and environs. The occurrence was higher in male (10.1 %) pigs than female (9.6 %) pigs (Table 1). It was also slightly higher in piglets (10.4 %) than adult (9.1 %) (Table 1). The breed prevalence was highest in the crossbred pigs (15.5 %), followed by the exotic (12.0 %) and lowest in the local pigs (5.9 %) (Table 1). There was a statistically significant relationship ($P > 0.05$) between the prevalence of rotavirus antigen and breed (Table 1).

The prevalence of rotavirus infection and risk factors

Table 1. Prevalence of porcine rotavirus antigen in relation to age, sex, breed and management system of pigs sampled in Zaria, Kaduna State, Nigeria

Variables	Number sampled	Number positive [%]	Odds Ratio [OR]	95 % CI on OR	P-Value
Age					
Pigs < 4 months	201	21 (10.2)	1.16	0.58—2.30	0.672
Pigs ≥ 4 months	175	16 (9.1)			
Sex					
Male	168	17 (10.1)	1.06	0.54—2.09	0.871
Female	208	20 (9.6)			
Breed					
Local	187	11 (5.9)	2.93	1.28—6.65	
Exotic	92	11 (12.0)	0.99	0.41—2.41	0.027*
Cross	97	15 (15.5)	Ref.		
Management system					
Intensive	107	15 (14.0)	1.83	0.91—3.682	0.086
Semi-Intensive	269	22 (8.2)			
Total	**376**	**37 (9.8)**			

OR — Odds Ratio; CI — Confidence Interval on OR; Ref — Reference Point, * — Significant

as the management was not significant (P > 0.05). The intensive system of management had a higher prevalence (14.0 %) than the semi-intensive system (8.2 %) of management (Table 1). There was a high prevalence seen in pigs from institutional farms (12.0 %) compared to those in the backyard farms (9.2 %) (Table 1).

The pigs fed on commercial feed revealed a higher prevalence of *Rotavirus* infection (11.8 %) than pigs that were fed home-made feed and also scavenged (9.2 %). The source of water also yielded a prevalence of 9.2 % and 11.8 % for borehole and dam respectively (Table 2). There was a slightly higher prevalence rate of 13.8 % in farms with other animal species within the pig premises compared to farms with animals far from the pig premises (9.5 %) (Table 2).

DISCUSSION

The overall prevalence of 9.8 % of rotavirus antigen in the faecal samples of pigs examined in this study, although seemingly low is of public health significance because of the risk of transmission of infection particularly to handlers and pig breeders owing to the fact that rotavirus infected pigs may serve as source of infection to humans [32]. Numerous case reports have indicated human infection incriminating animal strains of rotavirus [20, 33]. In this study, rotavirus antigen was isolated in apparently healthy pigs. This supports findings of [3, 9, 22, 29], who reported high viral shedding of rotavirus by asymptomatic pigs. This could be as a result of rotavirus being endemic in pig herds worldwide [28]. The presence of rotavirus antigen in asymptomatic pigs in this study is worthy of note as they could be a significant source of new emerging genotypes.

Some studies have recorded higher prevalences than obtained in this study, such as the findings by A n h et al. [3] in Vietnam, M i d g l e y et al [22] and S t e y e r et al. [29] both in Slovenia who recorded prevalence rates of 24.9 %, 18 %, and 20 %, respectively in mostly asymptomatic pigs. Lower prevalence rates were documented by W e i l e r et al. [34], in Germany and P a r r a et al. [25] in Argentina, with findings of 4 % and 3 %, respectively.

The variations in the prevalence of rotavirus infection could be attributed to the study duration, management system, number and age of pigs sampled and season amongst other factors. There was no significant association (P > 0.05) between the detection of rotavirus antigen in the faeces and the sex of the pigs sampled. This is an indication that both sexes might have fairly equal chances of being infected with the virus. The prevalence rate of rotavirus infection was higher in the pigs < 4 months (10.4 %) than in the pigs ≥ 4 months (9.1 %) (P > 0.05). The < 4 months

Table 2. Association between different risk factors and the prevalence of porcine rotavirus antigen in pigs sampled in Zaria, Kaduna State, Nigeria

Risk factors	Number sampled	Number positive [%]	Odds Ratio (OR)	95 % CI on OR	P-Value
Source of feed					
Home-made/Scavenging	284	26 (9.2)	0.74	0.351—1.568	0.433
Commercial	92	11 (11.9)			
Source of water					
Borehole	284	26 (9.2)	0.74	0.351—1.568	0.433
Dam	92	11(11.9)			
Other animals					
Present	29	4 (13.8)	1.52	0.499—4.64	0.51*
Absent	347	33 (9.5)			
Total	**376**	**37 (9.8)**			

OR — Odds Ratio; CI — Confidence Interval on Odds ratio; Ref — Reference Point, * — Significant

here include both the suckling and weanling piglets. High morbidity and mortality from rotavirus infection have been reported in nursing and weanling pigs [5, 30]. A m i m o et al. [2] also reported a prevalence of 78.7 % in asymptomatic nursing piglets.

The high prevalence of rotavirus antigen detected among the cross breeds and exotic breeds of pigs might be linked to the fact that most of these pigs kept and raised mainly under intensive management system, compared to the local breeds that were kept mostly under the semi-intensive or extensive system. Hence faecal-oral transmission of the virus is enhanced as these animals are in close contact with each other over a long period of time and thus infection of one animal can lead to infection of several animals through contamination of food, water, beddings and even contact with faeces of other infected pigs, and infected aerosols [8]. In addition, most of the intensively managed pig farms in the study area had no strict biosecurity measures in place to prevent cross infection between different pens. This is the likely reason for the high prevalence rate (14.0 %) found in the intensively kept animals in relation to the semi-intensive system. The observation showed that pigs on farms that sourced their water from a dam had an increased rotavirus infection. This agrees with findings of other authors where rotaviruses have been found in surface water samples at high concentrations, indicating that exposed water is likely to contribute to rotavirus transmission cycles on farms [3, 16].

The slightly higher prevalence rate of 13.8 % in pig farms with other animal species within the pig premises compared to farms with animals far from the pig premises (9.5 %), gives an indication that there is a possibility of interspecies transmission between these animals, as livestock animals kept on the farms may serve as sources of infection to the pigs and vice-versa. This trend of interspecies transmission of the virus has been reported previously [15, 20]. The high prevalence seen in pigs from institutional farms (12.0 %) compared to those in the backyard farms (9.2 %) might be due to compromised biosecurity in these farms. There is recirculation and spread of infections through contaminated boots from one farm into the other by the workers. Also, the improper cleaning and disinfection of pens and equipment of the farms lacking adequate biosecurity may result in high prevalence as seen here. The use of one feeder for so many animals can lead to the spread of infection when one of the animals gets infected.

The Giwa Local Government Area had the highest rate of infection in comparison to other Local Government Areas. Location of these animals had a statistically significant association with rotavirus infection. Pig farms in this area are raised under the intensive management system. A break in the biosecurity system can lead to transmission of the virus in the farm. The infection of one animal can lead to the transmission of the virus to other animals via contaminated materials in the pen.

CONCLUSIONS

This study demonstrates that rotavirus antigen is prevalent amongst pigs in the study area. There was an association between the prevalence of rotavirus antigen with breed and location. This study provides for the first time, data on the prevalence and risk factors of rotavirus infection in pigs in pig-raising communities and institutional farms in Zaria and environs, Kaduna state, Nigeria. The occurrence of rotavirus in pigs could lead to economic loss and may pose a zoonotic threat. Improved biosecurity measures and public enlightenment of the pig farmers on personal hygiene to prevent cross species transmission of rotavirus will aid in its prevention and control.

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PRE-INCUBATION AND ITS EFFECT ON THE DEVELOPMENT AND MALFORMATIONS OF THE CHICK EMBRYO

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ABSTRACT

This study was conducted to evaluate the effect of eggs stored with and without pre-incubation on chick embryos with emphasis on: embryo body, heart weight, malformations, and mortality. For this study, a total of 120 chick embryos were divided into three groups, based on the length of storage before hatching (3, 7 and 10 days). Observations of the weight of chick embryo bodies, chick embryo hearts, and the level of mortality and appearance of malformations were noted. With an increase in days stored, the chick embryo's weight decreased. The pre-incubation period had a positive effect on the weight of chick embryo, and chick hearts. Malformations, including: hydrocephalus, open body cavity and underdeveloped wings, were observed in all three groups, with the highest proportion seen in the pre-incubated hatching eggs stored for 10 days; this group also displayed the highest level of mortality. Non-pre-incubated eggs showed the most promise with better results in all experimental groups. In conclusion, the research suggests the optimal storage for chick embryos to be 3 days, with lowest levels of mortality, malformations and limited effects on the body and heart weight.

Key words: chick embryo; malformation; pre-incubation; storage

INTRODUCTION

Recent preventative medicine research has focused attention on studying the impact of external environmental factors on the growth of the individual, with potential teratogens and their effects on organism growth being largely unstudied.

Research into understanding their influence on physiological function and abnormal growth of an organism can lead to the identification and clarification of certain pathological conditions.

The animal model plays an important role in basic medical research with animal testing done in combination with *in vitro* methods in accordance with ethical, economic and scientific parameters. Alternative methods in animal

testing include: mathematical models, lower organism use and mammals in early stages of development; these reduce unnecessary stress and suffering to the model [13]. Chick embryos have been used as an alternative animal model for many types of experiments. It is also thanks to the fact that with chicken embryos the maternal metabolism does not have any impact. Therefore, we can better understand some of the processes underlying the chick embryo [12]. Chick embryos have also been used for the interpretation of morphogenesis in higher vertebrates as they require less space and storage requirements [4]. Hatching is a biological process of a fertilised egg, where the embryo develops into an individual. To ensure this process, the correct climate conditions including temperature, humidity and egg rotation are paramount. The success of this process is also impacted by: the biological value of the egg (correlated to storage time), the hatching technique used along with age of hen, egg weight and environmental conditions during the incubation. The time of the storage is mainly important for the biological value of the eggs. The highest hatching rate is observed in eggs stored within a 7 day period; as storage periods longer than 14 days there is a significant decrease in hatchability. Pre-incubating (preheating) the eggs can minimalize the negative effect of long storage, by providing uniform development for the eggs in storage. Preheating is helpful for a better uniformity of the development of the chicken embryo in the storage eggs, which will be incubated later together [9, 18]. Other factors that affect increased hatchability and chick quality are: genetic, and the environment through the storage and incubation (volume and gas exchange) [11]. Post-hatching, chick quality can be influenced by transportation induced stress [1]. With correct storage and pre-incubation, the impact of external factors on chick embryo development can be decreased. Therefore, we decided to evaluate the effect of the eggs storage with and without pre-incubation on chick embryos with emphasise on the embryo body, heart weight, malformations, and mortality.

MATERIALS AND METHODS

For this study, 120 eggs of Lohmann Brown chicken breed were obtained from the farm Parovske Haje (Nitra, Slovakia). These eggs were divided into groups according to their storage duration (3, 7 and 10 days at 15 °C and 75 % relative humidity). Approval was not needed to use 9-day-old chick embryos, as they are not included in the legislation on the protection of animals used for scientific purposes (2010/63/EU).

Each group contained 20 hatching eggs; half pre-incubated for 12 hours at room temperature (22—23 °C). All eggs were stored in the incubator under standard conditions for 9 days (37.5 ± 0.5 °C, 60 % humidity). On the 9th day, a hole was cut from the broad end of the egg and the embryo removed using a retractor. Foetal membranes were removed from the chick embryo using tweezers. The treatment was repeated twice.

Macroscopic changes were focused upon: malformations (open body cavity, beak deformations, upstretched wing/limb, two-headed embryos etc.) and haemorrhages. Subsequently, the bodies of embryos were weighted as well as the hearts of embryos were removed and weighted. Dead embryos were discarded without their use.

Statistical analysis of the data was performed using the programme GraphPad Prism 6.0. A two-pass ANOVA test and multiple comparison tests (Sidak test) were used for analysis. Values of $P < 0.05$ were deemed statistically significant.

RESULTS

This study concentrated on the effects of pre-incubation in relation to the incidence of embryonic mortality and malformations as well as its effect on embryonic weight. The results showed that storing hatching eggs although beneficial, repeatedly resulted in problems associated with premature embryonic mortality and variations in morphology and weight.

Embryonic mortality

Embryonic mortality was observed in all storage groups on days 3, 7 and 10. In the group stored for 3 days without pre-incubation, 10 % embryonic mortality was observed. However, in the case of pre-incubation of hatching eggs, mortality was not observed. In the group stored for 7 days without pre-incubation, 0 % embryonic mortality was observed; with pre-incubation, 10 % mortality was seen. The highest mortality occurred in eggs with 10 days of storage. In the group stored for 10 days without pre-incubation almost 32 % mortality was seen; with pre-incubation, 35 %

Table 1. Storage of fertilized eggs and effect of pre-incubation

Storage	No[2]	Live	Dead	Mortality [%]	Malformations	Weight [g]	SD[3]
3-day eggs	20	18	2	10.00	0	1.710	0.259
3-day eggs P[1]	20	20	0	0.00	1	1.835	0.298
7-day eggs	20	20	0	0.00	2	1.620	0.227
7-day egg P	20	18	2	10.00	1	1.731	0.235
10- day egg	19	13	6	31.00	3	1.534	0.226
10-day egg P	20	13	7	35.00	4	1.539	0.195
Total	**119**	**102**	**17**		**11**		

[1] — pre-incubation; [2] — number of chicken embryos; [3] — standard deviation

Table 2. Effect of pre-incubation on the development of the chick embryo

	Mortality [%]	Number of malformations	Body Weight [g]	Heart weight [g]
Eggs	13.00	3	1.632	18.770
Eggs with pre-incubation	14.00	2	1.744	18.890

Table 3. Effect of pre-incubation on heart weight of the chick embryo

Storage	Weight of heart [mg]	SD[2] [mg]
3-day eggs	18.0	3.9
3-day eggs pre-incubation	21.0	6.4
7- day eggs	18.6	5.2
7-day eggs pre-incubation	18.9	4.9
10-day eggs	18.1	3.9
10-day eggs pre-incubation	15.0[1]	3.5[1]

[1] — significant difference *P ≤ 0.05; [2] — standard deviation

mortality was recorded. Total embryonic mortality after 3 and 7 days of storage was 5 % and after 10 days of storage, the embryonic mortality was 33 %. For pre-incubation, total embryonic mortality in eggs without pre-incubation was 14 % and 15 % with pre-incubation. These results show embryonic mortality was not significantly influenced by pre-incubation. Embryonic mortality on the total number of hatching eggs represented about 14 % on average (Fig. 1, Tables 1 and 2).

Spectrum of malformations

Malformations were observed in all groups. Three-day storage without pre-incubation had no malformations present; however, with pre-incubation one case of single-

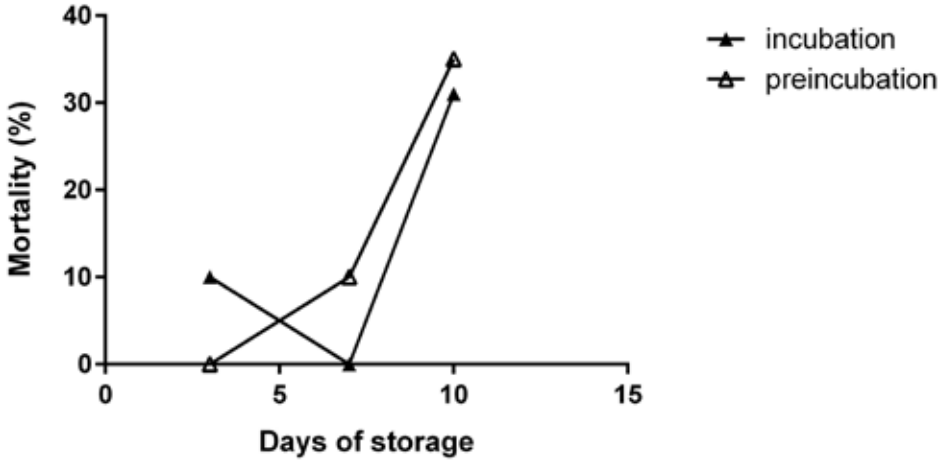

Fig. 1. Storage of fertilized eggs and its effect on the mortality

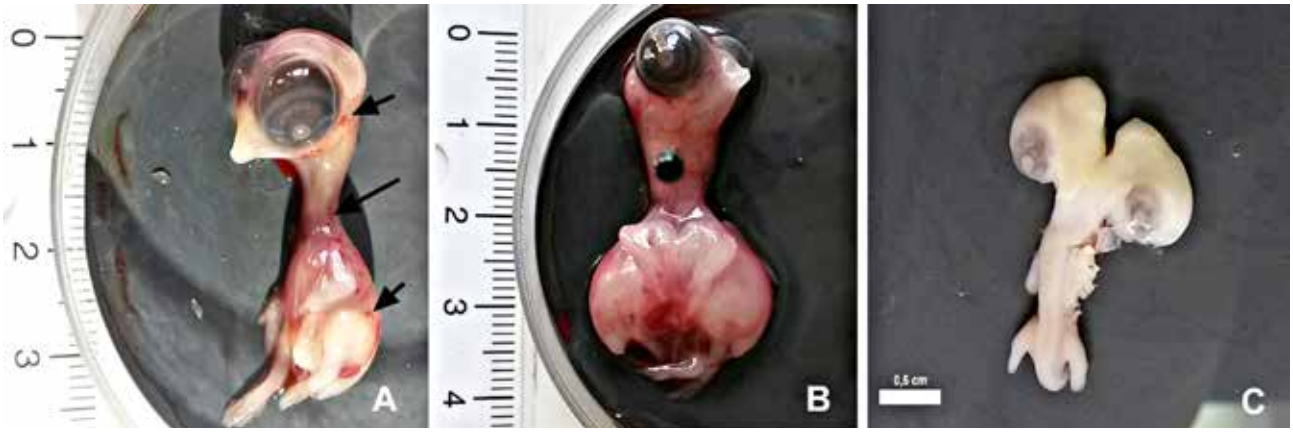

Fig. 2. Malformations observed on the embryonic day 9 in relation to the storage day of fertilized eggs
A) *Haemorrhage* (arrows) are present on the body after 3 days of storage; B) Double body of embryo after 3 days of storage and using pre-incubation; C) Double-headed embryo after 7 days of storage and using pre-incubation

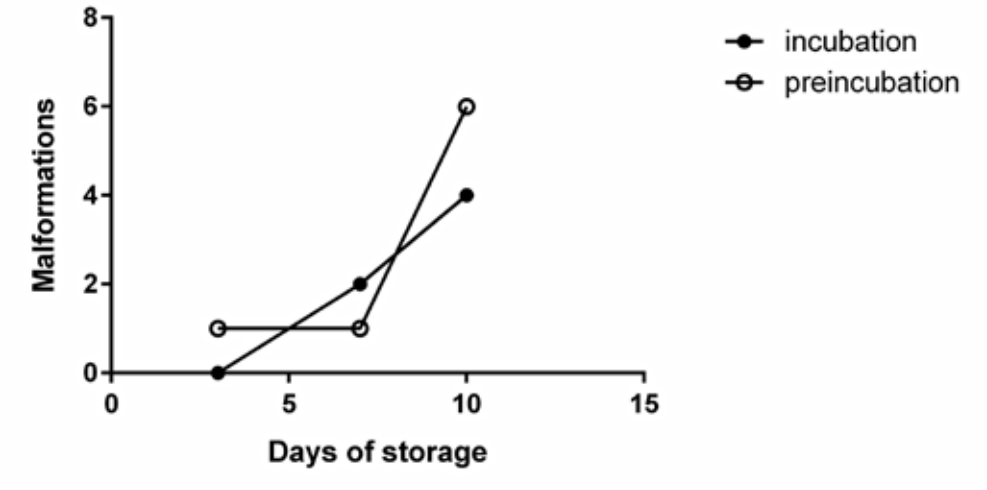

Fig. 3. Storage of fertilized eggs and its effect on the occurrence of malformations

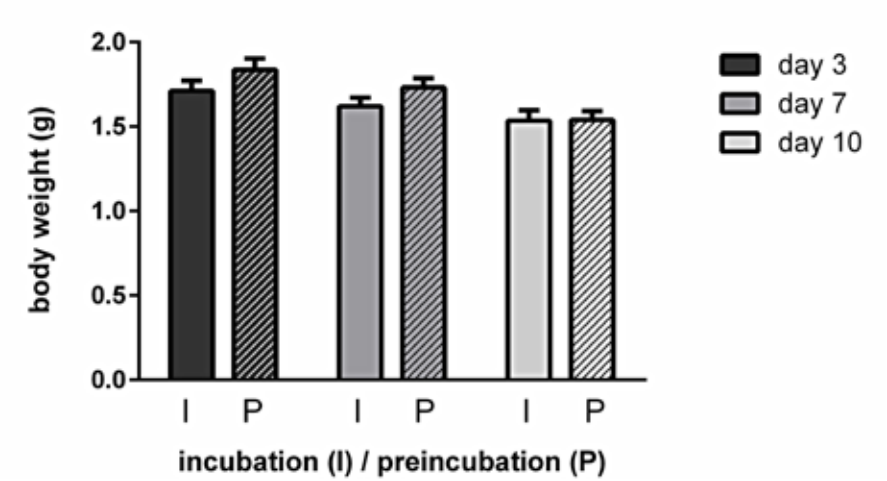

Fig. 4. Storage and pre-incubation of fertilized eggs and the body weight of the chick embryo

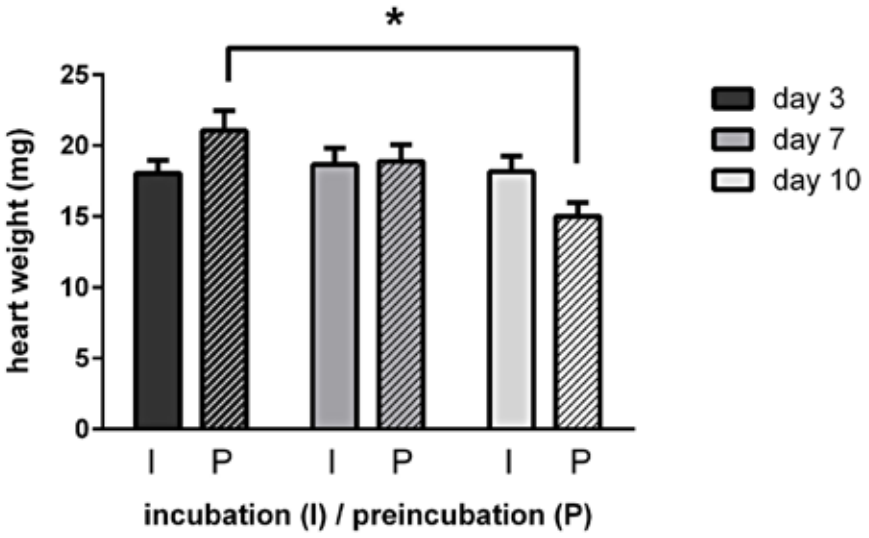

Fig. 5. Storage and pre-incubation of fertilized eggs affect the heart weight of the chick embryo

headed twins sharing organs and cardiomegaly was discovered. Twins weight reached up to 2.58 g and the heart weighed 0.037 g (Table 1).

Seven-day storage without pre-incubation had 2 malformed embryos, one with an open body cavity the other with an undeveloped wing; with pre-incubation one underdeveloped malformed embryo with two heads was found (Table 2).

The highest numbers of malformations were observed in the group of hatching eggs with a 10-day storage. Without pre-incubation, three embryos with malformations including cyanosis, open body cavity, deviations of limb and head were revealed. Hatching eggs with pre-incubation before the incubation contain 4 malformed embryos with malformations (lacked the wings and tail, open body cavity, double-headed embryo, haemorrhagically coloured body, undeveloped beak, and curled limbs (Figs. 2 and 3).

In all experimental groups of hatching eggs, various amounts of haemorrhaging was observed—mainly in the head and pelvic region. It cannot be ruled out that these haemorrhages were a result of removing the embryo from the egg.

Monitoring the difference in body weight of chick embryos

The average weight of all embryos without pre-incubation reached 1.62 g; with pre-incubation it was 1.70 g. Within the groups divided by days of storage, the weight average decreased with an increase in storage time. The greatest weight was reached by the eggs stored for 3 days, 1.835 ± 0.298 g with pre-incubation; 1.710 ± 0.259 g without. The mean average weight of hatching eggs was achieved by embryos with 7 days of storage (1.620 ± 0.227 g, with pre-incubation 1.731 ± 0.235 g). The lowest average weight was seen by the embryos of 10 days of storage, weighing only 1.534 ± 0.226 g without pre-incubation and 1.539 ± 0.195 g with pre-incubation (Table 1, Fig. 4).

Monitoring the difference in heart weight of chick embryos

Similarly, to the body weight correlation, the average weight of the chick hearts decreased in direct correlation with the increase in storage time. The lowest weights were observed in the chick embryos stored for 10 days. The average heart weight from the 3 day stored eggs were 18 ± 3.9 mg; with pre-incubation 21 ± 6.4 mg. The average weight from the 7 day stored eggs were 18.6 ± 5.2 mg; with pre-incubation 18.9 ± 4.9 mg. The 10 day stored eggs had the lowest average weight of 18.1 ± 3.9 mg, with pre-incubation of only 15 ± 3.5 mg (Table 3, Fig. 5).

In all examined hearts, those pre-incubated reached a higher weight than those that were not pre-incubated. The exceptions to this were those embryos retrieved from eggs stored for 10 days. Overall, we achieved the higher weight of all pre-incubated embryos in comparison to the weight of hatching eggs without pre-incubation.

DISCUSSION

Our results showed that eggs stored 7 days and longer had a decrease in hatching quality; the reason for this negative effect has not been resolved. Suggestion for this reduced hatchability could be due to the decreased viability of the embryo caused by the higher grade of cell death; another could be optimal time of pre-incubation could differ according to a length of storage (as the storage length correlates to embryo viability) [14]. The reduced heart and embryo weights could be the result of delayed re-initiation of development following longer storage. The storage of hatching eggs seven days or longer often induced cell death, which in turn led to: increased embryo mortality, decreased hatchability and decreased the development of the embryo metabolism. Embryo mortality of eggs stored at 4 days averaged reached 10.7 % increasing to 27.7 % for eggs stored at 14 days [6].

Chicken embryos from the broilers were examined to prove whether storage had any impact on the development of the chick embryo before incubation; results showed that the longer the egg was stored, the lower the embryo weight, which in turn lead to an increased embryonic mortality [5].

Hatching eggs from the broiler Ross 308 were divided into two groups and stored for 4 and 14 days at the same temperature and the humidity. The conclusion of this testing was that with the increased time of the storage the mechanisms of the apoptosis in the level of the blastoderm were activated. This may be an attributing factor to the reduced weight gain observed during incubation [3, 8].

Eggs stored for 14 days saw an increase in apoptosis, resulting in a growing number of abnormal embryos or dead embryos. These results clarified the importance of optimal conditions for normal growth and development [2].

Embryos retrieved from eggs stored for 7 and 14 days saw retarded growth after 42 hours, compared to the eggs that were not stored. It is likely not caused by late embryonic development but the reduced rate of embryonic growth, which was significantly lower in the first two days of incubation of the eggs stored for 14 days (compared to those not stored). No evidence of a relationship between the developmental stages of the embryo and time of incubation was apparent. A positive correlation was seen between the number of dead and malformed embryos and the storage length [10].

This study suggests eggs (from Cobb 500 broilers) pre-incubated 6 hours before storage provides better quality as it reduces the incubation period and level of embryonic mortality. The worst results were seen in eggs preheated for 12 hours then stored for 14 days; these results recommend that this method not to be used in commercial management [17].

The influence of pre-incubation, broiler breeder age and the impact of storage period on hatching efficiency were all evaluated. Eggs from the broilers Ross 344 x broiler Ross 308 (age of 28, 38 and 53 weeks) were stored follow-

ing 10 days in the storage room, others were removed on the fifth day of storage. They were pre-incubated firstly at 26 °C for 2 hours, 37.8 °C for 3 hours and finally at 26 °C for 2 hours. Eggs that were pre-incubated on the 5th day after 11 day storage had increased hatchability; research suggests this was due to the eggs coming from young broilers and being in the hypoblast stage at the start of the study [7].

Eggs stored longer than 7 days saw changes to: albumen characteristics, decreased the quality of incubation, increased the duration of the incubation period and halted embryo development. This study suggests longer storage times, conditions should be modified with the eggs thin pole ventral and the eggs rotated. In addition to these requirements, pre-incubation can be used which together should reduce the negative impacts seen in long storage [16].

When the hypoblast is completely formed (during the silent developmental state of an embryo), the embryo seems to be more resistant to the larger storage periods. Within the storage the differences in the albumen viscosity, pH of the yolk and albumen are visible. Optimal pH of albumen for the right development is 8.2. It is assumed that the embryo with completely developed hypoblast is able to make a stronger barrier between the embryo and its external environment, and/or it is more capable of producing enough amounts of CO_2, which reduce the pH in the microenvironment of the embryo for the optimal 8.2 [15].

Based on our results it can be concluded that the increased mortality is linked to increased storage periods of hatching eggs. Each studied group displayed malformations regardless of pre-incubation. Results also showed that pre-incubation increased overall bodyweight along with heart weight. The exceptions to this were the eggs stored for 10 days; to which pre-incubation did not play a significant role in the embryonic development. This research suggests ensuring good hatchability and chick quality after long periods of storage; the embryo should be in the stage of a completely formed hypoblast. This material may provide insight into enhancing the storage of experimental hatching eggs. It will allow avoiding false-induced malformations or mortality resulting from incorrect storage or pre-incubation of hatching eggs.

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ECOLOGICAL FACTORS OF TRANSMISSION, PERSISTENCE AND CIRCULATION OF PATHOGENS IN BAT POPULATIONS

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ABSTRACT

The existence of bats is crucial for all ecosystem units as they fulfil numerous ecological roles. However, they are also considered to be natural reservoirs of a wide range of zoonotic microorganisms, especially viruses. In this review article we briefly summarize current knowledge about various ecological factors that facilitate bat pathogen dispersal and about the current approaches to monitoring viral communities present within bat populations. On the basis of the cited papers, we suggest that the increased focus on complex viral populations in bats and their interactions with other populations and the environment is necessary to fully comprehend the relationship between emerging infectious diseases, the environment and their toll on human health.

Key words: bats; ecology; reservoir; viruses; zoonoses

INTRODUCTION

Bats belong to the mammalian order *Chiroptera*. Many species are ubiquitous; bats can be found on every continent except arctic regions and Antarctica [52]. Members of the order *Chiroptera* are the only known mammals capable of sustained flight. To this day, approximately 1,232 species of bats have been described [29, 49, 50].

The existence of bats is crucial for all ecosystem units, humans included. Bats fulfil numerous ecological roles [29]: they are important pollinators of fruit-bearing trees and play a role in the process of reforestation [29]. Insectivorous species are significant predators of insects, especially crop pests and nuisance insect species [29]. The guano of many bat species is collected due to its high nitrogen content and used as fertilizer [2].

However, bats are also considered to be natural reservoirs of a wide range of zoonotic microorganisms, especially viruses. These viruses include, but are not limited to, various species of the genus *Lyssavirus*, henipaviruses, coronaviruses (e. g. SARS-CoV) and filoviruses such as several species of the *Ebola* virus [7, 34, 39, 51, 54, 56];

Fig. 1. Hibernation of *Myotis dasycneme* together with *M. myotis*

there have been virus isolates from bats identified also as Rhabdoviruses, possibly a *Lyssavirus* labelled as Kotalahti bat *Lyssavirus* [38]. Novel viruses in bats are being identified quite often.

Bats are hosts also to a few pathogenic bacterial species, such as *Leptospira* spp. and *Bartonella* spp. [30]; various *E. coli* serotypes have also been isolated [1, 8, 11, 37]. In general, bats are not as prominent in carrying dangerous bacteria and transmitting them to humans, as they are in spreading dangerous viruses; however, these bacteria may constitute a potential threat to human health [2] whenever humans are exposed to bat carcasses or excretions.

In this review article we briefly summarize current knowledge about various ecological factors contributing to facilitate bat pathogen dispersal and the current approaches to monitoring viral communities present within bat populations.

The order *Chiroptera* and its ecological influence

The order *Chiroptera* is unique among all other mammalian orders. Their anatomy, physiology and behaviour are specifically suited to their unparalleled way of life and their function within the ecosystem of the Earth is conditioned by these features. In this article we summarize the factors contributing to the bats' surprisingly high ability to spread dangerous viral pathogens, while causing massive spillover events.

Colony size and gregarious social behaviour

Most bats live in groups with a high number of individuals at least for a certain period of time during one year (Figs. 1—2). Some authors describe a correlation between population size and persistence of pathogens in these populations [3, 41]; this has been observed in many cases. A study from 2012 [40], however, indicates that henipaviruses have been detected in an isolated bat population on an island near Africa, counting only a very few individuals. The number of individuals in this population does not reach a population threshold number necessary to keep the viruses circulating [40], nevertheless, it appears to persist in these bats.

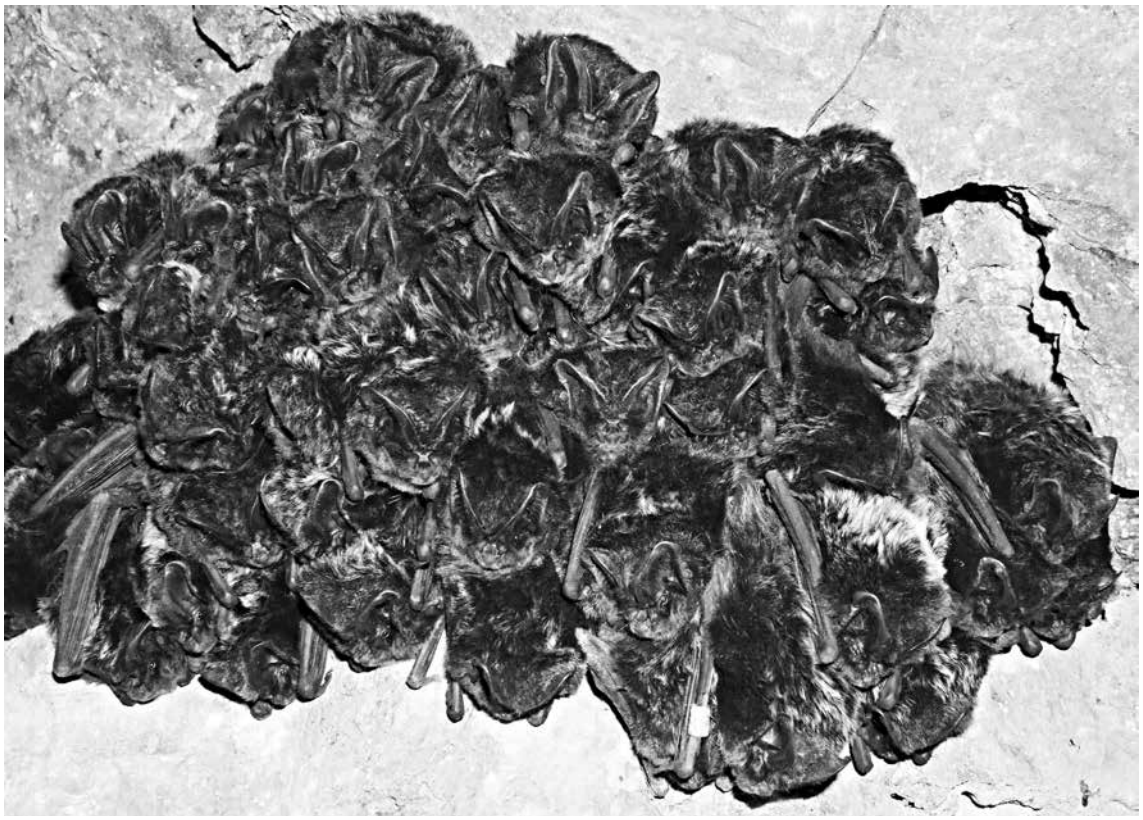

Fig. 2. A group of *Barbastella barbastellus*

Fig. 3. A cluster of hibernating *Myotis myotis*

Fig. 4. ***Pipistrellus pygmaeus***

Patagium

Bats are capable of sustained flight due to the presence of this specialized anatomical structure; it is a thin skin membrane extending between the phalanges and pelvic limbs. Bats also wrap themselves in it to steady their internal environment during hibernation and rest (Fig. 3). The ability to fly is probably one of the most important factors when it comes to pathogen dispersal; during migration, bats cover long distances [12—14], excreting viruses along the way. What is more, Brook and Dobson [5] have proposed an indirect way, in which the ability to fly increases viral spillover from bats to other species. These authors' work highlights the fact that bats are evidently immune to intracellular infections, while succumbing, even population-wide in some areas, to extracellular pathogens. Brook and Dobson [5] attribute this phenomenon to a special metabolic adaptation involving the mitochondria which apparently enables both flight and an increased resistance to viral infections.

Ways of obtaining nutrition

In order to be able to keep their aerodynamicity, bats need to ingest highly nutritious forms of sustenance, since they cannot afford to consume large quantities of food at once [36] (Fig. 4). For this purpose, some insectivorous bats only swallow a part of the insects, while dropping the rest down to the ground, where these remains—along with the bats' saliva— are ingested by other animals [18, 36]. Frugivorous bats only chew at the fruit, swallowing the sugary juice and dropping the rest on the ground as well [36]. Bat species feeding on blood can transmit diseases to bitten individuals, such as rabies [35, 36].

Anthropogenic and economic factors

The story of the frugivorous bats continues on the interface with humans. A fitting example of dangerous bat-human interface is date palm sap produce, date palm sap being a popular drink in Bangladesh [27]. Date palm sap is produced by tapping the date palm and collecting the sap in containers [27]. Frugivorous bats have learned to fly to these trees and lick the dripping sap, contaminating the raw product with urine [27, 33, 43]. Several preventive measures decreasing the risk of date palm sap have been proposed, on the other hand, their feasibility is questionable [27]. In many countries bats are sold at markets for

meat, which jeopardizes buyers, sellers, hunters and butchers alike [25]. Limiting the trade of bat meat is difficult, mainly due to economy in these regions [25, 55]. Bat guano collectors are also exposed to dangerous viruses present in the excretions [53].

Circannual cycle

All of the European bat species undergo hibernation, which is influenced by the outer temperature [44]. There have been findings connecting hibernation to decreased immune function [4, 19], which facilitates the persistence of viral pathogens in infected individuals [4, 5, 19].

Longevity

Bats can live for decades [12—14, 21]. The longer a reservoir organism lives and is active, the longer it is able to excrete pathogens; what is more, several authors [5, 47, 48] have connected their longevity with metabolic mechanisms facilitating the persistence of intracellular pathogens.

The importance of ecology in bat-borne pathogen research

W o o d et al. [57] in their work describe the interdependence of human health and the health of the ecosystem, and with an emphasis on bats as important reservoirs of emerging infectious diseases, these authors suggest a complex approach to studying zoonoses. Their idea is based on a case study involving bats as a source of RNA viruses transmitted to humans. The authors propose a comprehensive and holistic view of studying zoonoses based on systematic monitoring of the human-wildlife interface, i.e. the interactions between humans and wildlife, taking into account the dynamics of viral and reservoir species populations, but also other possible amplifiers of the diseases, such as livestock. They further emphasize the often neglected cultural and political background, which should support the research by spreading the news within the non-scientific public and issuing proper legislation. Preserving nature and reservoir species and protecting their environment while keeping to the proposed approach will, according to these authors, help minimize spillover of dangerous bat-borne viruses into humans.

Metagenomic analysis of viral populations in bats

Modern findings in the field of molecular biology enable us to analyse samples in bulk in a relatively short time. Metagenomic analysis facilitates the discovery of novel pathogens; it can be used to obtain genome sequences of complete viral populations from both environmental and biological samples [15, 28], such as mucosal swabs, blood clots etc. The main advantage of metagenomic analysis is the possibility to capture almost all (and often whole) viral sequences present in the analysed sample and thus to study viral populations (viromes) present in the infected organism. Such screening applied to bat guano samples, for instance, has the potential to reveal "old known" pathogens that have been the subject of chiropterologic studies in the past, along with completely novel (i. e. never before identified, or possibly new variations of known) viral taxa; these data may be useful for predicting the next potential grave spillover events [31]. There are several particular protocols for metagenomic analysis built on the same bioinformatic principle. With the advent of new generation sequencing (massively parallel sequencing methods), modifications were made to classic molecular-genetic methods in order to discover novel pathogens which had not been cultivated before [15, 22, 23, 26, 28, 32, 42, 46]. Sequence-Independent Single Primer Amplification [10] was developed by R e y e s and K i m [45]; it allows for fast amplification of a viral genome present in the analysed sample in only a few steps [17]. This method has been modified several times since its first implementation [16, 20, 58] together with the development of new sequencing methods, which simplified the process. Froussard's modification brought forth the so-called random-PCR, which combines reverse transcription and PCR using random hexamers labelled with known sequences used during the extension step; thus enough cDNA is produced for further cloning and sequencing [20, 58]. D e p e w et al. [16] modified this protocol so that viruses isolated on a single plaque are identifiable via HiSeq Illumina platform.

The influence of other species

Parasitism is present in many mammalian species, and bats are no exception (Fig. 5). Even though parasites of bats do not transmit to humans in such a degree as viruses—maybe with the exception of some ticks, e. g. *Argas vesperitlionis* [6]—bat parasites may have a direct impact on the ecology of bat and virus populations worldwide [24].

Some of the mentioned viral families have been detected also in small rodents [9], which may suggest another element of the viral ecological cycle.

Fig. 5. ***Rhinolophus hipposideros*** **attacked by tick**

CONCLUSIONS

The papers cited in our review focus the attention of the professional public on the importance of interspecies relationships in Earth's ecosystems with regard not only to animal species, but also in connection with populations of infectious agents and their circulation within the environment. Several authors cited in this review report findings of various animal and insect viruses in the insectivorous bats; the latter appear as a central meeting point connecting many arthropod, mammalian and other *Metazoan* species with the human race. It is plausible that by enabling dangerous viruses to persist in their populations, bats contribute to the spillover of these pathogens to species (including, but certainly not limited to, humans) for which they are highly virulent.

Based on the cited papers, we suggest that the increased focus on complex viral populations in bats and their in-

teractions with other populations and the environment is necessary to fully comprehend the relationship between emerging infectious diseases, the environment and their toll on human health. We assume that this necessity will increase as humans will continue to exploit parts of the world where these viruses have been circulating. Researchers studying viral populations should do so systematically, with a long-term focus on selected areas. There is currently no known method of exact prediction where or when another emerging infectious disease (EID) will appear [31]; however, with enough metagenomic data, it might be possible at least to presume and test which viral taxa are more or less likely to infect mammalian cells [31]. In order to achieve this goal, close cooperation of experts in ecology, zoology, microbiology, molecular biology, immunology and bioinformatics will be necessary; and last, but not least, also the cooperation of state legislation organs and public health officials.

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PREVALENCE AND ECONOMIC LOSSES DUE TO BOVINE TUBERCULOSIS IN CATTLE SLAUGHTERED AT BODIJA MUNICIPAL ABATTOIR, IBADAN, NIGERIA

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ABSTRACT

Bovine tuberculosis (BTB) is a zoonotic disease responsible for considerable economic losses; with consequent negative impact on both public health and the livestock industry. However, the burden of this disease and associated economic losses remain un-investigated among slaughtered cattle in Ibadan, the hub of livestock activities in south-western Nigeria. A cross sectional study was conducted over a three-month period to determine the prevalence and economic losses due to BTB among slaughtered cattle at the Bodija Municipal Abattoir, Ibadan, in south-western Nigeria. Individual slaughtered cattle were purposively inspected for the presence of tuberculous lesions, and representative sample of affected organs and associated lymph nodes from the BTB suspected animals were taken. The suspected lesions were processed based on Becton Dickinson digestion and decontamination procedure and the losses due to BTB were estimated mathematically, using a formula. The BTB prevalence of 9.3 % (38/408) was obtained with a significant statistical association (P ≤ 0.05) of the disease with the breeds of cattle slaughtered. Out of the 46 visceral organs condemned: 29 (63 %) were lungs, 12 (26.1 %) livers, 4 (8.7 %) hearts and 1 (2.2 %) kidney. The total estimated annual economic loss (direct and indirect) due to BTB was estimated as Nigerian naria NGN 703,980,070 (EUR 1,725,441.4). This study revealed BTB as endemic and a major cause of concern considering its ill-health and economic effects on both humans and the livestock industry. Efforts are therefore required: to control this disease along the beef value chain in order to safeguard human and livestock health; as well as to limit the economic losses associated with its prevalence.

Key words: bovine tuberculosis; economic loss; prevalence; zoonoses

INTRODUCTION

Bovine tuberculosis (BTB) is a chronic infectious and contagious disease of both domestic and wild animals as well as humans [28]. It is caused by *Mycobacterium bo-*

vis, a slow growing, non-photochromogenic and acid-fast bacillus. The disease is characterized by the formation of granulomas in tissues and organs, more significantly in the lungs, lymph nodes, intestines, liver and kidneys [32]. The BTB disease is a cause of concern for humans and others animals judging from its negative economic and health implications [6, 10]. Indeed, the disease induces high animal morbidity and mortality that eventually reduces the financial capital and increases production costs [9]. Bovine TB affects cattle health, impacts negatively on profitability and trade and can decimate years of genetic improvement towards desirable production traits [3]. Bovine TB is an important zoonotic disease, known to exist in all parts of the world. It is recognized in 176 countries as an important bovine disease that causes great economic loss [18, 24, 31].

Cattle infected with *M. bovis* are the main source of infection for other cattle and humans. *M. bovis* could be excreted through aerosol, sputum, faeces (from both intestinal lesions and swallowed sputum from pulmonary lesions), milk, urine, vaginal and uterine discharges, and discharges from open peripheral lymph nodes [29]. Domestic animals are significant reservoir hosts for human TB, caused by *M. bovis* [17]. The majority of the cases of human TB, both pulmonary and extra pulmonary in Nigeria are due to *M. tuberculosis* [6]. However, due to the endemicity of BTB in the cattle population, people are at risk of exposure to *M. bovis* from consumption of unpasteurized milk and milk products as well as the dearth of meat inspection services to identify infected animals in the food chain [5]. Transmission through consumption of milk and unpasteurized dairy products from infected cattle occurs mostly among the general public, whereas exposure through airborne infection remains highest among farmers, veterinarians, and slaughterhouse workers [25].

The diagnosis of TB in cattle is mainly through tuberculin testing, culture, and molecular genotyping [4, 36]. Culture is regarded as the gold standard for confirmation of BTB but most laboratories in the country are ill-equipped. Hence, routine diagnosis of BTB at the abattoir is based on identification of characteristic tuberculous lesions [1, 20]. The disease is a major cause of economic losses due to organ condemnation at the abattoir and slaughter slabs in Nigeria. Available reports showed that BTB causes direct loss of up to 10 % to 18 % decrease in milk and 15 % meat production [37]. Thus, BTB is of paramount importance to cattle breeders and public health authorities because of its economic and zoonotic implications [19]. This study aims at providing information on the current prevalence and estimated financial loss due to BTB among slaughtered cattle in Ibadan, located in south-western Nigeria.

MATERIALS AND METHODS

Study area

This study was carried out at Bodija Municipal Abattoir, a major abattoir in the city of Ibadan, where 250 to 300 heads of cattle are slaughtered on a daily basis. The abattoir is a major recipient of animals from different parts of the country especially the north and it is located in the geographical grid of reference longitude 3 °E, latitude 7 °N [14].

Sample size

The sampling method was employed to generate data on cattle presented for slaughter at the abattoir. Because of an expected 50 % prevalence, 408 cattle were examined in this study.

Sampling procedure and laboratory diagnosis

A total of 408 cattle were thoroughly examined at *post mortem*. Organs such as lungs, liver, heart and kidneys were visually examined, palpated and incised to detect the presence of granulomas; associated lymph-nodes were also palpated and incised, especially the bronchial and hepatic lymph-nodes. Each sample obtained was appropriately kept in sample containers and labelled. Data on breed, sex and body score of each sampled animal were collected and documented. Samples obtained were transported to the Tuberculosis Laboratory of the Department of Veterinary Public Health and Preventive Medicine, University of Ibadan, Nigeria for analysis. Suspected lesions were processed based on Becton Dickinson digestion and decontamination procedure [2]. The suspension obtained after the sample processing was inoculated onto Lowenstein-Jensen slopes with pyruvate and glycerol and incubated at 37 °C for between eight and twelve weeks. Cultures were considered negative if no colony growth was detected after 8 weeks.

Assessment of economic losses

The loss due to BTB in Bodija Municipal Abattoir was estimated mathematically, using a formula set

by Ogunrinade and Ogunrinade [27] as modified by Guadu et al. [16]. The total annual financial loss incurred due to BTB was computed by summing up the direct and indirect losses.

A = direct economic losses (annual loss from organs condemnation)
B = indirect economic losses (annual loss from carcass weight reduction)

Direct economic losses

The direct economic loss (A) was calculated based on condemned edible visceral. There were no documented data on annual slaughter rates at the abattoir, therefore it was estimated based on observations made during the study period; 250 to 300 heads of cattle were slaughtered on a daily basis, six [6] times a week, an average of 275 heads of cattle was estimated, which resulted into a mean slaughter rate of 85,800 head of cattle per annum. The percentage of each condemned organ was taken into consideration and the average market price of healthy lung, liver, heart and kidneys, which are 2000, 3000, 1500 and 700 NGN (Nigerian naria), respectively.

The economic loss is calculated as follows:

$$A = (MAS \times PLu \times CLu) + (MAS \times PLi \times CLi) + + (MAS \times PHr \times CHr) + (MAS \times PKi \times CKi)$$

MAS = mean annual cattle slaughtered at study abattoir
PHr = percentage of heart condemned
PLu = percentage of lung condemned
CHr = mean cost of a heart
CLu = mean cost of a lung
PKi = percentage of kidney condemned
PLi = percentage of liver condemned
CKi = mean cost of a kidney
CLi = mean cost of a liver

Indirect economic losses

To calculate the indirect economic losses due to BTB, a 36.6 % weight loss due to BTB was used as reported by Kwaghe et al. [22] and an average carcass weight of Nigerian cattle is estimated as 292.35 kg according to the report of Umar et al. [35] in order to obtain percentage weight of carcass reduction due to BTB. Beef sellers were orally interviewed and an average of NGN 800 was obtained to be the cost a kilogram of beef.

$$B = MAS \times PCW \times MCB \times P$$
$$B = MAS \times (292.3\,KG \times 36.6\%) \times MCB \times P$$

MAS = mean annual cattle slaughtered at study abattoir
PCW = percentage of carcass weight reduction
MCB = mean cost of 1 kg beef in Ibadan city butcheries
P = prevalence rate of bovine tuberculosis at study abattoir

Table 1. Prevalence of bovine tuberculosis in relation to breed, sex and body score

Variables	No. examined	No. infected	Prevalence [%]	X^2	P-value
Breed					
Bunaji	158	22	13.9	6.712	0.035
Rahaji	157	9	5.7		
Sokoto Ruhali	93	7	7.5		
Total	**408**	**38**			
Sex					
Male	60	4	6.7	0.800	0.777
Female	348	34	9.8		
Body score					
Emaciated	186	17	9.1	0.632	0.427
Moderate	222	21	9.5		

P — value is significant at $P \leq 0.05$
*significant, X^2 — chi square

Data Analysis

The data obtained were coded in Microsoft excel and subjected to descriptive statistics and chi-square in order to assess the magnitude of the difference of comparable variables using SPSS version 16.0 software. Statistically significant association between variables is considered to exist if the p-value $P \leq 0.05$.

RESULTS

A total of 46 lesions suggestive of tuberculosis were obtained from 408 slaughtered cattle examined, out of which only 38 were culture positive for *Mycobacterium* spp., giving a prevalence of 9.3 % (38/408). The Bunaji breed (22/158; 13.9 %) of cattle had the highest prevalence, followed by Rahaji (9/157; 5.7 %) and Sokoto Gudali (7/93; 7.5 %). The prevalence for sex were: male (4/60; 6.7 %) and female (34/348; 9.8 %) while the body score was emaciated (17/186; 9.1 %) and moderate (21/222; 9.5 %). The chi square test statistic revealed that the prevalence was significantly different ($P \leq 0.05$) for breeds, but not for sex and the body score (Table 1). The localization of tubercles (Table 2) was highest in the lungs (29/46, 63 %), followed by liver (12/46; 26.1 %), heart (4/46; 8.7 %) and least in the kidneys (1/46; 2.2 %).

The total annual economic loss due to bovine tuberculosis in the study abattoir was estimated by summing up losses calculated from organ condemnation (direct losses) and carcass weight reduction (indirect losses). The direct economic losses were calculated to be NGN 21,059,610 (EUR 51,616.7) and the indirect economic losses was calculated to be NGN 682,920 460 (EUR 1,673,824.7). A total annual economic loss (A + B) of NGN 703,980, 070 (EUR 1,725,441.4) was obtained in this study.

**Conversion rate:* NGN 408 is equivalent to 1 EUR.

Table 2. Disseminated tubercles of bovine tuberculosis in various organs

Organs	Frequency	Percentage
Lungs	29	63.0
Liver	12	26.1
Heart	4	8.7
Kidneys	1	2.2
Total	**46**	**100.0**

DISCUSSION

From this study, a prevalence of 9.3 % BTB was obtained among slaughtered cattle in Ibadan, in south-western Nigeria. The fact that BTB due to *M. bovis* is endemic and despite the global recognition of the disease as an animal and a zoonotic disease, substantial evidence suggests that its impacts on human health has been underestimated [26]. However, consumption of unpasteurized infected milk, a major factor that predisposes humans contracting zoonotic TB is still popularly practiced in Nigeria, hence the importance of the prevalence obtained in this study. The prevalence of 9.3 % obtained in this study is in line with previous studies on BTB conducted in south-western Nigeria with an established prevalence of between 8.8 % [6] and 10.5 % [7]. It is also similar to the prevalence of 8.8 % obtained by E j e h [12] in Benue State, north-central Nigeria and higher than 6.41 % reported in Borno State, north-east Nigeria by K w a g h e et al. [22]. The difference in prevalence might be as a result of varying detection abilities of the meat inspectors. The effective detection of BTB tubercles at *post mortem* is dependent on the thoroughness of the examination, training and experience of the meat inspectors [15, 23]. Based on this study, the slaughter hall layout and the ratio of meat inspectors to butchers are influential factors, because butchers slaughter and dress carcasses irrespective of whether meat inspectors get to inspect it or not. A working system can therefore be developed to maintain and improve meat inspection carried out in the country [11, 13, 34].

The lungs recorded the highest prevalence for tubercle localization, which can be attributed to the infection route of entry. Other studies carried out in other parts of the country reveal that lungs are the most affected organs [12, 22]. Pathogenesis studies suggest strongly that the route of transmission of bovine TB is largely via the respiratory system, which requires transmission via infectious aerosols [8]. The lungs are also regarded as the most exposed organ to different aggressions because of its anatomical and histological peculiarities. This finding therefore sug-

gests increasing risk of exposure of livestock workers to BTB due to aerosolization of tubercle bacilli from infected cattle.

The estimated total annual economic loss due to BTB from both direct and indirect losses obtained in this study was estimated to be NGN 703,980,070 (EUR 1,725,441.4) which is huge for a developing country considering the fact that decreased milk production and reproductive rate were not considered. The total annual economic loss obtained in this study is higher compare to the NGN 349,580,199.89 (EUR 856,814.2) reported by Kwaghe et al. [22]. This difference could be due to variation in the prevalence of the disease, mean annual slaughtered cattle and the retail prices of organs in different locations. A retrospective study by Ejeh et al. [12] to determine the direct economic loss between the years 2008 and 2012, revealed values that ranged from NGN 356,000 (EUR 872.6) to NGN 2,000,000 (EUR 4901.9) which are lower compare to NGN 21,059,610 (EUR 51,616.7) obtained in this study, thereby confirming the endemicity of BTB as a source of leakage to the buoyancy of the nation's economy.

Bovine TB has posed a tremendous impact on livestock's economic growth of Africa, as it affects the continent's involvement in the international trade of animals and animal products. The disease causes severe economic losses in livestock due to poor production, death and partial condemnation of organs or total condemnation of carcasses [33]. Many countries, including Nigeria do not implement any control or eradication programs. The BTB is an important economic challenge worldwide, most especially in developing countries and it can cause significant economic losses [37]. The zoonotic nature of BTB, coupled with the fact that it constitutes a significant economic burden to the agricultural industries [21] indicates the need to properly strategize a means to totally eradicate it. Slaughter houses have been proven to provide the opportunity for detecting diseases of both economic and public health importance [30]. It therefore becomes imperative that meat inspection activities in slaughter houses in Nigeria be scaled up in order to safeguard the health of the public and invariably limit the spread of the disease in both human and other animals with eventual prevention of unwarranted economic losses due to the disease in animals.

CONCLUSIONS

Bovine tuberculosis is still endemic in the cattle population in Nigeria and causes huge economic losses to the country. The consequent gross shortage of dietary animal proteins, due to condemnation of infected visceral organs and edible meat, as well as indirect weight loss and a potential health hazard to humans as result of its zoonotic nature are over-whelming. There is therefore a need for urgent government intervention in ensuring optimal control measures against BTB from the farm to the slaughter houses. The meat inspection at slaughter houses should also be stepped up by veterinary meat inspectors as this will go a long way to minimize the spread of bovine tuberculosis in the country.

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IN VITRO INHIBITION OF BIOFILM FORMATION BY *STAPHYLOCOCCUS AUREUS* UNDER THE ACTION OF SELECTED PLANT EXTRACTS

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ABSTRACT

In our study we investigated the ability of selected plant extracts to inhibit the formation of biofilms produced by *Staphylococcus aureus*. In the first phase, we focused on the optimisation of conditions for the correct method of an approach. For optimisation, we standardized the culture media and the bacterial culture in order to obtain interpretable results. The TSB (Tryptone Soya Broth) medium was used for the preparation of an inoculum from the bacterial suspension. For the *in vitro* tests of antibiofilm activity against the species *Staphylococcus aureus* CCM 3953, we used propylene glycol (PG) plant extracts from sage and rosemary, prepared in three different concentrations of 0.01 %, 0.05 % and 0.1 %. The tests were implemented in microtitre plates using crystal violet dye at 0.1 % concentration for visualization of the intensity of a biofilm. The results were obtained, by spectrophotometric measurements at a wavelength of 550 nm. Both rosemary and sage plant extracts had a significant effect on the formation of a biofilm by *S. aureus*. The antibiofilm activity was concentration-dependent as the formation of biofilm was reduced more effectively with increasing concentration of the extracts. The best antibiofilm activity was observed with 0.1 % rosemary extract resulting in 94 % inhibition of the biofilm formation.

Key words: biofilm; inhibition; plant extracts; *Staphylococcus aureus*

INTRODUCTION

Microorganisms living in nature, in many cases survive only through a special form of an association, called biofilms. A biofilm is a community formed by different species of microorganisms reversibly attached to substrates, interphases, or to each other, while they are inserted in a matrix produced by them [5]. The main skeleton of biofilm is a structure consisting of exopolysaccharides (EPS), proteins in the form of enzymes, DNA or RNA, microbial cells and up to 95 % of water. Some pathogenic microorganisms are capable of producing a biofilm as one of a number of

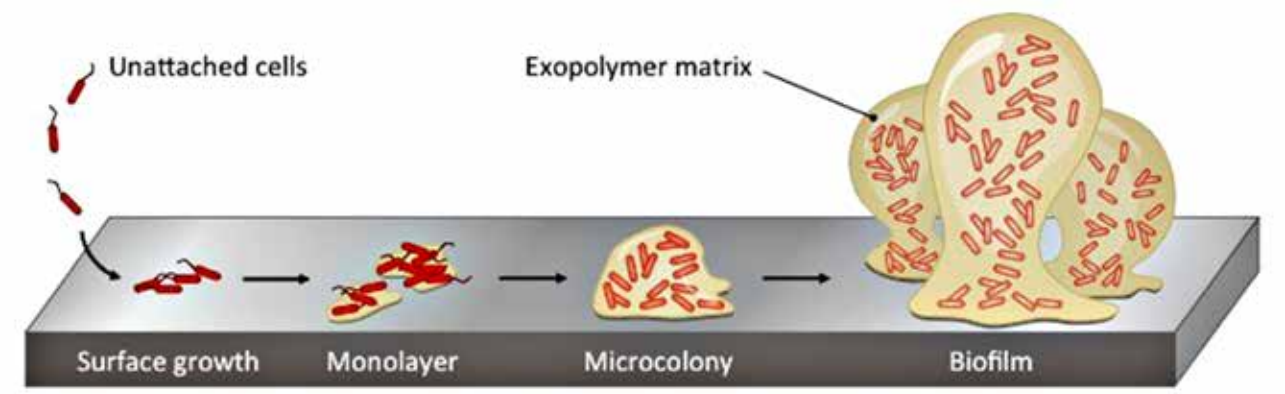

Fig. 1. Biofilm formation
Source: http://ib.bioninja.com.au/options/untitled/b3-environmental-protection/biofilms.html

defence mechanisms, resulting in their increased resistance to antimicrobials, particularly antibiotics [8]. Bacterial species important from the point of view of biofilm formation include those from the group "ESKAPE" (Enterococcus faecalis, *Staphylococcus aureus*, Klebsiella pneumoniae, Acinetobacter baumannii, *Pseudomonas aeruginosa* and *Enterobacter* spp.). These are bacteria that due to their multiresistance are frequently the agents of nosocomial infections. The infections with involvement of biofilm producing bacterial strains include for example periodontitis, valvular endocarditis, cystic fibrosis, urinary infections, dental plaques, infections of artificial joints and many others developing particularly in immunocompromised individuals [15]. Staphylococci belong to the bacteria that live in a natural habitat and are part of a microbiota of skin and mucous membranes of humans and other animals. On the other hand, pathogenic staphylococci may cause local or generalized infections and some of their strains have an ability to produce biofilms on both biotic and abiotic surfaces.

Biofilm formation is a very complex process during which the individually occurring cells form clusters and subsequently grow together. Formation of biofilms by non-motile and motile bacterial species differs. If the conditions are favourable, expression of adhesins on the surface of non-motile bacterial species increases. This results in the increase of cohesion forces among cells and, subsequently in improved adhesiveness to the surface. On the contrary, motile species lose their motility and produce an extracellular matrix owing to which they produce aggregates and stay together. The matrix is a dynamic system in which a homeostasis is achieved and the organisation of this system allows the cells to use available nutrients. Four basic phases are recognised in the process of biofilm formation. In the first phase, which involves attachment to the surface, microbial cells adhere to the surface by means of fimbria or flagella. For the attachment they can also use physical forces such as van der Waals forces or electrostatic interactions [9].

After attachment and stabilization, the process of cell division starts. The initial signal that is controlled genetically, is the production of EPS. Microcolonies consist usually of a large number of the so-called micro-communities. They communicate among themselves in many ways. The coordinated bacterial growth is controlled by the so-called quorum sensing system (QS) utilizing signal proteins and other molecules for stimulation of growth of the microcolonies [11]. The QS allows bacteria to sense their own density and, subsequently, modify their phenotype [2].

The third phase involves the production of three-dimensional structure—microcolonies—and their maturation. This is related to the increased expression of biofilm formation associated genes. The products of these genes are inevitable for the production of exopolysaccharide, the main biofilm component. Another important part of the maturation of this microcolony is the production of extracellular matrix that is initiated immediately after attachment of bacteria to the surface.

The last phase is the potential detachment of colonies. There is a sequence of reactions which support the conversion of a bacterial colony to the motile form. Saccharolytic enzymes are produced to facilitate detachment of the colony aimed at re-colonization. The cells increase their expression of proteins responsible for the production of flagella

so as to ensure motility of the detached bacteria. This detachment of the bacterial colony from the surface plays an important role in the spreading of the infections [9].

In staphylococci, the extracellular polysaccharide matrix with its principal component poly-N-acetyl-glucosamine is responsible for biofilm formation. The chemical structure of this polysaccharide corresponds to the structure of the polysaccharide intercellular adhesion (PIA). This was identified as the key component that enables the aggregation of staphylococci cells and their accumulation in the biofilm [17]. The synthesis of the PIA is encoded by the genes of the ica operone [13].

There exist substances, which are able to supress the production of a biofilm, even destroy the already formed biofilm. Metabolites of beneficial bacteria, disinfectants or plants extracts belong to the group of such substances.

Recently, some medicinal plants have increased the attention of researchers because of their potential and goal-directed ability to interfere with biofilms and thus facilitate the penetration of medicines to the bacterial agents. It was confirmed that, for example, lemon grass, oregano and Pimenta racemosa inhibit the formation of biofilms at concentrations ≤ 2 %. Also, rosewood, coriander, tea plant, Mentha × piperita and marjoram are effective at the same concentrations [7]. The components of *Humulus lupulus* L., such as lupulone, humulone and xanthohumol reduce the number of bacterial cells of *Staphylococcus aureus* released from biofilms. Extracts from *Melaleuca alternifolia* containing terpineole, terpinene-4-ol, or extracts from *Melissa officinalis* were more effective against biofilm produced by *S. aureus* and *Escherichia coli* than extract from *Lavandula angustifolia* containing lavender oil [3]. Currently implementation of nanoparticles acting as carriers of previously non-stabile essential oils help to interfere with bacterial biofilms. An example is a cinnamaldehyde obtained from cinnamon. When bound to nanoparticles, this aldehyde exhibits increased stability and thus also increased antibiofilm activity against methicillin-resistant *Staphylococcus aureus* (MRSA) strains and other bacteria [16].

The aim of this study was to investigate the antibiofilm activity of propylene glycol extracts of rosemary and sage, tested at various concentrations.

MATERIALS AND METHODS

The experiments were carried out using a reference, biofilm-producing strain *Staphylococcus aureus* CCM 3953. In order to obtain an inoculum, it was cultivated in meat-peptone broth (Himedia, India) for 18 hours at 37 °C with constant mixing. This way the prepared night culture was subsequently diluted with a modified tryptone soya broth (TSB; Himedia, India) to 1 % inoculum which was then used in an *in vitro* experiment.

We tested the antibiofilm activity of propylene glycol (PG) extracts prepared from the rosemary leaves (*Rosmarinus officinalis* L., PGR extract Certificate No. 310) and sage stems (*Salvia officinalis* L., PGS extract Certificate No. 309); both extracts were obtained from the company Calendula a. s., Nová Ľubovňa, Slovakia). We prepared the base solutions of extracts in dimethyl sulphoxide (DMSO; Sigma-Aldrich, USA) at concentrations of 1 %, 5 % and 10 % and diluted them 10-fold with TBS (Tris-buffered saline) to obtain concentrations 0.1 %, 0.5 % and 1 %.

The antibiofilm activity of the PG extracts were tested in standard 96-well microtitration plates (Greiner-Bio-One, Austria) using a modified staining method according to O' T o o l e [14]. In the first step, we pipetted into individual wells 180 µl of the bacterial suspension of staphylococci (1 % inoculum). To the wells with staphylococci suspension, we added 20 µl aliquots of the 10-fold diluted solutions of extracts (n = 4) to obtain the following effective concentrations of extracts: 0.01 %; 0.05 %; and 0.1 %. We prepared also the respective negative controls by using 20 µl 10 % DMSO (n = 4) instead of the diluted extracts. The plates were then incubated at 37 °C for 24 hours. After the incubation, the supernatant was removed by aspiration and the individual wells with the formed bacterial biofilm were washed with distilled water 3—5 times, as needed. After thorough but careful washing, the biofilm was stained with 0.1 % crystal violet solution (200 µl per well; 30 min, room temperature). Then the crystal violet solution was aspirated again and wells were washed thoroughly with distilled water (3—5 times). The following step consisted in a 10-min extraction of the stain using 200 µl of 30 % acetic acid per well. After the action of the acidic solution, the content of each well was mixed with a pipette. The quantity of the stain obtained in this way was determined by measurement of the absorbance of each solution at 550 nm employing a spectrophotometer Synergy HT (Biotek, USA).

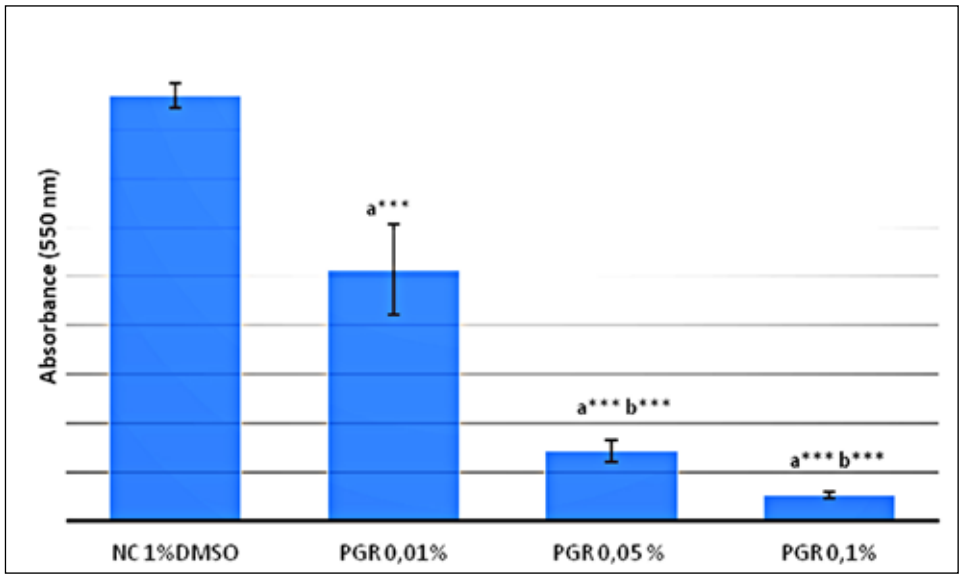

Fig. 2. Inhibition of *S. aureus* 3953
Biofilm by propylene glycol extract of rosemary (n = 4). PGR—propylene glycol rosemary extract; NC—negative control in DMSO; a—significantly different from NC; b—significantly different from 0.01 %; c—significantly different from 0.05 %; ***—P < 0.001

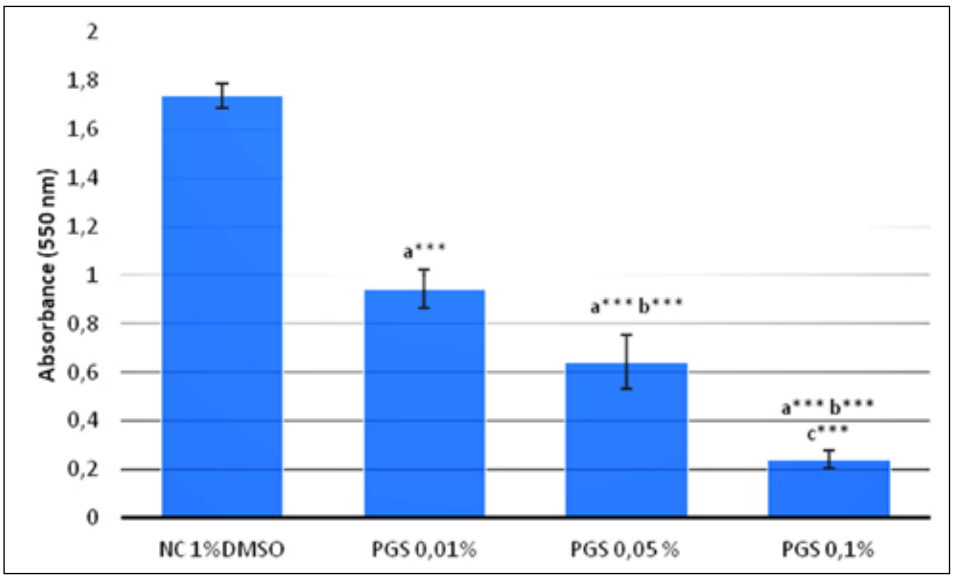

Fig. 3. Inhibition of *S. aureus* 3953
Biofilm by propylene glycol extract of sage (n = 4). PGS—propylene glycol sage extract; NC—negative control in DMSO; a—significantly different from NC; b—significantly different from 0.01 %; c—significantly different from 0.05 %; ***—P < 0.001

For statistical analysis of the results, a 1-way ANOVA was used. Significant differences between groups were assessed by Tukey's test. The difference between various concentrations of the plant extracts were determined by the unpaired t-test. P-values < 0.05 were considered significant.

RESULTS AND DISCUSSION

The results of our experiments indicated that the antibiofilm activity of the PG extracts of rosemary and sage was concentration dependent as shown in Figs. 2 and 3. With

increasing concentration of plant extracts the biofilm formation by *Staphylococcus aureus* decreased. The PG extracts of both plants showed the highest anti-biofilm concentration at a concentration of 0.1 %, followed by concentrations 0.05 % and 0.01 %; however, the antibiofilm action of the sage extract was lower compared to the rosemary extract ($P < 0.0001$). While the inhibition of biofilm formation by the sage extract ranged from 46 % (0.01 % concentration) to 86 % (0.1 % concentration), the rosemary extract caused 96 % inhibition at 0.1 % concentration and 46 % at 0.01 % concentration.

An antibiofilm activity of PG extract of rosemary was reported also by Oliveira et al. [6]. These authors observed that the extract significantly reduced the growth and multiplication of bacteria (CFU per ml) in monomicrobial biofilms of *Staphylococcus aureus*. The extracts of concentration 2 % significantly reduced biofilm within 5 minutes. Better results in comparison with biofilms produced by *S. aureus* were obtained with the strains Candida albicans and *Pseudomonas aeruginosa*. The above study used extracts prepared by similar procedure as used in our study. However, the highest inhibition of biofilm formation by PG extract of rosemary was reached with extract with a much lower concentration (0.1 %).

The extract of rosemary at a concentration of 25 $\mu l.ml^{-1}$ reduced the biofilm produced by *Staphylococcus epidermidis* by 57.1 %. At lower concentration (12.5 $\mu l.ml^{-1}$), rosemary decreased adhesion of cells by 49.21 % but reduced the formation of biofilm by only 29.05 % [10]. The rosemary extract tested in this study had an antibiofilm affect similar to that in our experiments but the inhibition ranged from 46 % to 86 %.

In the study by Ceylan [4], the antibiofilm activity of the rosemary extract against the biofilm produced by various bacteria including *S. aureus* was tested and the authors reported that the rosemary extract was the most effective specifically against *S. aureus* produced biofilm. The author used a longer cultivation of the inoculum (48 h) in comparison with our method. The inhibition observed was 60.76 %.

In addition to propylene glycol extract, other types of plant extracts also inhibited the formation of biofilms. Hydroalcohol extract of rosemary showed anti-adherent action and decreased the formation of biofilms [12].

Al-Bakri et al. [1] investigated the antibiofilm effect of ethanol extracts from sage and sage essential oil. Into individual wells of microtitration plates they added 100 µl of extract and 10 µl of staphylococci inoculum, so the final volume of the well content was smaller than that used in our study which explains the higher anti-biofilm activity reached by these authors. The extract from sage resulted in 86.2 % and 83.4 % inhibition of the production of biofilm by MRSA (methicillin-resistant *S. aureus*) strain and a 98.3 % inhibition of *S. aureus*.

The results of several studies are in agreement with our observations that indicated higher anti-biofilm activity of rosemary extract in comparison with sage extract. Also the percentage of inhibition achieved by rosemary extracts was similar or were in the range observed in our study taking into consideration the concentration of the tested extracts. However, standardization of the procedures appears important for reproducibility of results as different conditions of incubation and preparation of relevant inocula applied to microtitration plates influence the investigated activity.

CONCLUSIONS

From the medical point of view, the formation of biofilms by pathogenic microorganisms on both biotic and abiotic surfaces is an undesirable phenomenon with serious implications. The standardization of procedures is important in order to reach creditable interpretable results. Some compounds of natural origin, for example those in certain plant extracts, have the capacity to inhibit the formation of such biofilms. Our study confirmed the antibiofilm concentration-dependent effectiveness of rosemary and sage propylene glycol extracts with the highest activity of rosemary extract at concentration of 0.1 %.

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PREVALENCE OF ENDOPARASITES IN CARNIVORES IN A ZOO AND A WOLVES PARK IN GERMANY

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ABSTRACT

Endoparasites have the potential to cause significant health problems in humans and other animals. Consequently, particularly the endoparasites of a zoonotic nature are of great interest to health authorities and scientists. This study investigated the prevalence of different intestinal parasite species of carnivores kept in captivity. Altogether 36 pooled samples of faeces obtained from individual animal enclosures from the Neunkircher Zoo and Wolfspark Werner Freund in Germany were examined. The samples were analysed by means of a flotation concentration method with the use of Faust and Kozák-Mágrová solution. Out of a total of 36 samples, 19 were positive for endoparasites (52.78 %). Furthermore, 13 out of 19 positive samples were protozoans (68.42 %).

Key words: carnivores; endoparasites; wolves park; zoo

INTRODUCTION

Zoological gardens and animal parks display wild animals for recreational and educational purposes but also play an important role in the conservation of species, particularly the rare and endangered ones [18]. In their natural habitat, wild animals have large areas available to them. Their exposure to parasitic infections is, therefore, fairly low and they have consequently a low genetic resistance against parasitic infections. When groups of these wild animals are kept in confined space and many captive animal species are housed in close proximity to each other, the problem of parasitic infections can become aggravated and pose a serious threat to the animals; occasionally causing sudden local fatalities [16].

The occurrence of parasites in animals housed in zoos varies according to the type of husbandry, parasite prophylaxis and type of parasitic treatments. Usually, captive animals in zoos do not show alarming signs of parasitism if deworming is carried out regularly [18]. In addition, captivity animals are often under considerable stress, which

further diminishes their resistance to parasitic infections. One should also consider the impact of environmental changes on the zoonotic disease risk, which is mostly the subject of speculation and lacks a coherent framework for understanding environmental drivers of pathogen transmission from animal hosts to humans [3].

Endoparasites have the potential to cause a significant health problem in humans and other animals. Particularly endoparasites with a zoonotic nature are of great interest to health authorities and scientists [3, 6].

In the study by K h a t u n et al. [10], 60 % of the animals (72.7 % of carnivores) at Rangpur Recreational Garden and Zoo in Bangladesh were found positive for gastrointestinal parasites. Other authors reported similar [18], higher [5, 17] or lower [14] prevalence, but the prevalence always ranged between 40.4 and 76.6 %. In all animals, except primates, the prevalence of helminth infections was higher than the prevalence of protozoan infections, similar to other studies [18].

In this study, faecal samples of carnivores from a zoo and a wolves park in Germany were investigated for intestinal parasites in order to obtain information on the prevalence of endoparasites in captive-held carnivores in these facilities. Such an assessment should involve the consequences the parasite has on the host itself, its transmission abilities and its role in public health [13]. As environmental factors play an important role in the occurrence of parasites [20], the cleaning and feeding regime of the animals should also be mentioned.

In the zoo, various species of carnivores were kept: yellow-throated martens (*Martes flavigula*), slender-tailed meerkats (*Suricata suricatta*), raccoons (*Procyon lotor*), snow leopards (*Uncia uncia*), asiatic wild dogs (*Cuon alpinus*) and bush dogs (*Speothos venaticus*).

The Wolfspark Werner Freund kept 4 Canadian wolves (*Canis lupus lycaon*), 4 Mongolian wolves (*Canis lupus chanco*), 3 Swedish wolves (*Canis lupus lupus*) and 10 arctic wolves (*Canis lupus arctos*), which were divided into one 4-wolf and two 3-wolf packs. All of the groups had their separate enclosures, spread across 7 hectare of the forest. A pronounced focus of this park is to keep the animals under as natural conditions as possible.

An important difference between the animals in the zoo and the wolves park is, that the zoo animals have contact with a higher number of different people (zoo keepers), while the wolves are only in direct contact with two people.

Another difference is that the zoo animals receive anti-parasitic treatment every spring and autumn while the wolves are treated with anti-parasitic as puppies and as adults, they will only receive such treatment when specific symptoms indicate a higher infestation with parasites, causing health problems to the animals.

The aim of the study was to get an impression of the current endoparasitic situation in relevant and often-overlooked animals, as those can potentially reflect a zoonotic risk. As a result, a more precise anti-parasitic treatment can be instituted, helping to protect the environment, the animals and counteract resistance development against anti-parasitic products, hence improving the management of the endoparasites.

MATERIALS AND METHODS

In this study, 36 pooled faecal samples of carnivores from the Neunkircher Zoo and the Wolfspark Werner Freund in Merzig in Germany were investigated for intestinal parasites.

The feeding and cleaning regimens

The yellow-throated marten was fed a diet composed of small mammals, birds, insects, nuts and fruits, once a day. The enclosure was cleaned every 2—3 days. The slender-tailed meerkats received three times a day mainly an insectivorous diet with an addition of small mammals and some plant material. Their enclosure was cleaned once a day. The raccoons were fed once a day, mainly an insectivorous and plant based diet with fruits and nuts. Occasionally, also fish and eggs might be included in their diet plan. As with the martens, the enclosure was cleaned every 2—3 days. The snow leopards were fed once a day as other solitary mammals and birds. They were not fed one day a week. The enclosure was cleaned once a week. The pack of Asiatic wild dogs were fed mainly mammals. Additionally, they also receive some insects and fruits. As with snow leopards, they were fed once a day, while they did not receive any food for one day a week. Their enclosure was cleaned every 1—2 weeks. The bush dogs were fed mammals, particular rodents and birds. As with the previous 2 animals, they did not receive any food one day a week and their enclosure was cleaned every week once.

The wolves were kept as close to their natural condi-

tions so they were not fed every day. The time span between their meals depended on the size of the previous meal. They received animals from hunters and forest rangers (mainly deer), farmers (cattle, sheep, goats) and some local privately owned animals such as horses. Pigs were not fed.

Collection and processing of samples

The samples were collected in April 2017 and November 2017. Multiple samples were collected from each individual enclosure in a plastic bag and stored individually at 4 °C. The pooled samples were evaluated within 24 hours. For analysis of all samples, we used the flotation concentration test with zinc sulphate solution (Faust solution—specific gravity 1.18 g.cm^{-3}) for the presence of protozoan cysts (*Giardia duodenalis*) and Kozák-Mágrová solution for the presence of protozoan oocysts and helminth eggs [12].

For the flotation concentration method with zinc sulphate solution we mixed 5—10 grams of a composite faecal sample with 20—30 ml of water to make a semi-solid suspension. The suspension was filtered through a plastic sieve into a beaker and the filtrate was transferred to two tubes which were centrifuged for 2 min at 2500 rpm. Subsequently, the supernatant was poured off from both tubes. To the remaining pellet on the bottom of one tube we added Faust solution (zinc sulphate solution) up to 1/3 of the tube volume and to the second tube we added Kozák-Mágrová solution. The content of both tubes were mixed and centrifuged at the same setting as above. Using a parasitological loop, we picked up 3 drops from the solution surface, transferred them onto a clear glass slide and examined under a light microscope Olympus Model BX41 (Japan). For detection of cysts of Giardia duodenalis the drops were covered with a cover slip and also observed under a light microscope.

RESULTS

Out of a total of 36 samples, 19 were positive for endoparasites (52.78 %). The results are summarized in Table 1. Thirteen out of 19 positive samples were of protozoan origin (68.42 %).

Out of 6 samples from the yellow-throated martens, 1 was positive for *Ancylostoma* spp. (Fig. 1). All 6 samples from the slender-tailed meerkats, were positive for *Cystoisospora* spp. From the 6 samples obtained from raccoons, 3 were positive for *Cystoisospora* spp. (Fig. 2). The 5 samples from the snow leopard, included 3 positive samples for *Toxocara cati* (Fig. 3) and 1 for *Ancylostoma* spp. Of the 3 samples from the asiatic wild dog, 2 were positive for *Sarcocystis* spp. Two samples originated from the bush dog (*Speothos venaticus*) and both were positive for *Sarcocystis* spp. From a total of 8 samples obtained from wolves only 1 sample was positive for *Trichuris vulpis*.

Table 1. Number of positive samples for each animal and species of endoparasites found

Animal Species	Number of samples	Number of positive samples	Species of endoparasites
Yellow-throated marten (*Martes flavigula*)	6	1	*Ancylostoma* spp.
Slender-tailed meerkat (*Suricata suricatta*)	6	6	*Cystoisospora* spp.
Raccoon (*Procyon lotor*)	6	3	*Cystoisospora* spp.
Snow leopard (*Uncia uncia*)	5	3 1	*Toxocara cati* *Ancylostoma* spp.
Asiatic wild dog (*Cuon alpinus*)	3	2	*Sarcocystis* spp.
Bush dog (*Speothos venaticus*)	2	2	*Sarcocystis* spp.
Wolves (*Canis lupus*)	8	1	*Trichuris vulpis*
TOTAL	**36**	**19**	

Fig. 3. ***Ancylostoma*** **sp. egg with larva in a yellow-throated marten. Magn. ×100**

Fig. 2. ***Cystoisospora*** **sp. oocyst in a raccoon. Magn. ×100**

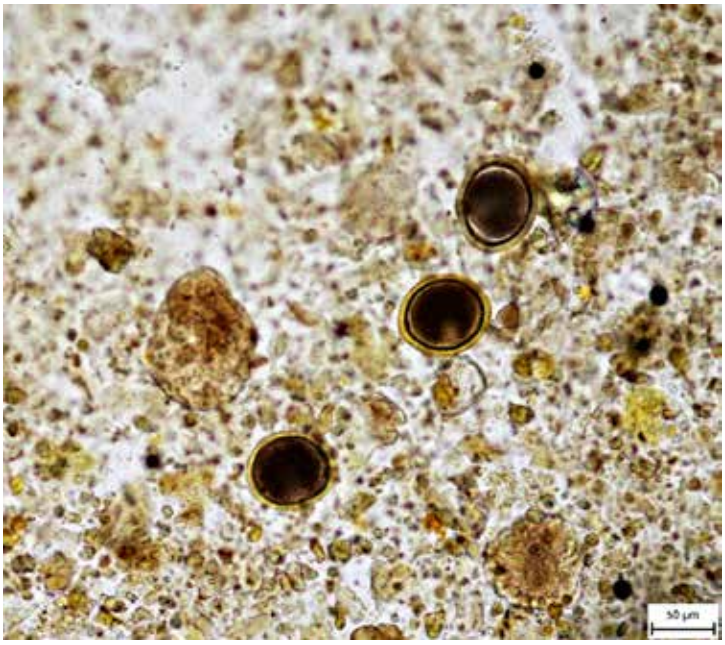

Fig. 3. ***Toxocara cati*** **eggs in a snow leopard. Magn. ×20**

DISCUSSION

A total of 52.78 % of the samples were positive for one or more endoparasitic species, from which 68.42 % belonged to protozoa. In the study by K h a t u n et al. [10] 72.7 % of the carnivores were found positive for gastrointestinal parasites. Higher (97.3 % and 89.3 %) and lower (50 %) infection rates were found by other authors [14, 15]).

It is interesting to notice that all samples from the meerkats were positive for *Isospora* spp. This can be explained by the fact that in general higher occurrences of this parasite appear in younger animals [8, 11], which correlates with the constitution of the clan, as more than half were only 14 months old at the time of the collection of the samples.

With regard to the zoonotic risk, particularly *Ancylostoma* spp., *Toxocara* spp. and *Sarcocystis* spp. should be noticed. *Ancylostoma* spp. is commonly known as a hookworm. Pathogenesis arises from mature adults, grasping

plugs of intestinal mucosa with their toothed buccal capsule, to feed from blood (approximately 0.1 ml blood/day) [22]. Pathogenesis is displayed as acute or chronic haemorrhagic anaemia, particularly in young animals as older animals can often compensate for the anaemia to a certain degree. Yet, after a prolonged period of time, the animal will experience iron deficiency, leading to microcytic hypochromic anaemia [22].

Also some species such as *Ancylostoma braziliens* and *Ancylostoma caninum* pose a zoonotic risk, causing eosinophilic enteritis and cutaneous larva migrans in humans [22]. Humans can be infected via the third stage larvae (filariform), penetrating the skin [19].

Toxocara spp. can be found worldwide in the small intestine of its final host. Humans can become a dead end host, resulting in larva migrans visceralis. Commonly affected is the liver, resulting in hepatomegaly and eosinophilia [21]. If larvae escape the circulation, they can end up in other organs, notably the eye, commonly referred to as larva migrans ocularis [21]. The body's immune system will respond with proliferative inflammation, resulting in granuloma formation around the larvae, typically sitting on the retina, rarely on the optic disc. While a total loss of vision is uncommon, partial loss with endophthalmitis or granulomatous retinitis is more commonly reported [21].

Humans can serve as a final host in *Sarcocystis hominis* and *S. suihominis*, when consuming undercooked, infected meat. Additionally, humans can also function as intermediate host for *S. nescbitti* by ingesting faecally contaminated food, water or simply from the environment. Clinical signs might appear as headaches, fever and myalgia [7].

We detected a low occurrence of parasites in the wolves park compared to the zoo. As the number of samples was low, such a trend might be simply due to the chance of parasites shedding irregularly or only in small numbers at the time, and thus they might not have been detected [1]. Yet, it could also be explained by the less confined environment of the wolves, compared to the zoo [2]. For the zoo animals might be more likely to re-infect themselves after treatment, particularly if the enclosures are not properly cleaned after administration of the drugs. On the other hand, the wolves were in closer contact with wild animals (birds, rodents), which they regularly eat. However, they did not receive regular anti-parasitic treatments, which speaks against such a trend. The decreased use of prophylactic anti-parasitic drugs, which would in the long term result in the development of resistance to antiparasitics [4, 9], might explain the observed trend.

For future study, it would be interesting to investigate the parasitic burden of the zoo animals before and after the treatment in order to investigate the current resistance status of the parasites on the property.

CONCLUSIONS

Despite the low number of samples, the data can be used to compare the changes in endoparasite burden with regard to species and annual occurrence. This can be expected due to climate change and agricultural influences. Hence, regular epizootiological investigations of captive wild animals will help to increase our insight of not only the prevalence of such parasites but also its transmissibility between wild as well as domesticated animals and humans.

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CONTAMINATION OF SANDPITS WITH SOIL-TRANSMITTED HELMINTHS EGGS IN AN URBAN ENVIRONMENT

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ABSTRACT

The aim of this study was to monitor the occurrence of the propagative stages of intestinal endoparasites in dog excrements collected within the close proximity of sandpits in an urban environment (Košice, Slovakia) and to determine the level of sandpits contamination with soil-transmitted helminths (STHs). A total of 201 dog faecal samples were examined for the presence of helminth eggs with 10.95 % of the samples being positive. In faeces the most prevalent eggs were those of Toxocara canis (7.46 %). The contamination of sand with STH eggs in 84 sandpits was also investigated. Toxocara spp. eggs were found in 21.43 % of the sandpits. The eggs from the family Ancylostomatidae and Toxascaris leonina were also present. Taenia type eggs and Trichuris sp. eggs occurred less frequently. In some samples, not only monoinfection but also co-infection with eggs of 2–3 helminth species were detected. In conclusion, the environmental contamination of sandpits with STHs eggs might pose a significant threat to the public health.

Key words: dog excrements; sandpits; soil-transmitted helminth eggs

INTRODUCTION

Dogs are the most popular pet animal. They play many roles in human society such as: guard dogs, hounds, sheepdogs, tracker dogs, guide dogs, and are also used in canistherapy, life-saving actions, transport, and last but not least, for fun and research [2, 8, 14]. On the other hand, intestinal nematodes affecting dogs have a relevant health-risk impact not only for animals but also for human beings. Both dogs and humans are typically infected by ingesting the infective stages, (i. e. larval eggs or larvae) which are present in the environment [15]. Dogs faeces are often infected with the cysts of intestinal parasitic protozoa (*Entamoeba histolytica, Giardia* spp.), the eggs of tapeworms (*Dipylidium* sp., *Echinococcus* sp.) and parasitic nematodes [11]. Dogs and cats are also major sources of environmental contamination by helminths eggs (e. g. *Toxocara* spp., *Ancylostoma* spp.). These helminths are able to cause para-

sitic infections also in man [9]. For example, *Ascaris lumbricoides, Trichuris trichiura, Ancylostoma duodenale* and *Necator americanus* (soil-transmitted helminths—STHs) are included in the World Health Organization list of Neglected Tropical Diseases [18]. The STHs infections are the most prevalent human infections worldwide [15, 17]. The most vulnerable to helminth infections are children [3, 4,12, 13]. The important source of parasitic infections are children playgrounds with sandpits [9, 10, 11, 16].

The aim of this study was to monitor the occurrence of propagative stages of intestinal endoparasites in dogs excrements collected within close sandpits proximity in the city of Košice, and to determine the level of contamination of sandpits with soil-transmitted helminths (STHs).

MATERIALS AND METHODS

Characterization of the locality

Košice (48° 43' 12" N, 21° 15' 29" E) is the second largest city in the Slovak Republic. It is situated in the valley of the river Hornád in the Košice basin at the eastern reaches of the Slovak Ore Mountains, near the border with Hungary. The population is approximately 240,000. It lies at 206 m above sea level. The climate is moderately warm with average temperatures of 19 °C in July and –3 °C in January. The average annual rainfall is 608 mm.

The town is divided into four districts: Košice I (covering the central and northern parts), Košice II (covering the southwest), Košice III (east), and Košice IV (south), and into 22 boroughs (wards). The survey was carried out from March to December 2018 in Košice III district, borough of the Dargov heroes. The borough consists of the local housing estates, built mainly in the form of panelled blocks of flats. Currently, 26,000 people live in this borough. There are little to no industrial structures or activities there and serves primarily as housing. In the period of investigation there were about 700 dogs registered in the borough.

Dog excrements

A total of 201 faecal samples of unknown dogs were collected at random from the public areas. After collection, the faecal samples were stored at 4 °C and examined for the presence of propagative stages of endoparasites within 24 hours. A flotation method with the Shaeter's flotation solution (specific gravity 1.3 $g.ml^{-1}$) was used for the coprological examinations. A total of 3 grams of a faecal sample were centrifuged with 10 ml of water for 5 minutes at 1200 rpm. After pouring off the supernatant, the Shaeter's flotation solution was poured up to 2/3 of the tube with the sediment, stirred and centrifuged once again. After 5 minutes, the test tube was replenished with the flotation solution until a meniscus formed and the tube was covered with cover glass. The cover glass was removed and put on the mount glass after an hour of egg flotation. The samples were examined under a light microscope (Leica, Germany) with ×20 and ×40 magnification.

Sand samples

A total of 84 sand samples were collected from children's sandpits. The sand samples were examined according to Kazacos [5]. To 100 g of pooled sand samples, 100 ml of water and 0.5 ml of the Tween 40 was added and decanted for 10 minutes. Then the sample was passed through a sieve and replenished with 100 ml of water. After an hour of sedimentation, the sand sample was centrifuged and then floated with Sheather's flotation solution and examined as described above.

RESULTS AND DISCUSSION

Out of 201 dog faecal samples collected around sandpits in Košice III district, 22 were positive for the presence of the propagative stages of endoparasites, representing the prevalence of 10.94 %. In some samples not only monoinfection but also co-infection with eggs of 2—3 helminth

Table 1. Occurrence of dog endoparasites in excrements collected close to sandpits

Helminth eggs	p/n	P [%]
Toxascaris leonina	3/201	1.49
Toxocara canis	15/201	7.46
Trichuris vulpis	5/201	2.48
Family Ancylostomatidae	3/201	1.49
Capillaria aerophila	1/201	0.50

n — number of examined samples; p — number of positive samples
P — prevalence

Table 2. Presence of soil-transmitted helminth eggs in fenced and unfenced sandpits

Helminth eggs	p/n	P (%)	Fenced		Unfenced	
			p/n	P [%]	p/n	P [%]
Toxascaris leonina	7/84	8.30	4/53	7.55	3/31	9.68
Toxocara spp.	18/84	21.43	11/53	20.75	7/31	22.58
Taenia type eggs	1/84	1.19	0/53	0	1/31	3.23
Family ***Ancylostomatidae***	9/84	10.71	6/53	11.32	3/31	9.68
Trichuris spp.	1/84	1.19	1/53	1.89	0/31	0

n— number of examined samples; p — number of positive samples; P — prevalence

species were detected. In the examined samples the eggs of Toxocara canis, Trichuris vulpis, Toxacaris leonina, Capillaria aerophila and eggs from the family Ancylostomatidae were detected. The occurrence of parasitic species is summarized in Table 1. Our results corresponded with those of S z a b o v á et al. [14], P a p a j o v á et al. [11] and A n t o l o v á et al. [1].

The high prevalence of intestinal helminths in the dog populations indicated a contamination of the environment in which the animals move. Epidemiologically, toxocariasis caused by nematodes *T. canis* and T. cati is considered to be one of the most serious parasitic diseases of humans. Humans became infected usually per os by ingestion of soil with embryonated *Toxocara* eggs. *Toxocariasis* manifests itself in two distinct forms: visceral, larva migrans visceralis, and ocular, larva migrans ocularis [6, 7]. Soil contamination seems to be the most direct indicator of the risk to human populations, mainly to children. For this reason, also STHs contamination of sandpits in Košice III district were also studied. Totally 84 sand samples were collected from the children's sandpits from areas with frequent movement of dogs and examined for the presence of the parasites. The occurrence of parasitic eggs in the sandpits was as follows: *Toxocara* spp., *Toxascaris leonina, Trichuris* spp, *Taenia* type eggs and eggs from the family *Ancylostomatidae* (Table 2). The sandpits were classified as fenced (53) or unfenced (31). The unfenced sandpits were found to be significantly more contaminated than the fenced sandpits. In unfenced sandpits 43.39 % prevalence of the parasites were recorded, compared with the fenced sandpits where 28.30 % positivity was observed (Table 2). In some samples not only monoinfection but also co-infection with eggs of 2—3 helminth species were detected. Similar to samples of the soil around sandpits, co-infection with eggs of 2—3 helminth species were detected in some of sand samples. Our results correspond with those of the other authors. In the Slovak Republic, S z a b o v á et al. [14] reported eggs of *Toxocara* spp. in 28.3 % of the sand samples from sandpits in Košice. In previous study P a p a j o v á et al. [11] found STHs eggs in 7.40 % examined sand samples from sandpits in the same town. O n d r i s k a et al. [10] detected *Toxocara* sp. eggs in 27.0 % of the sandpits in Bratislava and in smaller towns in 6.8 % of the sandpits.

CONCLUSIONS

This study demonstrated that the environmental contamination of sandpits with STHs eggs might pose a significant threat to public health. The occurrence of intestinal parasites in dog's excrements indicate the necessity of the veterinary care for dogs and control of hygienic measures for dog owners.

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EFFECT OF LOW-DOSE EXPOSURE TO TOXIC HEAVY METALS ON THE REPRODUCTIVE HEALTH OF RATS A MULTIGENERATIONAL STUDY

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ABSTRACT

The aim of this investigation was to evaluate the effects of the exposure to low doses of lead, mercury and cadmium dissolved in drinking water (200× above maximal permissible dosage) on the reproductive potency of 200 Wistar rats (100 males and 100 females of F1 generation) and their progeny. Ten groups of rats were formed according to their exposure to heavy metals, including one control group without exposure. The females gave births between weeks 13 and 78 of the experiments. Reproduction parameters, such as number of litters, total number of newborns, number of newborns per litter, and number of weanlings were assessed weekly. The results demonstrated that the number of litters and newborns were higher after exposure to mercury and lower after exposure to lead. The number of weanlings and their share from newborns were the highest after exposure to cadmium and the lowest after exposure to mercury. A sex-specific effect of metals was related to the reproductive success.

Key words: low-level exposure; heavy metals; rat; reproduction effect; reprotoxicity; several generations; sex-specific effect

INTRODUCTION

We live in a toxic world [25, 34]. Every day we are exposed to hundreds of toxic metals and chemicals including mercury, lead, cadmium, aluminium, food additives, pesticides, radiation toxins and many more. Heavy metal poisoning and chemical toxicity result in the accumulation of toxins in our tissues and organs, causing nutritional deficiencies [35], hormonal imbalances [26], neurological disorders, and can even lead to autoimmune disorders, cancer [17], and other debilitating chronic conditions [14]. Reproductive hazards from metal exposure is nowadays one of the fastest growing areas of concern in toxicology [12]. Exposure to various heavy metals causes irreversible toxic insult to both male and female reproductive systems [28, 29]. Heavy metals produce cellular impairment of the reproductive system at structural and functional levels.

While exposure to toxic (high-level) doses of heavy metals was examined thoroughly, relatively little data is available with regard to chronic exposure, particularly life-long conditions [24, 30]. There are almost no references in the literature regarding the effects of chronic exposure to very low doses of heavy metals involving multigenerational studies [18]. The problematic issue is the contamination of water and food with very low concentrations of these toxic heavy metals [10]. With chronic exposure to low doses, it is difficult to predict the consequences not only for those who are directly exposed, but also for future generations.

This fact motivated us to conduct multigenerational studies of chronic exposure to sub-toxic (low-level) doses of heavy metals such as in the reproductive experiments presented here. In this study we monitored the reproductive success of rats from the first filial generation whose parents were also exposed to low doses of heavy metals by comparing the number of litters, the number of new-borns, and the number of weanlings.

MATERIALS AND METHODS

Animals

This investigation was carried out on 200 Wistar rats (100 males and 100 females of F1 generation) aged four weeks—(F1) generation of rats and their progeny to age of 28 days. Ten groups of rats were formed according to exposure to heavy metals, including one control group without exposure. The rats were exposed to low doses of heavy metals in the drinking water.

After 28 days, the rats were transferred to another study site as representatives of the second filial (F2) generation. The rats were kept in polyethylene cages, one male and one female with free access to water and food in an air-conditioned animal house at a temperature of 22 ± 2 °C with steady humidity (50 %) and 12 : 12 h light:dark cycle. The experiments were terminated at 78 weeks. The experiments were done at the Central Animal Laboratory of the Faculty of Medicine, Pavel Jozef Šafarik University in Košice, which is accredited for breeding and testing on laboratory animals in compliance with the relevant legislation. The study was approved by the Ethical Committee of the Faculty of Medicine and the State Veterinary and Food Administration of Slovak Republic (No. Ro-7879/04-220/3).

Experimental protocol (Table 1)

All animals received standard food for laboratory animals (Larsen diet, commercially prepared by Velaz Praha, Czech Republic), with the content of heavy metals not exceeding the level of the natural environmental load. The animals were divided to 10 groups (10 females and 10 males, 1 pair in each cage). The first group C (control, n = 20) received pure water only. The second group Pb (n = 20) received drinking water containing basic lead acetate (Lachema, Brno, Czech Republic) in a concentration of 100 mmol.l^{-1} (20.0 mg.l^{-1} of lead in the drinking water), corresponding to 200 times the maximum allowable concentration (MAC) in water. The third group PbF (n = 20) comprised 10 males not exposed to Pb and 10 female offspring of parents exposed to lead that received daily 2.35 mg.kg^{-1} body weight (bw) Pb by gavage, equivalent to the daily dose of their parents. The fourth group PbM (n = 20) comprised 10 females not exposed to Pb and 10 male offspring of parents exposed to lead that received daily 2.35 mg.kg^{-1} bw Pb by gavage, equivalent to the daily dose of their parents. The fifth group Hg (n = 20) received drinking water containing mercuric chloride (Lachema, Brno, Czech Republic) in a concentration of 1 mmol.l^{-1}(0.2 mg.l^{-1} of mercury in drinking water), corresponding to 200X the MAC in water. The sixth group HgF (n = 20) comprised 10 males not exposed to Hg and 10 female offspring of parents exposed to mercury that received daily 0.022 mg.kg^{-1} bw of Hg by gavage, equivalent to the daily dose of their parents. The seventh group HgM (n = 20) comprised 10 females not exposed to Hg and 10 male offspring of parents exposed to mercury that received daily 0.022 mg.kg^{-1} bw Hg by gavage, equivalent to the daily dose of their parents. The eighth group Cd (n = 20) received drinking water containing cadmium chloride dehydrate (Lachema, Brno, Czech Republic) in a concentration of 20 mmol.l^{-1} (i. e., 2.0 mg.l^{-1} of cadmium in drinking water), corresponding to 200X the MAC in water. The ninth group CdF (n = 20) comprised 10 males not exposed to Cd and 10 female offspring of parents exposed to cadmium that received daily 0.17 mg.kg^{-1} bw Cd by gavage, equivalent to a daily dose of their parents. The tenth group (CdM; n = 20) comprised 10 females not exposed to Cd and 10 male offspring of parents exposed to cadmium that received daily 0.17 mg.kg^{-1} bw Cd by gavage, equivalent to a daily dose of their parents.

All groups were monitored daily and evaluated for the following parameters: animal weight, food intake, and wa-

Table 1. Subdivision of rats into the groups (200 rats—10 females and 10 males/group)

Group	Mark	Sex	Exposure	Parents
1	C	F, M	Control, not exposed	Unexposed
2	Pb	F, M	Received drinking water containing 20.0 mg.l^{-1} of lead in drinking water	Exposed as their offspring
3	PbF	F	Received daily 2.35 mg.kg^{-1} bw of lead by gavage, equivalent to a daily dose of their parents	Received drinking water containing 20.0 mg/l^{-1} of lead in drinking water
		M	Unexposed	Unexposed
4	PbM	F	Unexposed	Unexposed
		M	Received daily 2.35 mg.kg^{-1} bw of lead by gavage, equivalent to a daily dose of their parents	Received drinking water containing 20.0 mg/l^{-1} of lead in drinking water
5	Hg	F, M	Received drinking water containing 0.2 mg/l^{-1} of mercury in drinking water	Exposed as their offspring
6	HgF	F	Received daily 0.022 mg.kg^{-1} bw of mercury by gavage, equivalent to a daily dose of their parents	Received drinking water containing 0.2 mg/l^{-1} of mercury in drinking water
		M	Unexposed	Unexposed
7	HgM	F	Unexposed	Unexposed
		M	Received daily 0.022 mg.kg^{-1} bw of mercury by gavage, equivalent to a daily dose of their parents	Received drinking water containing 0.2 mg/l^{-1} of mercury in drinking water
8	Cd	F, M	Received drinking water containing 2.0 mg/l^{-1} of cadmium in drinking water	Exposed as their offspring
9	CdF	F	Received daily 0.17 mg.kg^{-1} bw of cadmium by gavage, equivalent to a daily dose of their parents	Received drinking water containing 2.0 mg/l^{-1} of cadmium in drinking water
		M	Unexposed	Unexposed
10	CdM	F	Unexposed	Unexposed
		M	Received daily 0.17 mg.kg^{-1} bw of cadmium by gavage, equivalent to a daily dose of their parents	Received drinking water containing 2.0 mg/l^{-1} of cadmium in drinking water

F — female; M — male; bw — body weight

ter intake. Every week toxicological parameters were assessed: intake of heavy metal per kg of body weight of a rat, average daily dose (ADD) in the weight of heavy metal/kg bw/d during the experiments. Every week the number of litters, the number of newborns (determined on the date of birth), and the number of weanlings (determined on the 28th day after birth) were assessed.

Statistical methods

The statistical significance was examined by the Student's t-test or one-way analysis of variance (ANOVA) with the Newman-Keuls *post hoc* test. The significance was set to $P < 0.05$.

RESULTS

Our research encountered some problems with the evaluation of its results. It was difficult to determine whether the exposure was low-, medium-, or high-dose exposures or whether it was acute or chronic exposures. According to L u k a č i n o v á et al. [19], exposure to heavy metals in drinking water in our experiment should be classified as a low-dose exposure, as it is at a level normally found in the environment. Toxicological exposure parameters are presented in Table 2. Exposure assessment and the subsequent health risks are the most important steps in environmental toxicology. The basic unit is ADD (average daily dose). The reproductive period (the period with litters) in this inves-

Table 2. Basic toxicological parameters in week 78 of the trial

Parameter	Pb	Hg	Cd
Total dose received during the experiment in mg.kg^{-1} bw	1.23	12.9	93.5
% LD50	26.4	34.8	41.6
ADD in mg^{-1}.kg^{-1}.d^{-1}	2.35	0.022	0.17

Pb — exposure to lead (20 mg Pb.l^{-1} in drinking water or daily 2.35 mg.kg^{-1} bw of lead by gavage, respectively); Hg — exposure to mercury (0.2 mg Hg.l^{-1} in drinking water or daily 0.022 mg.kg^{-1} bw of mercury by gavage, resp.); Cd — exposure to cadmium (2.0 mg Cd.l^{-1} in drinking water or daily 0.17 mg.kg^{-1} bw of cadmium by gavage, respectively); LD50 — 50 % lethal dose; ADD — average daily dose; bw — body weight.

Table 3. Reproductive parameters in the week 78 of the trial

Group	Number of litters	Number of newborns	Number of newborns/litter	Number of weanlings	% W
C	99	766	7.73	698	91.1
Pb	82*	725*	8.84*	530*	73.1**
PbF	91*	787*,+	8.65	548*,+	69.6**
PbM	82*	735*,x	8.96*	653+,x	88.8*,x
Hg	101	772	7.64	471*	61.0*
HgF	103	773	7.50	482*	62.4*
HgM	108*,+,x	797*,x	7.38	625+,x	78.4**,++,xx
Cd	95	752	7.92	677	90.0
CdF	101+	762	7.54	717*,+	94.1*,+
CdM	105*,+,x	768	7,31	687x	89.5xx

Pb — exposure to lead (20 mg Pb.l^{-1} in drinking water or daily 2.35 mg.kg^{-1} bw of lead by gavage, respectively); Hg — exposure to mercury (0.2 mg Hg.l^{-1} in drinking water or daily 0.022 mg kg^{-1} bw of mercury by gavage, respectively); Cd — exposure to cadmium (2.0 mg Cd.l^{-1} in drinking water or daily 0.17 mg kg^{-1} bw of cadmium by gavage, respectively); W — percentage of weanlings from the total number of newborns; bw — body weight; * — significance $P < 0.05$ against to C group; ** — significance $P < 0.0001$ against to C group; + — significance $P < 0.05$ against to Pb, Hg, and Cd group, respectively; ++ — significance $P < 0.0001$ against to Pb, Hg, and Cd group, respectively; x — significance $P < 0.05$ between F and M in same exposed groups; xx — significance $P < 0.0001$ between F and M in same exposed groups.

tigation lasted from week 13 to week 78. Reproductive parameters are shown in Table 3.

The number of litters in the groups exposed to mercury and cadmium were higher compared to the control, whereas in the groups exposed to lead, the number of litters were lower. The dynamics of the litters showed that the number of litters in the control group gradually increased up to week 39 and then declined. The number of litters increased up to 26 weeks after birth in the Hg, HgF, HgM, and Pb groups, and in this period the number of litters were greater than in the control group. After week 26, the number of litters declined. The reproductive period in the Pb, PbM, Hg, and HgF groups lasted until week 65. The number of litters in the PbM group peaked on week 13 and then declined. Although the number of litters is itself a little probative, in the context of other reproductive parameters, it is suitable for complex assessment of the reprotoxicity of heavy metals.

The number of newborns was the highest in the groups exposed to mercury and was also significantly higher in the group in which only females were exposed to lead. In the group exposed to cadmium, the number of newborns was similar to that of the control group. Interestingly, in the group with only females exposed to mercury, the number

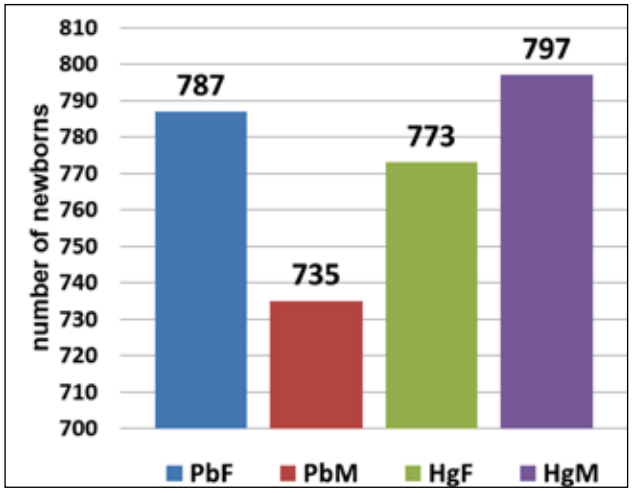

Fig. 1. Number of newborns during the reproductive period (P < 0.05 between PbF, PbM, HgF and HgM)

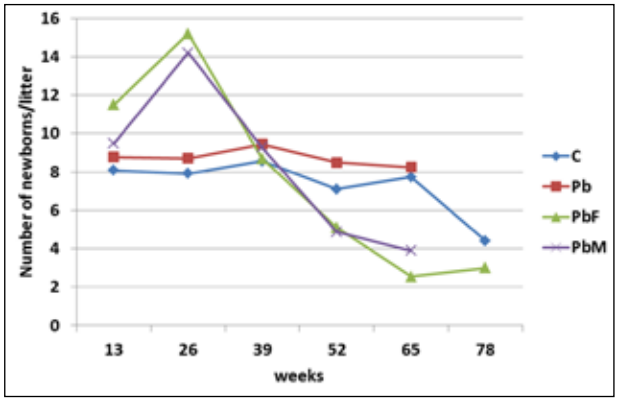

Fig. 2. The newborns/litter during the reproductive period

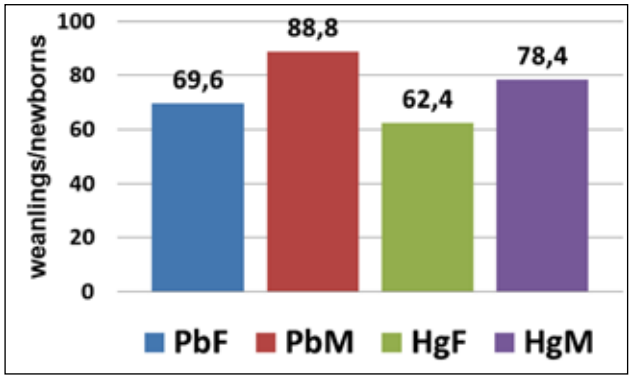

Fig. 3. The percentage of weanlings from newborns during the reproductive period (P < 0.05 between F and M)

of newborns was lower but in the group with only males exposed to mercury, the number of newborns was higher. The opposite was seen in the groups exposed to lead, where in the PbF group the number of newborns was higher in compared to the PbM group, where it was lower (Fig. 1).

The number of newborns per litter and the percentage of weanlings from newborns during the reproductive period is shown in Figs. 2 and 3.

DISCUSSION

The data indicated a difference in the sensitivity to various toxic heavy metals between the sexes [11, 31, 33]. The higher numbers of litters and newborns have led us to believe that in this case there is some adaptive response [7, 23] to the increased background of toxic heavy metals in the environment in environmentally compromised populations. These adaptive responses are well known in bacteria [8], plants and lower animals [3, 4, 22]. Other authors also observed an increased number of offspring followed by a high mortality during the first two weeks of their life after chronic exposure to high doses of cadmium [24, 30].

We assume that exposure to low concentrations of heavy metals activate biological mechanisms leading to maintenance of the species (particularly the activation of reproductive function), and the negative effects begin to occur after exceeding a certain exposure (dose) to the heavy metal.

The number of newborns per litter is one of the most commonly used indicators of reprotoxicity [9]. It was surprising that the highest number of newborns per litter was observed in the group exposed to lead, even though the number of litters was the lowest. Compared to the control group, only in Pb and PbM groups the difference was significantly ($P < 0.05$) higher; the other groups did not differ significantly (Fig. 2). In our opinion, this is related to a hormetic effects after exposure to low doses of heavy metals [5, 15, 16]. The nature of hormesis was described in detail by C a l a b r e s e [6].

A very important parameter of reproductive toxicity is the number of weanlings (individuals who live up to day 28 of life) and especially the percentage of weanlings from the number of newborns in the litter. At the end of the experiment, the lowest numbers of weanlings were in groups exposed to mercury, followed by those in groups exposed to lead. The number of weanlings in groups exposed to cadmium was comparable to that in the control group.

The highest percentage of weanlings was found after exposure to cadmium, which was comparable to the control group. The lowest percentages of weanling were found after exposure to mercury and lead ($P < 0.05$). After exposure to mercury and lead in groups with only male exposure, unexposed females took care of the offspring and may account for the significantly higher percentage of weanlings ($P < 0.05$) compared to groups with only female exposure or with both male and female exposure. The exposed females are less effective at taking care of the offspring (Fig. 3). This phenomenon could involve epigenetic and neurobiological mechanisms [2]. In contrast, after exposure to cadmium, there was a higher percentage of weanlings, which may be the result of the epigenetic phenomena of adaptation [20, 21, 32]. The high percentage of weanlings after exposure to cadmium can be attributed to the fact that low doses of cadmium may have the character of essential elements [1, 13, 27].

CONCLUSIONS

The knowledge obtained during our study allows us to state that the reproductive success after chronic (lifetime) exposure to low doses of toxic heavy metals in drinking water may involve a number of biological mechanisms:

1. Adaptation to a toxic environmental background results in an increase in the number of litters and newborns (vulnerable populations increase reproductive activity).
2. There exist sex differences in the reproductive success of exposed individuals as certain toxic metals act more on the reproductive capabilities of males while other affect the females more.
3. Unexposed mothers take better care of their offspring than the exposed ones.
4. Low doses of certain toxic heavy metals, including mercury and lead, may have hormetic effects.
5. Some heavy metals, including cadmium, may exhibit essential characteristics related to reproductive success in animals.
6. There is an indication that epigenetic mechanisms are involved in the adaptation to a background with low levels of toxic heavy metals.

Further studies are required to support the above conclusions. Obtaining detailed knowledge of the relevant processes and their precise mechanisms can significantly contribute to the reproductive success of vulnerable populations.

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