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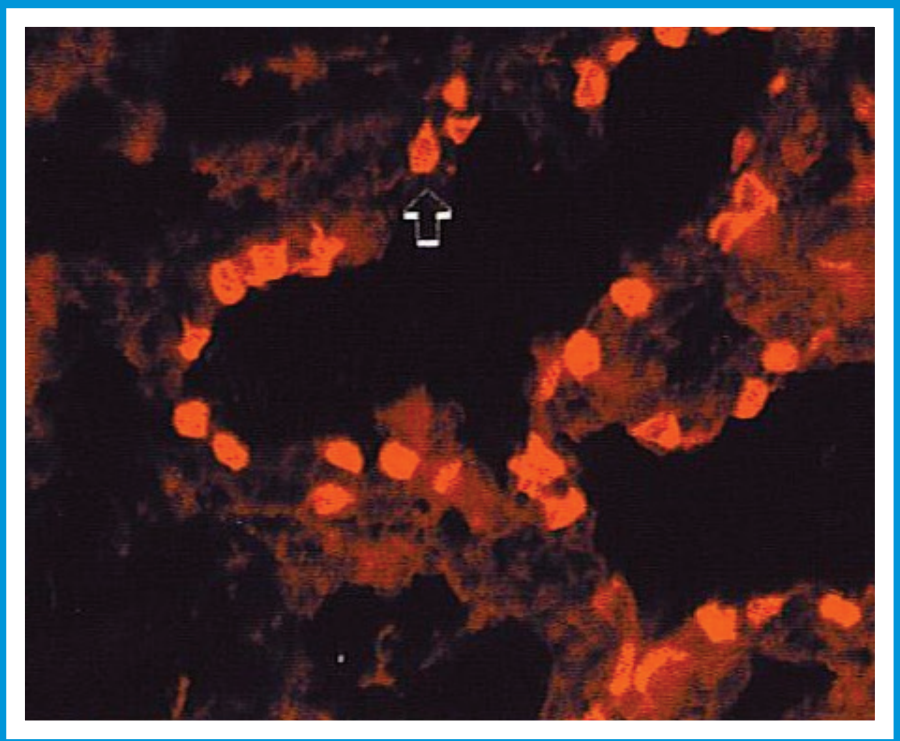

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Original Scientific Article

CYTOKERATIN EXPRESSION IN MOUSE MAMMARY GLAND DURING FIRST FIVE WEEKS POST-PARTUM

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Summary: Changes during mammary gland development can be detected with methods using specific antibodies directed against specific cell structures. In the present study, the expression pattern of a type of intermediate filament called cytokeratins (CKs) was evaluated in tissue samples from mice mammary glands during the first five weeks post-partum (pp). Animals were divided into 5 homogeneous groups with 8 mice in each. Immunofluorescence and immunohistochemical staining procedures were used to determine various characteristics of different cells in the mammary gland. Several CKs were analyzed with specific markers and immunohistochemistry methods: CK5, CK7 and CK14 were detected in all weeks pp, although in different cell types; CK8 was positive in all periods except at week 1 pp; CK6, CK16 and CK19 were partially identified; CK1 and CK13 were not observed during the trial; and vimentin was detected in fibroblasts and fatty cells. It is known that CK expression varies with physiological and pathological changes, and it has been reported to mark different epithelial cell lineages; its evaluation is therefore of considerable importance for studies of breast cancer of a stem/progenitor cell origin, both in humans and animals. Our trial provides additional knowledge relative to the use of specific antibodies and techniques as valuable tools to detect CKs during early post-partum (pp) mice mammary gland development (weeks 1 to 5 pp), emphasizing the role of CKs as markers of mammary epithelial differentiation.

Key words: NMRI mouse; mammary gland; intermediate filaments; cytokeratin; vimentin

Introduction

The most resistant elements inside the cytoskeleton are the intermediate filaments. They form a cytoplasmic network, provide mechanical strength to cells, interact with other components of the cytoskeleton, and regulate protein localization and intracellular signaling (1). They are prominent in the cytoplasm of cells that are subjected to mechanical stress (2, 3), indicating that intermediate filaments are essential to cell growth and size because they regulate protein synthesis.

This multigene family is composed of more than sixty components, which can be subdivided into six categories classified into specific cell types according to their sequence homology, gene structure and assembly properties; the fifth category has its own characteristic features (4).

Cytokeratins (CKs), a set of polypeptides of different molecular weights, comprise the main type of intermediate filaments in epithelial cells and provide scaffold structures within cells (5). Immunohistochemical methods have been used to

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study the cellular expression and distribution of CKs, as well as to determine whether progression of the epithelium is accompanied by changes in these cytoskeletal structures. Furthermore, although murine strains do not show differences in CK expression, it is known that animal species can exhibit different CK polypeptides, and clear differences have been reported between CKs in mouse and rat hepatocytes, in contrast to epidermis CKs (6).

CK composition is extremely heterogeneous and depends on various factors such as level of differentiation or anatomical location. Little is known about intermediate filaments in the mammary gland, which is composed of four cellular types (7): myoepithelial cells, luminal alveolar cells, luminal ductal cells and basal cells. Mammary gland arises from the surface ectoderm during embryogenesis, relying on reciprocal epithelial-mesenchymal interactions for morphogenesis. Following birth, although mouse mammary gland grows isometrically with the body, it is rapidly expanded via branching during puberty from week 3 post-partum (pp) (8) and it develops the lactating function from week 5 pp (9) what culminates in a definitive mature gland at week 12 pp (8). Moreover, CK expression can be analyzed according to the different stages of mammary gland development and can be modified by the loss of normal tissue architecture, such as occurs in tumor progression and metastasis (10, 11), which can be useful as a diagnostic test using anti-CK antibodies (12, 13). In fact, gene expression studies have shown that basal-like breast tumors are associated with expression of basal-type CKs, such as CK5, CK6 or CK14 in humans (12, 14), and that CK8, CK18, CK19 and vimentin can alter their expression profiles during tumor development in animal models (5). Expression of specific CKs has been found to mark different epithelial cell lineages; therefore, their analysis can be particularly useful in studies of breast cancers of a stem/progenitor cell origin, both in humans and animals (8).

CK1 and CK10 are synthesized in the epidermis (15), CK3 and CK12 (16) as well as the CK complex 8/18 (17) in corneal tissue, CK4 and CK13 in the esophagus, CK5 and CK14 in the basal layer of stratified epithelia, CK6 in terminal end buds (18), CK7, CK8 and CK19 in simple epithelia, and CK13 in non-keratinized stratified epithelia (13). Due to morphological and biochemical changes in mammary glands, CK expression can be lost, but can be detected by immunohistochemistry in areas of myoepithelial proliferation, as well as enhanced expression of vimentin in proliferative areas with osseous or chondroid metaplasia (19). Immunohistochemical procedures using paraffin-embedded specimens are the method of choice to evaluate protein expression at a cellular level while preserving tissue architecture in normal and neoplasic tissues (20), and can be enhanced by immunofluorescence techniques in order to improve the identification of some structures (8).

In the present study, we used immunohistochemical and immunofluorescence methods to analyze and compare patterns of CK expression during mammary gland development from weeks 1 to 5 pp in experimental mice models.

Materials and methods

Animals and experimental design

The present study was carried out on 40 female Naval Medical Research Institute (NMRI) mice, selected because of their high reproductive capacity and low incidence of spontaneous mammary tumors before week 52 pp. NMRI mice were weighed one day before the beginning of the study and divided according to their average weight (20-40 g) into 5 homogeneous groups (weeks 1 to 5 pp) of 8 mice in each:, given that lactating mammary glands are developing from week 5 pp in accordance with Mínguez-González (9), and these weeks were established as different stages. Animals were housed in plastic cages measuring 180 cm^2 and maintained in a temperature controlled room (22-23ºC) on a twelve-hour light/dark cycle. Food and water were available ad libitum throughout the experiment. No clinical signs of parasitic or infectious diseases were observed, and faecal analyses (flotation, sedimentation and larval migration) were negative in all mice at the beginning of the tests.

Groups were sacrificed by cervical dislocation followed by immediate exsanguination. The study was carried out in accordance with the VICH guidelines for "Technical requirements for registration of veterinary medicinal products". The protocol of the experiment was approved by the ethics committee of the Faculty of Veterinary Sciences (León, Spain), where the trial was conducted.

Table 1: Cytokeratin (CK) and vimentin expression during weeks 1-5 post-partum (pp) in mouse mammary gland

ANTIBODY USED AND CYTOKERATIN REVEALED	WEEKS POST-PARTUM				
	1	2	3	4	5
Anti-CK5	++	++	++	++	++
LLO01 (CK14)	+	+	+	+	-
LP1K (CK7)	+	+	+	+	+
LP2K (CK19)	+/-	-	-	-	+/-
LLO20 (CK6)	-	-	-	-	+
Anti-CK6 (CK6)	-	-	-	-	+
LLO25 (CK16)	+/-	-	+/-	-	+
TROMA 1 (CK8)	-	++	++	++	++
Anti-CK1	-	-	-	-	-
Anti-CK13	-	-	-	-	-
Vimentin	+*	+*	+*	+*	+*

Key: (-) no stain; (+) positive stain in 10-50% cells; (++) positive stain in more than 50% cells; (+/-) variable result in the mammary tissue studied; (+*) positive stain in the mammary stroma.

Immunofluorescence technique

Left and right abdominal and thoracic glands were taken for this experiment. Samples from mammary glands were dissected and embedded in "Tissue-Tek ® CRYO-OCT" compound (Fisher Scientific, Spain), frozen in liquid nitrogen and cut into 5 μm-thin sections using a "Leitz 1720 Cryostat Microtome" (Wetzlar, Germany).

Frozen sections were subjected to indirect immunofluorescence in accordance with the protocol described by Mínguez-González (9). The expression of CK subtypes was evaluated using different antibodies on frozen tissue sections by means of indirect immunofluorescence (IFI). The following primary antibodies were used: anti-CK1 (rabbit, 1:500), anti-CK5 (rabbit, 1:500), anti-CK6 (rabbit, 1:500), LP1K anti-CK7 (mouse, 1:1), TROMA 1 anti-CK8 (rat, 1:4), anti-CK13 (rabbit, 1:500), LLO01 anti-CK14 (mouse, 1:1), LL025 anti-CK16 (mouse, 1:1) and LP2K anti-CK19 (mouse, 1:1) (Jackson ImmunoResearch Labs., USA), and clone LN-6 anti-vimentin (mouse, 1:100) (Sigma-Aldrich, Spain). Secondary incubation was performed according to the protocol described by Sun et al. (8), although Fluorescein-Isothiocyanate-Fluorochrome (FITC)-conjugated goat anti-rat, Texas-Red (TR)-conjugated anti-mouse and anti-rabbit secondary antibodies (Jackson ImmunoResearch Labs., USA) were used here. In addition, vimentin was examined using a streptavidin-biotin peroxidase complex commercial kit (Santa Cruz Biotechnology, Spain) on tissue embedded in paraffin wax.

The expression of CK subtypes was also evaluated by double-IFI. This technique was carried out to detect CK8 (TROMA 1 anti-CK8, rat, 1:2) with CK5 (anti-CK5, rabbit, 1:250) and CK6 (anti-CK6, rabbit, 1:250). The entire primary incubation was performed with TROMA 1/anti-CK5 or TROMA 1/anti-CK6, and the secondary incubation was performed with FITC-conjugated goat anti-rat for TROMA 1 and with TR-conjugated anti-rabbit for anti-CK5 and anti-CK6.

Slides were mounted in "Antifade medium" (Vector Labs., Cambridgeshire, UK), and images were taken using a "Leitz Diaplan Microscope" (Wetzlar, Germany) equipped with automatic fluorescein and rhodamine filter sets, with a "Wild MPS 51S" camera with a special "Kodakcolor VR 400S" film for immunofluorescence.

Immunohistochemical staining procedures

Left and right abdominal and thoracic glands were also taken for this method. Samples from these mammary glands were excised, fixed in 10% buffered formalin for at least 24 hours at

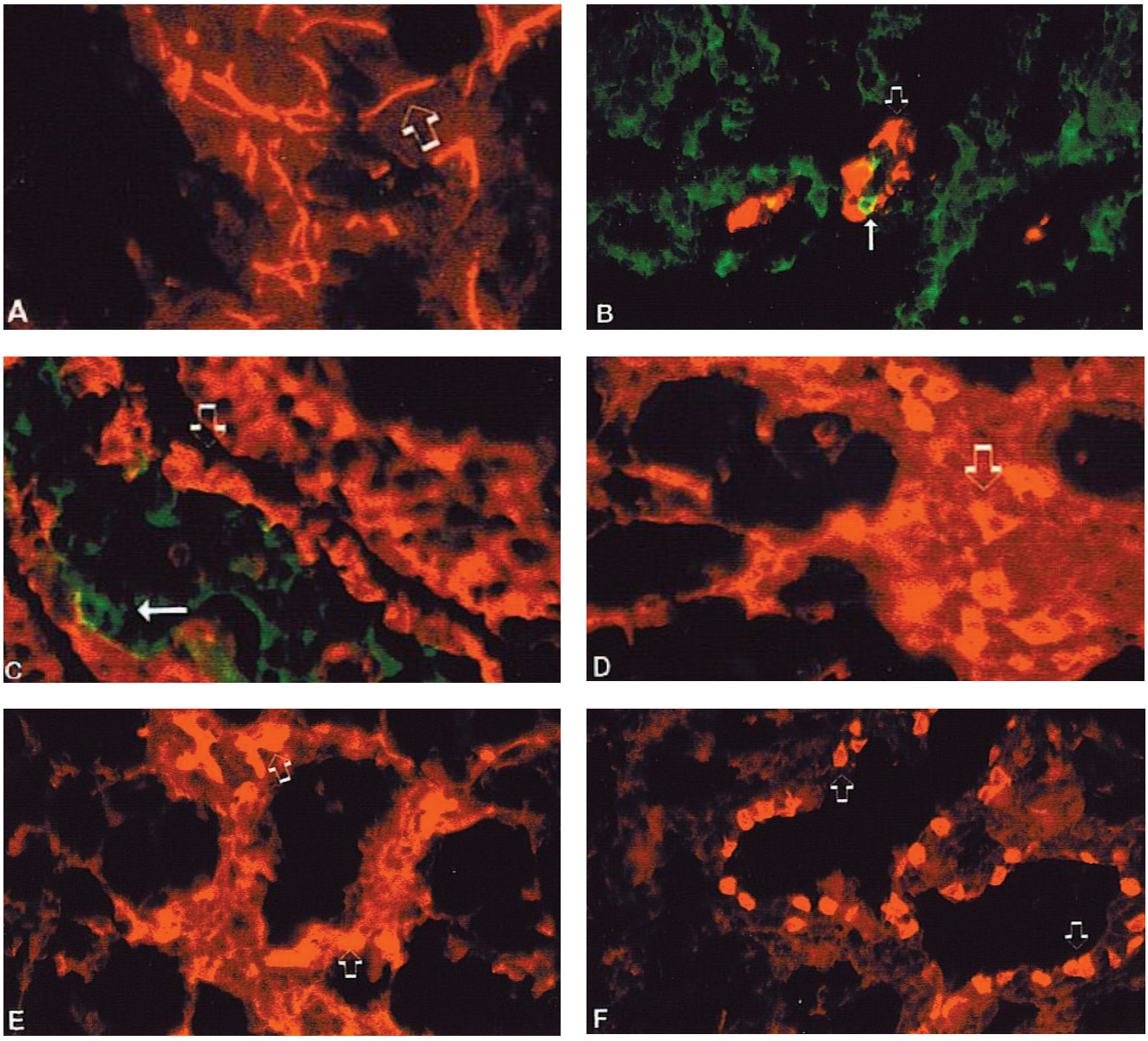

Figure 1: A. Expression of CK5 in mouse mammary gland during week 3 post-partum (pp). The thick arrow (⇒) indicates myoepithelial cells. IFI [anti-CK5 / 25x], **B.** Mammary tissue during week 3 pp. Luminal alveolar cells expressing CK8 are shown in green and indicated by a thin arrow (→). Myoepithelial cells around mammary alveoli expressing CK5 are shown in red and indicated by a thick arrow (⇒). Double-IFI [TROMA 1/anti-CK5 / 40x], **C.** Alveolus during week 3 pp. CK5 is shown in red and indicated by a thick arrow (⇒). CK8 is shown in green and indicated by a thin arrow (→). Double-IFI [TROMA 1/ anti-CK5 / 40x, **D.** CK7 expression in cells from lactating mammary glands, indicated by a thick arrow (⇒). IFI [LP1K / 40x], **E.** CK7 expression in scattered cells, indicated by a thick arrow (⇒). IFI [LP1K / 40x], **F.** Luminal ductal cells with CK7, indicated by a thick arrow (⇒). IFI [LPIK / 40x]

room temperature, embedded in paraffin wax, cut into 4 μm-thin sections using a "Leitz 1512 Rotatory Microtome" (Wetzlar, Germany) and examined for reactivity to the same panel of immunohistochemical markers as described for the immunofluorescence technique and using a streptavidin-biotin peroxidase complex commercial kit (Santa Cruz Biotechnology, Spain), in accordance with Rabanal and Else (21) and Mínguez-González (9), scoring the slides as negative (-) for no reactivity, (+) for weak reactivity, (++) for moderate reactivity and (+++) for strong reactivity (Table 1). Images were taken using an "Olympus AX70 Upright Compound Microscope" (Hamburg, Germany) with a "Multi Control Box U-MCB" equipped with "Kodakcolor VR 100S" film for immunohistochemistry.

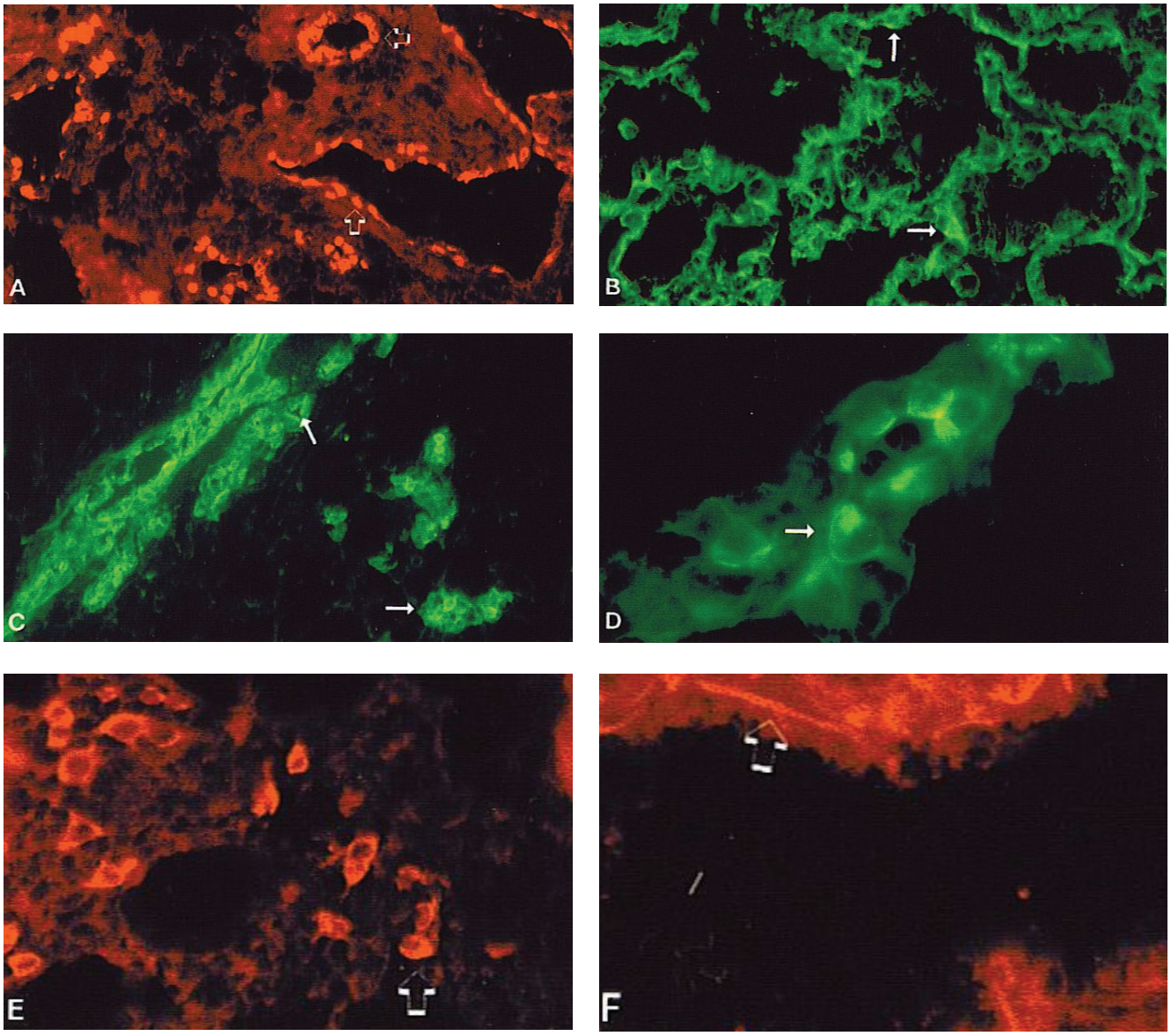

Figure 2: A. Tubular cells with CK7, indicated by a thick arrow (⇒). IFI [LP1K / 40x], **B.** Mammary gland during week 3 pp. CK8 expression in luminal alveolar cells, indicated by a thin arrow (⇒). IFI [TROMA 1 / 25x]. **C.** Mammary ducts stained with TROMA 1 antibody, indicated by thin arrow (→). IFI [TROMA 1 / 25x]. **D.** Mammary ducts stained with TROMA 1 antibody, indicated by a thin arrow (→). Intensity is more evident in the luminal segment of the cells. IFI [TROMA 1 / 100x], **E.** CK16 expression in isolated cells of a lactating mammary gland, indicated by a thick arrow (⇒). IFI [LL025 / 40x], **F.** CK14 expression in a mammary gland during week 1 pp. Myoepithelial cells surrounding mammary alveoli are indicated by a thick arrow (⇒). IFI [LLO01 / 40x]

Both immunofluorescence and immunohistochemical techniques were performed with negative controls where no primary antibody was added to the sections.

Results

CK5 was recognized by polyclonal antibody anti-CK5 and its expression pattern was similar from weeks 1 to 5 pp. Myoepithelial cells reacted with great intensity to the anti-CK5 and their characteristic cytoplasmic projections ("basket cells") surrounding the mammary alveoli could be observed (Figures 1A, 1B and 1C). Basal cells were positive in all samples (Table 2).

Regarding CK14, the LLO01 antibody produced a moderate reaction in myoepithelial (Figure 2F), luminal ductal and luminal alveolar cells, whereas basal ductal cells were negative in all the stages analyzed. During week 5 pp, only a few myoepithelial cells were detected (Table 2).

Table 2: CK expression in mouse mammary gland – Cellular types

CELLULAR TYPE				
Post-partum	Myoepithelial cells	Luminal alveolar cells	Luminal ductal cells	Basal cells
Week 1	CK5 and CK14	CK7, CK14 and CK16	CK7, CK14 and CK19	CK5
Week 2	CK5, CK7 and CK14	CK7, CK8 and CK14	CK7, CK8 and CK14	CK5
Week 3	CK5 and CK14	CK7, CK8, CK14 and CK16	CK7, CK8 and CK14	CK5
Week 4	CK5 and CK14	CK7, CK8 and CK14	CK7, CK8 and CK14	CK5
Week 5	CK5 and CK14	CK6, CK7, CK8 and CK16	CK7, CK8 and CK19	CK5

Some luminal alveolar and luminal ductal cells expressed CK7 in lactating mammary glands (Figure 1D). During week 5 pp, some isolated alveoli reacted positively to CK7 (Figure 1E) and only two mice showed this CK in every luminal ductal cell (Figures 1F and 2A). An increase was observed in the number of positive cells for CK7 as the stages progressed. LP1K antibody appeared in isolated myoepithelial cells during week 2 pp, as well as during weeks 1 to 5 pp in luminal alveolar and luminal ductal cells (Tables 1 and 2).

While CK19 was partially identified with LP2K during weeks 1 and 5 pp, CK6 was slightly positive with LLO20 and anti-CK6 during week 5 pp, and CK16 was detected with LL025 in some cells during weeks 1, 3 and 5 pp (Figure 2E) (Tables 1 and 2).

CK8 was recognized by the monoclonal antibody TROMA 1. There was a clear response, distributed in the same way in both luminal alveolar and luminal ductal cells (Figures 2B, 2C and 2D) in the mammary gland during weeks 2, 3 and 4 pp. There was an intense reactivity in luminal ductal cells in mammary samples from week 5 pp (Tables 1 and 2).

In contrast, CK1 and CK13 were negative in all stages, while vimentin was present in fibroblasts and fatty cells (Tables 1 and 2).

Discussion

Several CKs are lineage markers within the mammary epithelium (8) and can be analyzed by immunohistochemical and immunofluorescence procedures, providing relevant information on differentiation processes and cellular interaction dynamics (22). In histological sections it can be observed that the mouse mammary gland is mainly composed of adipose tissue with ducts and alveolar lobules scattered within it. Lobule-alveolar development reaches its peak during pregnancy and lactation; after these situations the mammary gland changes towards small ducts and terminal branches (23). Therefore, cellular differentiation implies obvious changes in stain, related to the pattern of CK expression, the nature of which is conserved throughout the mammary tree (20).

Luminal and basal cells express differentiation markers that gradually increase during mammary morphogenesis (24). During puberty and later, myoepithelial cells can be distinguished from luminal cells thanks to the expression of CK5. In the present study, myoepithelial cells expressed both CK5 and CK14 (typical for stratified epithelia) in all stages, and basal cells expressed CK5. These results agree with previous reports for mice (18, 25), dog (26) and human (27, 28) mammary glands. Furthermore, Sun et al. (8) detected CK5 expression in embryonic and prepubertal mammary glands. CK5 and CK14 usually appear in the basal layer of the epidermis (2). Moreover, the expression of these two CKs should be asynchronous during mammary gland development; this could be due to their link to other CKs through different binding proteins (29).

CK7, an important marker of ductal differentiation, was detected in all the stages analyzed, whereas CK19 expression was unclear. This finding is similar to those reported in previous experiments on human mammary glands (30, 31). It is known that CK19 is a typical marker of cholangiocytes with specific localization in the mammary gland, and it has been widely reported in cases of fibrosis provoked by helminthes such as *Fasciola hepatica* (32). An increase was observed in the number of cells positive for CK7

as lactation progressed, confirming that changes in cellular cytoskeleton are only detectable by immunohistochemistry, and not by conventional histology.

Our results showed that few cells expressed CK6 during week 5 pp, and CK16 was expressed during weeks 1, 3 and 5 pp. After using IFI in rat mammary samples, Lichtner et al. (7) failed to detect CK6, as did García et al. (33) and Mikaelian et al. (20) in mouse mammary glands; whereas Sapino et al. (18) identified CK6 in the mouse mammary tubular end buds. Using immunofluorescence, Smith et al. (34) detected the presence of CK6 in tubular end buds and in some cells from intralobular ducts, leading scientists to consider it as a marker (35, 36) for mammary pluripotent cells. CK6 is associated with epidermal proliferation and is expressed in epidermal hyperplasia in conjunctiva, oral mucosa and in some carcinomas (37, 38). In addition, Sun et al. (8) reported the presence of CK6 in most regions of embryonic and early postnatal mammary glands, and Grimm et al. (39) reported that the cross sections of nearly all embryonic mammary gland cells stained positive for this CK.

In contrast to the findings of previous studies on mice and rats (7, 40), we did not detect CK8 during week 1 pp. This may have been due to alterations in the cellular cytoskeleton structure as a result of physiological changes induced by hormonal status during week 1 pp, rendering CK8 inaccessible to the specific marker. Luminal cells have also been reported to be positive in other studies on human and animal models (8, 41, 42).

In contrast to humans, CK13 is not expressed in mouse mammary glands. Something comparable occurs with CK1 and CK10, which are only expressed in carcinogenic processes (43), particularly in transgenic mice and in human carcinomas (44). These results are similar to the negative detection of CK1, CK10 and CK13 at any developmental stage of the mouse mammary gland reported by Mikaelian et al. (20). Vimentin expression was similar to that found in the assay reported by Asch and Asch on mice (40) and others on dog (26) and human (45).

In conclusion, this report presents a comparative analysis of CK expression during the first five weeks pp. CK expression varies with physiological changes and tumor cells. Our study explored the use of specific CKs as markers of mammary epithelial differentiation in mice as an experimental model. By means of immunofluorescence techniques with specific antibodies for each CK, we detected several differences in the expression of these proteins among different cells from the same specimen. In addition, inter-species comparisons indicate similarities in relation to the pattern of CK expression.

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IZRAŽENOST CITOKERATINOV V MLEČNIH ŽLEZAH MIŠI V PRVIH PETIH TEDNIH PO KOTITVI

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Povzetek: Spremembe v mlečni žlezi med njenim razvojem lahko opazujemo z uporabo specifičnih protiteles proti posameznim celičnim strukturam. V opisani raziskavi smo ugotavljali izraženost vmesnih filamentov, imenovanih citokeratini (CK), v vzorcih tkiva mišje mlečne žleze pet tednov po kotitvi. Preiskovane miši so bile razdeljene v pet skupin po osem živali, v njihovih mlečnih žlezah pa smo s pomočjo imunofluorescenčne imunohistokemične metode ugotavljali značilnosti tkiva mlečne žleze. Ugotavljali smo izraženost različnih tipov CK. Prisotnost CK5, CK7 in CK14 smo zaznali pri vseh časovnih točkah, vendar pa so bili izraženi v različnih tipih celic. CK8 smo zaznali pri vseh časovnih točkah, razen prvi teden po kotitvi, CK6, CK16 in CK19 smo zaznali samo v nekaterih vzorcih tkiva, izraženosti CK1 in CK13 pa nismo zaznali v nobenem vzorcu. Poleg CK-jev smo v vseh preiskovanih vzorcih ugotovili tudi izraženost beljakovine vimentin, in sicer v maščobnih in vezivnih celicah (fibroblasti). Znano je, da je izraženost različnih CK-jev različna v različnih tkivih, da se lahko spreminja glede na fiziološko stanje tkiva in da so nekateri tipi CK-jev specifično izraženi v posameznih stopnjah razvoja epitelijskih celic. Poznavanje izraženosti posameznih tipov CK je zato pomembno za razumevanje razvoja posameznih celičnih linij epitelijskih celic mlečne žleze, ki lahko predstavljajo tudi izvor rakastih celic. Naša raziskava tako predstavlja prispevek o uporabi specifičnih protiteles kot metode za ugotavljanje izraženosti različnih tipov CK v času razvoja mlečne žleze pri miših po kotitvi (od prvega do petega tedna) in kaže na uporabnost različnih tipov CK kot označevalcev razvoja epitelijskhi celic mlečne žleze.

Ključne besede: miši NMRI; mlečna žleza; vmesni filamenti; citokeratin; vimentin

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Original Scientific Article

CONTENT OF MACRO- AND MICROELEMENTS IN THE MILK OF CROATIAN COLDBLOOD MARES DURING LACTATION

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Summary: The concentrations of macro- and microelements in the milk of Croatian Coldblood mares were determined throughout the lactation phases by inductively coupled plasma-optical emission spectrometry. Element contents during days 10–180 of lactation were in the ranges (mg/kg): Ca 585–772, K 547–687, Na 131–165, Mg 56.8–71.0, Cu 0.085–0.14, Fe 0.013–0.41, Zn 1.86–2.15, Se 0.003–0.029. Variation trends for elements were found throughout the lactation stages. However, significantly higher Fe concentrations were found on days 10 and 40 of lactation than on day 60 ($p < 0.05$, both). There were no statistically significant differences between concentrations of other elements during lactation. Moderate correlations were found between the elements: Ca: K, Mg, Se; Mg: K, Cu; Se: Cu, Fe. The element concentrations in horse milk, with the exception of Ca and Cu, fell within the ranges previously reported for different breeds of dairy horses. Cu and Fe contents were lower, while the Zn content was similar to those obtained in different dairy horse breeds. Concentrations of elements obtained in horse milk were lower in comparison to cow and goat milk. Generally, levels of Ca and Mg were higher, Na and K were similar, but Cu, Fe and Zn were lower than those in human milk.

Key words: milk; lactation; horse; micro elements; macro elements; ICP-OES; Croatia

Introduction

Worldwide, hundreds of different breeds of horses are used for milk production. In the European Union, horses have been used for dairy production in Hungary, Austria, Bulgaria, Germany, Belarus and Ukraine (1, 2). The composition and quality of horse milk is attributed to the genetic, physiological, zoohygienic and feeding conditions, and varies among species with regard to the nutritional requirements of their newborns. Horse milk is suitable for human consumption due to the following similarities and differences compared to human milk: similar composition of major protein and immunoglobulins, high levels of polyunsaturated fatty acids, low nitrogen, low cholesterol contents, lower fat content, similar lactose content and higher vitamin C content (1, 3, 4). It has been estimated that more than 30 million people worldwide consume equine milk regularly, and that figure is showing significant annual increases (5).

Milk production in horses depends on the breed and is high during the first week of lactation and increases to a maximum between the first and

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third months (1, 6). Lactation in mares may be of different durations, though it usually lasts one year. The composition of horse milk is sufficient for the foal's demands in terms of nutritional requirements and providing the necessary elements (7). Element content variations were determined during the different lactation stages (5, 8). The elements represent different functions. Calcium and phosphorus have a fundamental role in the rapid skeletal development of the foal, while magnesium plays a part in bone mineralization (4, 9). Furthermore, Na^+ is an important cation in blood and extracellular fluid bathing cells and K^+ is a monovalent cation significant to the maintenance of fluid integrity within the cell.

The element content in horse milk has been studied in different countries (6, 8, 10-13). Differences were found in the element composition between different breeds of horses (4). The largest autochthonous horse breed in Croatia is the Croatian Coldblood (14, 15). Today, this breed is raised in many other parts of the Croatia, though the majority are found in Sisak-Moslavina County and Zagreb County. Throughout much of the year, they are kept in the open countryside. In the past, before the advent of mechanization, the Croatian Coldblood had great economic value as an agricultural labourer. Today, it is used for recreational and tourism purposes and in preserving both the cultural heritage and natural diversity in Croatia (16). In 2008, a total of 2778 Croatian Coldblood mares were registered in Croatia (15).

The aim of this study was to determine the macro- and micro mineral composition of Ca, K, Na, Mg, Cu, Fe, Zn, Mn and Se in the milk of nursing mares of the Croatian Coldblood breed throughout the lactation phases

Materials and methods

Sample collection

Six mares of the Croatian Coldblooded breed reared at horse farms in Lonjsko Polje Nature Park (Central Croatia) were included in this study. Mares were aged from 5 to 11 years and weighed between 650 and 750 kg. Mares were kept under similar conditions of snow barn and were at pasture from spring to autumn, with supplemental feeding of 3 kg oats per day when necessary. Winter feeding was 3 kg hay, 2 kg concentrate and *ad libitum* straw daily. Mares bore foals from late January to early May. Animals were kept in the stalls of owners, while during summer some remained in the nature park, where milk samples were collected. Milk samples (80–100 ml) were collected from February to October 2011 on days 10, 40, 60, 120 and 180 of lactation. Milk was collected by hand milking from a single mammary gland, in the presence of the foal that had been prevented from suckling.

Samples were placed into clean, acid-washed polyethylene bottles, labelled and stored at -18°C until analysis.

Reagents and standards

Analytical reagent grade HNO_3 and H_2O_2 were purchased from Kemika, Croatia. Ultra-pure water (18 MΩxcm) was generated by the purification system NIRO VV UV UF 20 (Nirosta d.o.o. Water Technologies, Osijek, Croatia). Plastic and glassware were cleaned by soaking in diluted HNO_3 (1/9, v/v) and by subsequent rinsing with double deionised water and drying prior to use. In the calibration process, stock standard solutions with the concentrations of Ca, K, Na, Mg, Cu, Fe, Zn and Se (Perkin Elmer, USA) of 1 g/L prepared with diluted HNO_3 (0.5%) were used.

Sample preparation

Milk samples were weighed (2 g) in a PFA digestion vessel and 1 ml of H_2O_2 and 6 ml HNO_3 were added. Acidic digestion of samples were performed by microwave oven Multiwave 3000 (Anton Paar, Ostfildern, Germany) using a two-step digestion program: step I power 800 W, ramped 15 min, 800 W for 15 min; step II power 0 W for 15 min.

Digested samples were diluted to the final volume of 50 ml with ultra-pure water. All samples were run in batches that included blanks, a standard calibration curve and two spiked specimens. The limits of detection were calculated according to three times the standard deviation of ten blank samples (mg/kg): Ca 0.01, Na 0.01, K 0.025, Mg 0.02, Cu 0.01, Fe 0.005, Zn 0.005 and Se 0.001.

Skim milk powder (BCR-063, IRMM, Belgium) was used as certified reference materials for checking the quality of results. The reference material was treated and analysed under the same conditions as the samples. The results showed

good accuracy with certified reference materials and the recovery results for elements were (%): Ca 98.3, K 98.5, Na 96.1, Mg 93.7, Cu 97.7, Fe 94.6 and Zn 98.9. To calculate the recovery percentage for Se, five milk samples were spiked with known amounts of elements. The quality of data showed good accuracy with a recovery rate of 96.9%.

Analysis of elements

An inductively coupled plasma optical emission spectrometer (ICP-OES) with axial and radial viewing plasma configuration Model Optima 8000 (Perkin Elmer, Waltham, Massachusetts, USA) operating at a 40 MHz free-running ratio-frequency and equipped with S 10 autosampler was utilized. The instrumental operating conditions used are shown in Table 1.

Statistical analysis

Statistical analysis was calculated using the Statistica 6.1 software (StatSoft® Inc., Tulsa, USA). Concentrations were expressed as mean ± standard deviation, minimum and maximum values. One-way analysis of variance was used to test for differences in element levels in milk samples. Differences between results were considered significant at $p < 0.05$. Association between variables was examined by calculating simple linear correlations. Significant correlations were declared weak ($r < 0.3$), moderate (r from 0.3 to 0.7) or strong ($r > 0.7$).

Results and discussion

Descriptive statistics of the concentrations of Ca, Na, K, Mg, Cu, Fe, Zn and Se in horse milk are presented in Table 2. For all elements studied, except Zn, the highest mean values were determined at early lactation on day 10 postpartum. Variation trends for all elements were found throughout the lactation stages. Significantly higher Fe concentrations were found on days 10 and 40 of lactation than those measured on day 60 ($p < 0.05$, both). However, there were no statistically significant differences during the lactation phases between concentrations of other elements.

The correlations between the measured macro- and microelements in horse milk were investigated, and moderate and significant positive correlations were found between: Ca and K (r=0.35, $p<0.05$), Ca and Mg (r=0.61, $p<0.001$), Ca and Se (r=0.52, $p<0.01$), Mg and K (r=0.64, $p<0.001$), Mg and Cu (r=0.43, $p<0.01$), Se and Cu (r=0.41, $p<0.05$), Se and Fe (r=0.64, $p<0.001$). In previous reports, moderate to strong positive correlations were found among macroelements, except between Na and Mg in horse milk (5).

Element concentrations in horse milk in different lactation stages obtained in different countries are presented in Table 3. The results obtained in the present study, with the exception of lower Ca and Cu values, fell within the ranges previously reported for different breeds of dairy horses (5, 7, 12, 17-19, 32) (mg/kg): Ca, 544.2–1220; K, 413.1–928.6; Na, 120–320; Mg, 43.8–139.7; Cu, 0.19–1.06; Fe, 0.34–1.58; Zn, 0.21–2.95.

Table 3 clearly shows that the content of Ca, K, Na and Mg decreased throughout the lactation period in mares of different breeds in Hungary (Hungarian Draught, Haflinger, Breton, & Boulonnais mares; 17, 32) and in Italy (Haflinger mares; 5, 7, 18, 19). Also, variation and an irregular decrease of elements throughout the lactation period was determined in Thoroughbred mares from USA and New Zealand and in Italian Saddle mares from Italy (9, 12, 18). The present study also determined the trend of variation of content of Ca, K, Na and Mg during lactation. In contrast to the significant decline in the concentration of these elements towards the end of lactation in previous studies, in this study no significant decreases in concentrations were identified at the end of lactation. According to previous available data, this can be explained as a consequence of the differences between horse breeds.

Cu and Fe contents determined in the present study were 1.8–4.8 and 4.2–6.7 times lower than values measured in mares form Hungary (7, 17). On the other hand, Zn values measured by these authors were similar to those in the present study. It was previously determined that the concentrations of Cu, Zn and Fe in the milk of late gestating and lactating mares were not influenced by supplementation with higher dietary trace element levels (20).

In previous reports, there are no data regarding the Se content in horse milk. The results obtained in the present study ranged from 0.003–0.029 mg/kg. The mean Se content was similar to concentrations measured in goat (0.0129 mg/kg) and human (0.0141 and 0.0152 mg/kg) milk but lower than in bovine milk (0.0215–0.4 mg/

kg) (2, 21-24). Element concentrations in cow, goat and human milk from different countries are reported in Table 4. Studies have shown that Ca concentrations in horse milk were approximately three times higher than in human milk (251 mg/kg) and about two times lower than in cow and goat milk (21, 23, 24, 26-28). Mg concentrations ranged from 56.8 to 71.0 mg/kg, i.e. they were 2.5-3.5 times higher than the values 24–40 mg/L reported for human milk (2, 21), but 1.5-2 times lower than concentrations measured in cow and goat milk (23, 24, 26-29). Na and K contents were similar to those in human milk (2, 21), though they were approximately three times lower than the values reported in cow and goat milk (23, 24, 26-29). Furthermore, concentrations of Cu, Fe and Zn determined in horse milk in the present study were lower than those in cow, goat and human milk.

Table 1: Working conditions for ICP-OES.

Element / Parameter	Ca, Na, Mg, K	Fe, Cu, Zn, Se
Plasma viewing mode	Radial	Axial
Read time	1-5 s	1-5 s
Measurement replicates	3	3
RF incident power	1000 W	1300 W
Plasma argon flow rate	8 L/min	15 L/min
Nebulizer argon flow rate	0.85 L/min	0.55 L/min
Auxiliary argon flow rate	0.2 L/min	0.2 L/min
Sample uptake rate	1.5 mL/min	1.5 mL/min
Inner diameter of the torch injector	2.0 mm	2.0 mm
Nebulizer type	Concentric glass (Meinhard)	Concentric glass (Meinhard)
Spray chamber type	Glass cyclonic spray chamber	Glass cyclonic spray chamber

Table 2: Concentrations of elements in milk of Croatian Coldblooded mares

		Days postpartum				
Element (mg/kg)	Statistics	10 (*n*=6)	40 (*n*=6)	60 (*n*=6)	120 (*n*=6)	180 (*n*=6)
Ca	Mean±SD Min-max	772 ± 265 543-1246	598 ± 178 331-820	608 ± 165 426.4-910.8	585 ± 195 387.2-885.1	674 ± 144 557-942
K	Mean±SD Min-max	687 ± 145 531-840	547 ± 167 366-860	561 ± 108 429-676	634 ± 93.6 529-807	677 ± 135 510-800
Na	Mean±SD Min-max	165 ± 54.2 106-261	138 ± 33.7 94.9-168	138 ± 13.4 125-157	131 ± 73.5 15.9-245	138 ± 12.6 123-151
Mg	Mean±SD Min-max	71.0 ± 17.9 50.5-92.6	56.8 ± 19.8 30.9-85.2	63.8 ± 11.7 46.1-80.2	68.6 ± 17.7 47.5-96.6	69.8 ± 17.4 49.9-92.7
Cu	Mean±SD Min-max	0.14 ± 0.079 0.021-0.25	0.11 ± 0.048 0.040-0.17	0.12 ± 0.032 0.077-0.16	0.13 ± 0.032 0.076-0.17	0.085 ± 0.042 0.019-0.14
Fe	Mean±SD Min-max	0.41 ± 0.35 0.082-1.12	0.33 ± 0.31 0.084-1.02	0.13 ± 0.047 0.066-0.21	0.15 ± 0.082 0.082-0.285	0.18 ± 0.16 0.072-0.51
Zn	Mean±SD Min-max	1.86 ± 0.47 1.44-2.44	2.01 ± 0.19 1.64-2.23	1.99 ± 1.13 0.83-3.27	2.11 ± 1.44 0.85-4.85	2.15 ± 0.98 1.05-4.39
Se	Mean±SD Min-max	0.029 ± 0.041 0.003-0.11	0.004±0.002 0.002-0.008	0.0027±0.003 0.002-0.008	0.0027±0.004 0.001-0.011	0.007±0.004 0.006-0.011

Table 4: Concentrations of elements in cow, goat and human milk from different countries

Elements	Brazil (30) (mg/L)	Greece (29) (mg/kg)	Iceland (24) (mg/kg)	Italy (22) (mg/kg)	Spain (21) (mg/L)	Spain (26, 28) (mg/kg)	Sweden (31) (mg/L)	Tenerife (23) (mg/kg)	Source: 2007 (2) (mg/kg)	Source: 2009 (27) (mg/kg)
Ca		G 1320	C 1140-1260	C 1263	H 251	G 1586 (26) G 1940 (28) C 1135.8 (26) C 1936 (28)		G 1340	H 330 G 1340	C 1220
K		G 1520		C 1096				G 1240	H 550 G 1810	C 1520
Na		G 594	C 399-401	C 441	H 164			G 510	H 150 G 410	C 580
Mg		G 158.7	C 96.0-99.8	C 118	H 24	G 129.2 (26) G 178.2 (28) C 94.0 (26) C 150.1 (28)		G 120	H 40 G 160	C 120
Cu	H 0.54	G 0.80	C 0.041-0.046		H 0.311	G 0.42 (26) C 0.14 (26)	H 0.12	G 0.18	H 0.60 G 0.50	C 0.6
Fe	H 1.72	G 0.60	C 0.20-0.27	C 0.3	H 0.388	G 1.5 (26) C 0.9 (26)	H 0.29	G 0.70	H 2.0 G 0.70	C 0.8
Zn	H 6.97	G 3.7	C 3.89-4.33	C 3.6	H 3.8	G 5.28 (26) G 4.46 (28) C 4.63 (26) C 4.03 (28)	H 0.46	G 3.20	H 3.8 G 5.6	C 5.3
Se			C 0.0215-0.0263	C 0.4	H 0.0141			G 0.0129	H 0.0152 G 0.013	C 0.0096

C – cow milk; G – goat milk; H – human milk

*The number in parentheses refers to the reference

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VSEBNOST MIKRO- IN MAKROELEMENTOV V MLEKU HRVAŠKIH HLADNOKRVNIH KOBIL V ČASU LAKTACIJE

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Povzetek: Vsebnost makro- in mikroelementov v mleku hrvaških hladnokrvnih kobil v različnih fazah laktacije je bila ugotavljana s pomočjo indukcijske plazmonske optične emisijske spektrometrije. Vsebnost elementov med 10. in 180. dnevom laktacije je bila v mejah (mg/kg): Ca 585–772, K 547–687, Na 131–165, Mg 56,8–71,0, Cu 0,085–0,14, Fe 0,013–0,41, Zn 1,86–2,15 ter Se 0,003–0,029. Koncentracije vseh elementov so se v času laktacije spreminjale, vendar pa so bile razlike statistično značilne samo pri železu, ki ga je bilo 10. in 40. dan statistično značilno več kot 60. dan laktacije ($p < 0,05$). Korelacijska analiza je pokazala zmerno korelacijo med elementi Ca, K, Mg, Se, Mg, K, Cu, Se, Cu in Fe. Razen za Ca in Cu so bile koncentracije vseh ostalih elementov znotraj meja, o katerih so že prej poročali za različne pasme konj. Koncentracije vseh elementov v konjskem mleku so bile nižje, kot so običajno v kravjem ali kozjem mleku. V primerjavi s človeškim mlekom pa so bile koncentracije Ca in Mg višje, Na in K podobne, koncentracije Cu, Fe in Zn pa nižje v konjskem kot v človeškem mleku

Ključne besede: mleko; laktacija; konj; mikroelementi; makroelementi; ICP-OES; Hrvaška

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Original Scientific Article

EFFECTS OF LACTATIONAL EXPOSURE TO NON-PLANAR PCB-155 AND PLANAR PCB-169 ON BODY WEIGHT GAIN AND CRANIOFACIAL GROWTH IN RAT OFFSPRING

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Summary: The adverse effects of two polychlorinated biphenyls (PCBs), non-planar PCB-155 and planar PCB-169, individually and in combination, on body weight gain and craniofacial growth in rat offspring lactationally exposed to PCBs during the early postnatal period were investigated. Lactating adult Wistar rats (n=15) were intraperitoneally administered a total of 12 mg/kg b.w. PCB-155 (group 1, n=4), 3 mg/kg b.w. PCB-169 (i.e., 90 mg toxic equivalents (TEQ)/kg b.w.) (group 2, n=4), or PCB-169 and PCB-155 together (group 3, n=3). The fourth group (n=4) served as a control. Offspring were sacrificed on postnatal days (PNDs) 9 and 22. Body weights and craniofacial dimensions were recorded. On PND 9, all of the exposed offspring weighed less ($p \leq 0.001$) than the control group, and group 2 ($p \leq 0.001$) and 3 ($p \leq 0.01$) weighed less than group 1. On PND 22, only groups 2 and 3 weighed less than the control group ($p \leq 0.001$). Narrower neurocranium was observed on PND 9 in groups 1 ($p \leq 0.01$), 2 ($p \leq 0.05$) and 3 ($p \leq 0.001$). Skulls were shorter in groups 2 ($p \leq 0.001$) and 3 ($p \leq 0.01$), and this reduction persisted until PND 22 ($p \leq 0.001$). On PND 22, rounder skulls were observed in groups 2 ($p \leq 0.001$) and 3 ($p \leq 0.05$), and mandibular length was reduced ($p \leq 0.001$). The data suggest that PCB-155 may have an additive effect with PCB-169 on growth reduction. In conclusion, lactational exposure to PCB-155 and PCB-169 negatively affected body weight gain and craniofacial growth in rat offspring until PND 9, while the adverse effects of PCB-169 were more potent and persisted until PND 22.

Key words: polychlorinated biphenyls; lactational; body weight; craniofacial growth; rat

Introduction

Polyhalogenated aromatic hydrocarbons (i.e., polychlorinated-biphenyls (PCBs), -dioxins) are persistent and widespread environmental pollutants. Being lipophilic, they accumulate in animal and human tissues, where they can cause a wide range of biological and toxicological effects (1). Planar dioxin-like PCBs exhibit physicochemical properties, environmental distributions, and toxicity profiles that are different from their non-planar homologues (2). The effects of planar PCBs are mediated through the aryl hydrocarbon receptor (AhR), a cytosolic receptor protein with high affinity for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (3). Predominant exposure to PCBs is via ingestion of food, and they also cross the placenta and are excreted in milk (2). Concern has been raised about breast-fed infants, whose daily intake of polyhalogenated aromatic hydrocarbons on a body weight basis may be one or two orders of magnitude higher than in adults (4). Importantly, organisms are not fully developed at birth, and thus, a process of maturation continues postnatally for an extended *period*. Tissues and organs are most sensitive during this critical period of intensive growth, and therefore, an

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insult/interference posed to suckling offspring in early postnatal life may influence their postnatal growth and development (5).

Evidence for PCB-induced deficits in growth and development includes studies on exposed human populations that show reduced growth rates in children (1, 6-8) and skull abnormalities with irregular calcifications and wide open sutures and fontanelles (9). Other evidence suggests that PCBs may increase the susceptibility to weight gain and obesity (10). Alterations in bone growth and composition have previously been described in experimental animals exposed to PCBs (11-17) as well as in wild animals environmentally exposed to polyhalogenated aromatic hydrocarbons (18-23). Experimental exposure to TCDD caused growth suppression (24), impaired skull growth in adult rats (25), and osteolysis in maxilla and mandibles in minks (26); similar adverse effects were induced with planar PCB-126 (14). In developmental studies, fish exhibited dysmorphogenesis in craniofacial structures (27) and decreased growth with more juvenile cranial characteristics (17) in response to TCDD and PCB-126, respectively. In rodents, offspring that were perinatally exposed to PCB mixtures showed growth suppression, although in some groups, only transient (28, 29) and facial malformations were observed (28). Alterations were mainly driven by the dioxin-like PCBs, although the contribution of the non-planar PCBs to the exposure outcome could not be excluded (30). Perinatal exposure to TCDD decreased body weights at higher doses (31), caused smaller and less mineralised skulls (32), and affected the shape and size of the mandible (33). Considering the potential toxicity of PCBs and the limited understanding of factors that govern their adverse effects, it is imperative to pursue these studies further.

In our previous studies, different lactational transfer of non-planar PCB-155 and planar PCB-169 to lactationally exposed lambs (34) and their enrichment in lamb mandibular bone (35) were observed. The objective of this study was to therefore examine the adverse effects of two different PCBs on body weight gain and craniofacial growth in rat offspring that were lactationally exposed during early postnatal life, which in rats can be divided into the following periods: presuckling (first 6 hours after birth), suckling (until PND 17) and weaning (from PND 17 until 28). The last starts when offspring ingest food other than maternal milk (5). We administered two hexa-chlorobiphenyls, non-planar PCB-155 and planar PCB-169, to actively lactating rats. For dioxin-like PCB-169, a toxic equivalency factor (TEF) of 0.03 is proposed (36), representing its toxic potency evaluated in comparison to that of TCDD. To explore possible interactions between individual PCBs, concomitant exposure was also performed. Offspring were sacrificed on PNDs 9 and 22 so that the effects of PCBs on different periods of early postnatal development, i.e., the suckling and weaning periods, could be studied. On PND 9, there is the first peak of daily weight increments, while a decrease is reported around PND 16. Milk consumption, which peaks between PND 17 and 19, decreases with the intake of water and solid food. Maternal milk composition also changes substantially as the fat and protein content is three- and two-fold lower on PND 20 (weaning period) compared to PND 10 (suckling period) (5).

Materials and methods

Animal care and PCB administration. Sexually mature 8-10 week old adult female Wistar rats (n=15), weighing between 230 and 250 g, were obtained from Lek d.d. (Ljubljana, Slovenia). They were housed under standardised conditions at the Veterinary Faculty in Ljubljana. They received standard pellet feed (M-K 02) and tap water *ad libitum*. After mating and delivery, the lactating mothers were randomly assigned to four groups. The first group (n=4) was administered a single dose of 6 mg/kg b.w. PCB-155 (2,2',4,4',6,6'-hexachlorobiphenyl) in olive oil after delivery via an intraperitoneal injection, followed by three maintenance doses of 2 mg/kg b.w. PCB-155 on days 6, 12, and 17 day after delivery; the total amount administered was 12 mg/kg b.w. PCB-155. The second group (n=4) was administered a single dose of 2 mg/kg b.w. PCB-169 (3,3',4,4',5,5'-hexachlorobiphenyl) in olive oil after delivery via an intraperitoneal injection, followed by two maintenance doses of 0.5 mg/kg b.w. PCB-169 on days 6 and 14 day after delivery; the total amount administered was 3 mg/kg b.w. PCB-169. The combined regime of the PCB-155 and PCB-169 administrations was used in the third group (n=3). On the day of delivery, mothers received a single dose of 2 mg/kg b.w.

PCB-169 and 6 mg/kg b.w. PCB-155, followed by maintenance doses as described above for the first and second groups. The fourth group (n=4) served as a vehicle control; lactating mothers were administered 0.5 mL olive oil after delivery by an intraperitoneal injection, and on days 6, 12, 14, and 17, an additional 0.15 mL olive oil. Offspring were exposed to PCB-155 and/or to PCB-169 via the mother's milk.

To achieve equal concentrations of both PCBs in milk, the maintenance doses of PCB-155 and PCB-169 were determined according to the results of our previous study (34). The excreted amount of the PCB-169 in mothers' milk was more than three times higher than the amount of PCB-155; therefore, the administered loading and maintenance doses of PCB-155 were higher and more frequent. Standards of PCBs were purchased from Promochem (Wesel, Germany). IUPAC numbers were used for assigning the PCB congeners (37). All procedures involving the experimental rats complied with the Prevention of Cruelty to Animals law that is consistent with the European Community Directive 86/609/EC and were approved by the Slovenian Veterinary Administration and its Ethics committee (Permits No. 323-02-206/00 and 3440-165/2006).

PCB analysis, measurement of body weights and craniofacial dimensions. From each group, the same number of male and female offspring per litter was sacrificed on PNDs 9 and 22. On PND 9, 19 offspring were sacrificed from the PCB-155 group, the same number was sacrificed from the PCB-169 group, 14 offspring were from the PCB-155+169 group, and 15 from the control group. On PND 22, 17 offspring were sacrificed from the PCB-155 group, 19 from the PCB-169 group, 14 from the PCB-155+169 group, and 14 from the control group. All offspring were weighed (to the nearest 0.01 g) on PND 9, and those not sacrificed on PND 9 were weighed also on PND 22. At the end of the experimental period, offspring were sacrificed under deep general inhalation anesthesia induced by CO_2 followed by exsanguination. Blood samples for PCB analysis were collected from the ophthalmic plexus into tubes containing lithium heparin as an anticoagulant and stored at −20°C until analysis. PCB residues were determined using the solid phase microextraction technique and gas chromatography with electron capture detection (GC-ECD) as previously described (34). Number of animals used for each chemical analysis was smaller than for the assessment of body weights and craniofacial dimensions, as insufficient blood volume samples were not analysed. Skulls and mandibles were dissected, the muscle and soft tissue were removed, and the linear craniofacial dimensions were measured under a stereomicroscope using a Vernier sliding caliper (to the nearest 0.05 mm) by one inspector who was blinded to the treatment. The skull length was measured as the distance between the occipitointerparietal suture and the anterior margin of the nasal bone, and the neurocranium width was measured immediately caudal to the zygomatic arches; the mandibular length was measured as the distance between the foramen mentale and condylar processus as schematically shown in Fig. 1.

Statistical analysis. Data were expressed as the mean±SD. Analysis of variance (ANOVA) with the Tukey post-test was used to explore differences in

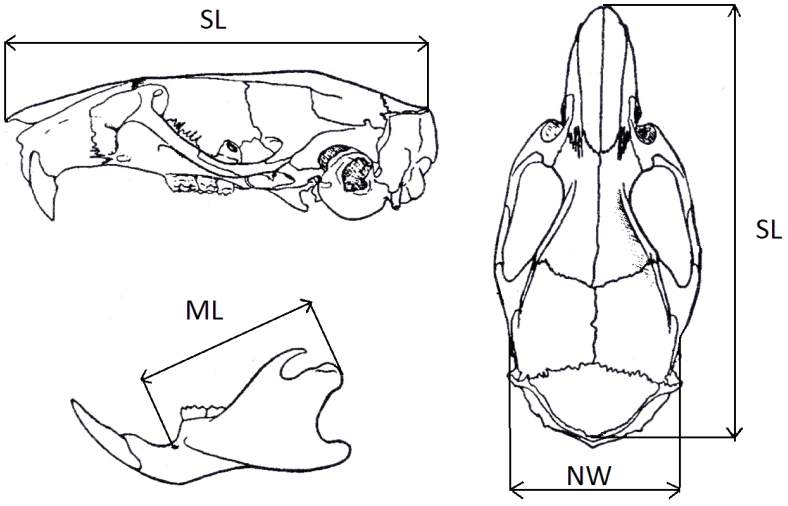

Figure 1: Schematic presentation of rat skull and mandible showing the measurements used to assess craniofacial growth.
SL - skull length; NW - neurocranium width; ML - mandibular length

Table 1: Blood PCB concentrations (mean±SD) of Wistar rat offspring on PNDs 9 and 22 in differently exposed groups

	PCB concentrations (ng/mL)			
	PND 9		PND 22	
Treatment	PCB-155	PCB-169	PCB-155	PCB-169
PCB-155	8.0±5.4 (5)	/	10.3±3.1 (10)	/
PCB-169	/	1.3±0.1 (5)	/	3.2±0.1[a] (11)
PCB-155+169	8.2±2.6 (5)	1.7±0.3 (5)	14.8±3.6[b] (3)	1.9±0.1[c] (3)

Number of animals used for each analysis is given in parentheses.
[a] - significantly different from PND 9 ($p \leq 0.001$)
[b] - significantly different from PCB-155 group on PND 22 ($p \leq 0.001$)
[c] - significantly different from PCB-169 group on PND 22 ($p \leq 0.001$)

Table 2: Body weights and craniofacial dimensions (mean±SD) of Wistar rat offspring at PNDs 9 and 22 in differently exposed groups

	Treatment			
Variable	Control	PCB-155	PCB-169	PCB-155+169
Body weight (g)				
PND 9	21.12±2.73[155,169,155+169] (29)	18.93±1.25[c,169,155+169] (36)	17.14±1.49[c,155] (38)	16.49±3.30[c,155] (28)
PND 22	61.07±3.61[169, 155+169] (14)	58.91±3.16[169,155+169] (17)	48.47±3.75[c,155] (19)	48.25±9.84[c,155] (14)
Skull length (mm)				
PND 9	24.79±0.65[169,155+169] (15)	24.27±0.55 (19)	23.85±0.45[c] (19)	23.52±0.91[c] (14)
PND 22	33.28±0.71[169, 155+169] (14)	33.24±0.39[169,155+169] (17)	30.87±2.44[c,155] (19)	31.07±1.24[c,155] (14)
Neurocranium width (mm)				
PND 9	13.57±0.43[155,169,155+169] (15)	13.03±0.21[c] (19)	13.18±0.25[c] (19)	12.97±0.31[c] (14)
PND 22	15.76±0.42[155+169] (14)	15.74±0.45[155+169] (17)	15.65±0.26[155+169] (19)	15.34±0.33[c,155,169] (14)
Skull length / neurocranium width				
PND 9	1.82±0.04 (15)	1.86±0.03[169,155+169] (19)	1.80±0.01[155] (19)	1.81±0.03[155] (14)
PND 22	2.11±0.05[169,155+169] (14)	2.11±0.05[169,155+169] (17)	1.97±0.15[c,155] (19)	2.01±0.05[c,155] (14)
Mandibular length (mm)				
PND 9	10.93±0.41[155+169] (15)	10.69±0.21 (19)	10.62±0.23 (19)	10.21±0.75[c] (14)
PND 22	14.93±0.30[169,155+169] (14)	15.04±0.19[169,155+169] (17)	13.88±0.19[c,155] (19)	14.05±0.55[c,155] (14)
Skull length / body weight (mm/g)				
PND 9	1.16±0.13[155,169,155+169] (15)	1.29±0.07[c,169] (19)	1.35±0.07[c,155] (19)	1.46±0.27[c] (14)
PND 22	0.54±0.03[169,155+169] (14)	0.56±0.03[169,155+169] (17)	0.64±0.06[c,155] (19)	0.67±0.12[c,155] (14)
Neurocranium width / body weight (mm/g)				
PND 9	0.63±0.07[169,155+169] (15)	0.69±0.04[169] (19)	0.75±0.04[c,155] (19)	0.81±0.16[c] (14)
PND 22	0.26±0.01[169,155+169] (14)	0.26±0.01[169,155+169] (17)	0.32±0.02[c,155] (19)	0.33±0.07[c,155] (14)
Mandibular length / body weight (mm/g)				
PND 9	0.51±0.06[169,155+169] (15)	0.57±0.04[169] (19)	0.60±0.03[c,155] (19)	0.63±0.09[c] (14)
PND 22	0.24±0.01[169,155+169] (14)	0.25±0.01[169,155+169] (17)	0.29±0.02[c,155] (19)	0.31±0.06[c,155] (14)

Number of animals used for each measurement is given in parentheses.
[c] – significant differences in the parameters measured between the control group and differently exposed groups ($p \leq 0.05$); [155] – significant differences between the PCB-155 group and differently exposed groups ($p \leq 0.05$); [169] – significant differences between the PCB-169 group and differently exposed groups ($p \leq 0.05$); [155+169] – significant differences between the PCB-169+155 group and differently exposed groups ($p \leq 0.05$) as evaluated by one-way analysis of variance (ANOVA)

body weights and linear craniofacial dimensions among different groups. Two-tailed independent-samples t-test was used to compare PCB blood concentrations and treatment effects between PND 9 and PND 22. A p-value of ≤0.05 was considered statistically significant. The data were analysed using the SPSS 12.0 statistical software package for Windows (SPSS Inc., Chicago, Ill, USA).

Results

Exposure of offspring. To confirm offspring PCB exposure via mothers' milk, PCB blood concentrations on PNDs 9 and 22 were determined (Table 1). The data show that on PND 22, the concentrations of PCB-169 in the blood samples were higher (p≤0.001) than on PND 9. The concentrations of PCB-155 were also higher on PND 22, but not significantly. On PND 22, the concentrations of PCB-155 were lower (p≤0.001), and those of PCB-169 were higher (p≤0.001) in offspring exposed to single PCB compared to offspring exposed to the combination of PCB-155 and PCB-169.

Body weight gain. The body weights of differently exposed offspring are summarised in Table 2. The mean body weight of Wistar offspring on PND 1 (n=142) was 6.83±0.64 g. On PND 9, the body weights of exposed offspring were significantly (p≤0.001) lower compared to the non-exposed (control) offspring. The PCB-155 group gained more weight than the PCB-169 group (84.7% and 72.1% of that of the control group, respectively; p≤0.001) or than the group exposed to the combination of both PCBs (67.6%; p≤0.01). On PND 22, the PCB-169 group and the group exposed to the combination weighed less (p≤0.01) than the control and PCB-155 groups. The offspring of both sexes showed similar patterns of response to PCB treatment.

Craniofacial dimensions. The craniofacial dimensions of the offspring on PNDs 9 and 22 are presented in Table 2. Data from male and female offspring were combined for the statistical evaluation, as no significant sex-by-treatment interactions were observed. On PND 9, the PCB-169 group had significantly shorter skulls (p≤0.001) and narrower neurocranium (p≤0.05) than the control group. This reduction was observed in the more mature stages of PND 22 for skulls and mandibles (p≤0.001), but not for neurocranium. Therefore, on PND 22, PCB-169 exposed offspring had smaller ratios of skull length/neurocranium width (i.e., rounder skull) (p≤0.001) compared to the control group. On PND 9, the PCB-155 group had narrower neurocranium (p≤0.01) than the control group, while no significant treatment-related differences were observed for other craniofacial dimensions, and no differences were observed on PND 22. The group exposed to the combination of both PCBs had shorter skulls (p≤0.01), narrower neurocranium (p≤0.001), and shorter mandibles (p≤0.05) than the control group on PND 9. On PND 22, the reduction was still observed in the dimensions of skull and mandible length (p≤0.001) and neurocranium width (p≤0.01), and the skulls were rounder (p≤0.05) than in the control group. Compared to the PCB-155 group, on PND 9, only rounder skulls (p≤0.001) were detected in the PCB-169 group, but on PND 22, the PCB-169 group had shorter and rounder skulls and shorter mandibles (p≤0.001).

On PNDs 9 and 22, significantly longer skulls, wider neurocranium, and longer mandibles relative to body weight than in the control group were observed for the PCB-169 group (p≤0.001) and the group exposed to the combination of both PCBs (p≤0.01). The skull length/body weight ratio was higher (p≤0.01) in PCB-155 exposed offspring on PND 9, but this increase was not observed on PND 22.

Discussion

In the present study, we examined the adverse effects of two hexachlorobiphenyls corresponding to the non-planar (PCB-155) and planar (PCB-169) structure, individually or in combination, on the body weight gain and craniofacial growth of lactationally exposed rat offspring. PCB-169 adversely affected body weight gain and craniofacial growth until weaning on PND 22, alone and in combination with PCB-155 (Fig. 1 and Table 2). On PND 22, skulls and mandibles of the offspring exposed to PCB-169 and to the combination of both PCBs were shorter. In addition, their skulls were rounder, mimicking features expected in less-developed or younger animals. This result of the adverse effects of the dioxin-like PCB-169 on body weight gain and craniofacial growth is similar to results reported in previous studies where TCDD was administered (25, 31-33). TCDD treatment dose-dependently decreased body weight, mandibular length (31), and produced smaller skulls in rat offspring (32).

Likewise, TCDD exposure affected the shape and reduced the size of mandible in mouse offspring (33). In the study of Alaluusua and coworkers (25), TCDD was shown to impair growth resulting in shorter and narrower skulls in young adult rats.

Until PND 9, the PCB-155 exposed group gained less weight, although the weight gain was more than in the PCB-169 group (Table 2), and individual PCB-155 exposure decreased neurocranium width. No adverse effects of PCB-155 on body weight gain and craniofacial growth were observed from PND 9 to PND 22, and until PND 22, the PCB-155 group already gained 96.0% of the control group weight.

On PND 9, the group exposed to the combination of both PCBs gained less weight than the group exposed to PCB-169 alone, and craniofacial dimensions were reduced more when the offspring were exposed to both PCBs (Table 2). This trend suggests that PCB-155 in combination with PCB-169 may have an additive negative effect on growth rate, but was not sufficient to generate significance. This observation is consistent with results reported by Chu et al. (24), who found that growth suppression of adult rats was more pronounced in the group receiving TCDD in combination with a mixture of PCBs than with the TCDD alone.

The observed effect of PCBs on craniofacial bone growth is in agreement with several previous studies where evidence for PCB induced bone effects was found. After accidental exposure of human infants to PCBs and polychlorinated dibenzofurans (PCDFs), growth retardation and skull abnormalities were reported (9). In wildlife studies, Baltic seals exposed to polyhalogenated aromatic hydrocarbons such as PCBs suffered from severe skull bone loss (19). Furthermore, exposed adult herring gulls from heavily contaminated Great Lakes had shorter femurs, with lower mineral content and density (22). Similarly, chicks of tern collected from that area showed growth retardation, and cases of weak ossification and shortened mandibles, even lack of jaw or skull bones were reported (18). Embryos and chicks from eggs laid by hens that consumed a diet containing PCB contaminated carp had malformed brain cases and poorly ossified skull bones, as well as feet and leg deformities (12). Additionally, chronic exposure of Baltic seals (20) and deer mice to PCBs (23) was associated with reduced bone mineral density. In another study on East Greenland polar bears (21), subcutaneous adipose tissue residues of polyhalogenated aromatic hydrocarbons, including total PCBs, were negatively associated with skull bone mineral density. Furthermore, Hodgson and coworkers (38) found a negative association between serum planar PCB-118 concentration and bone mineral density in Swedish men. On the other hand, the sum of the three most abundant non-planar PCBs was positively associated with bone mineral density.

In the environment, PCBs are always present as mixtures. It is not known if it is the planar or non-planar components in the mixture that cause adverse effects or whether there are synergistic or antagonistic effects of the simultaneous exposure to different compounds (30). Maternal exposure to commercial technical PCB mixture Aroclor 1254, where PCBs are present as mixtures of planar and non-planar congeners, among other organochlorines, resulted in growth suppression in rat offspring (28) and induced shorter, thinner and weaker bones in perinatally exposed rats (30). However, different adverse effects were produced after exposure to a complex mixture of 14 PCBs and 11 organochlorine pesticides, based on blood levels reported in exposed humans, where facial malformations characterized by a rounded skull and underdeveloped snout and lower jaw were observed (28). Thus, different kinds of PCB mixtures may have dissimilar effect on growth/bone, even though it was suggested that effects are mainly driven by the planar congeners (30).

In the present study, adverse effects of individual hexachlorobiphenyls were investigated, with non-planar (PCB-155) and planar (PCB-169) structure. Fewer studies have tested the effects of individual PCB congeners on bone growth and development. Exposure to planar PCB-126 was shown to result in decreased bone growth in nestling American kestrels (11), shorter bone length, impaired bone strength, and increased organic content in experimental rats (13). In addition, juvenile diamondback terrapins exposed to PCB-126 were smaller, with more juvenile cranial characteristics, their skulls had a higher organic content, and their femora had reduced bone mineral density (17). Decreased growth and differences in cranial form are similar to those found in the present study. But adverse bone effects for different types of PCBs are not yet well described. As an example, perinatal exposure to the planar PCB-118, as well as to the non-

planar PCB-153 has been reported to reduce bone length and affect bone composition in lambs (16). Another study demonstrated that perinatal exposure to PCB-153 resulted in altered bone composition in goat offspring (15). In contrast, planar PCB-126 did not induce marked bone effects (15). In the present study, adverse effects were mainly observed after exposure to the planar congener PCB-169. They were also elicited by the non-planar PCB-155, but less effectively (Table 2).

In the current study, lactating dams were exposed from PND 1. The total amount of administered PCB-169 was 3 mg/kg b.w. The corresponding concentration of toxic equivalents (TEQs), an estimate of the total TCDD-like activity (36) calculated by the concentration of PCB-169 multiplied by its TEF, was 90 μg TEQ/kg b.w. In a study with a comparable design, a similar dose of 50 μg TCDD/kg b.w. administered on PND 1 to lactating dams caused smaller and less mineralised skulls on PND 22 (32). In another study, in which TCDD was administered to dams on gestation day 9, just 0.5 μg TCDD/kg b.w. produced a significant decrease in mandible size and altered mandible shape in their offspring (33). However, an extremely high single dose of 1000 μg TCDD/kg b.w. administered to adult rats was required to cause impaired body weight gain and skull growth (25). Exposure from PND 1 onwards was selected in the present study to test the hypothesis that PCB lactational exposure would be harmful on offspring growth.

We examined ratios between craniofacial dimensions and body weight to determine which studied parameter i.e., body weight gain or craniofacial growth, is more susceptible to PCB exposure. On PNDs 9 and 22, due to their smaller body weights, all measured craniofacial dimensions relative to body weight were larger in the PCB-169 exposed group and the group exposed to the combination of both PCBs (Table 2). The results suggest that craniofacial growth was less affected by PCB-169, alone or in combination with PCB-155, than body weight gain. This finding is in contrast to results obtained by Hoffman and coworkers (11) on nestling American kestrels, where exposure to doses of PCB-126 that did not significantly decrease body weight, exhibited significantly shorter long bone length.

When the offspring were exposed to PCB-169 alone, skull and mandibular length were reduced more on PND 22 than on PND 9 (Table 2). The differences in adverse effects between PNDs 9 and 22 could also be attributed to PCB accumulation after long lactational PCB exposure. The blood levels of both PCBs increased from PND 9 to PND 22 in all three experimental groups (Table 1), which implies that accumulation of PCBs exceeded the growth of the offspring. The blood levels of the metabolically more stable and super lipophilic planar PCB-169 increased more than the levels of PCB-155. On PND 22, the offspring exposed to the combination of both PCBs had blood PCB-155 levels higher than the levels with exposure to individual PCBs and PCB-169 levels lower than those with exposure to individual PCBs. These results support the finding that when administered together, non-planar PCBs decrease the serum levels of dioxin-like organochlorines (39).

In conclusion, lactational exposure to PCB-169 negatively affected body weight gain and craniofacial growth in rat offspring until PND 22, alone and in combination with PCB-155. Until PND 9, PCB-155 also decreased neurocranium width and body weight gain, but did so less effectively than PCB-169. Evidence is also presented that PCB-155 in combination with PCB-169 may have an additive negative effect on growth rate in early postnatal life based on body weight gain and craniofacial measurements.

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VPLIV NEPLANARNEGA PCB-155 IN PLANARNEGA PCB-169 V MATERINEM MLEKU NA RAST TELESNE MASE IN KRANIOFACIALNEGA PODROČJA PRI SESNIH MLADIČIH PODGANE

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Povzetek: V raziskavi smo ugotavljali škodljive vplive dveh polikloriranih bifenilov (PCB) v materinem mleku, neplanarnega PCB-155 in planarnega PCB-169, posamič ali v kombinaciji, na rast telesne mase in kraniofacialnega področja pri sesnih mladičih podgane, ki so bili izpostavljeni vplivom polikloriranih bifenilov v zgodnjem postnatalnem obdobju. Odraslim podganjim samicam seva wistar (n=15) smo po kotitvi intraperitonealno vbrizgali skupno 12 mg/kg t.t. PCB-155 (skupina 1, n=4), 3 mg/kg t.t. PCB-169 (kar ustreza 90 pg toksičnega ekvivalenta (TEQ)/kg t.t.) (skupina 2, n=4), ali kombinacijo PCB-169 in PCB-155 (skupina 3, n=3). Četrta skupina (n=4) je bila kontrolna. Mladiče smo žrtvovali 9. in 22. dan po skotitvi. Izmerili smo telesne mase in dolžine kraniofacialnega področja. Vsi izpostavljeni mladiči so bili 9. dan po skotitvi lažji ($p \leq 0.001$) od mladičev v kontrolni skupini, skupini 2 ($p \leq 0.001$) in 3 ($p \leq 0.01$) sta bili lažji kot skupina 1. V starosti 22 dni sta bili lažji kot kontrolna skupina le skupini 2 in 3 ($p \leq 0.001$). V starosti 9 dni smo izmerili ožji nevrokranij pri skupinah 1 ($p \leq 0.01$), 2 ($p \leq 0.05$) in 3 ($p \leq 0.001$). Lobanje so bile krajše pri skupinah 2 ($p \leq 0.001$) in 3 ($p \leq 0.01$), škodljivi vpliv na rast lobanje je bil viden tudi v starosti 22 dni ($p \leq 0.001$). Pri 22 dneh so imeli mladiči bolj okrogle lobanje v skupinah 2 ($p \leq 0.001$) in 3 ($p \leq 0.05$) in tudi krajše mandibule ($p \leq 0.001$). Na podlagi predstavljenih rezultatov je mogoče sklepati, da se škodljivi vplivi PCB-155 in PCB-169 na rast seštevajo. Dobljeni rezultati kažejo, da je izpostavljenost PCB-155 in PCB-169 preko materinega mleka negativno vplivala na rast telesne mase in kraniofacialnega področja pri sesnih mladičih podgane do 9. dneva po skotitvi. Škodljivi vpliv PCB-169 je bil močnejši in je trajal vse do 22. dneva.

Ključne besede: poliklorirani bifenil; materino mleko; telesna masa; rast kraniofacialnega področja; podgana

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Original Scientific Article

SEX-SPECIFIC BEHAVIORAL EFFECTS OF FLUOXETINE TREATMENT IN ANIMAL MODELS OF DEPRESSION AND ANXIETY

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Summary: There are strong sex differences in clinical characteristics and in responses to treatment of several psychiatric diseases. Depressive and anxiety disorders are 2 to almost 3 times more common in women, but the majority of experiments examining the biological basis of these disorders and pharmacological agents for treatments are conducted in male animals. Several studies suggest that females respond better than males to the action of selective serotonin reuptake inhibitors (SSRIs), suggesting that gonadal hormones modulate mood and the response to these drugs. The beginning of clinical use of SSRI fluoxetine (Prozac) in late 80-ies was the frst major breakthrough in the treatment of depression since the introduction of tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) nearly 30 years earlier. Fluoxetine is today widely prescribed for the treatment not only of depression but also of some anxiety related disorders. Animal models of depression and anxiety represents a useful tool for the investigation of sex differences of pharmacokinetics and pharmacodynamics of antidepressants. In this review the animal models of depression/anxiety using three most common performed acute stressor behavior tests (forced swim test – FST, elevated plus maze – EPM and open field – OF) will be introduced, followed by presenting behavior alterations after fluoxetine treatment in male and female rodents. In addition, data from our lab in C57BL/6J mice of both sexes on the behavioral effects of chronic fluoxetine treatment in comparison to other studies will be presented. Given the overlap between human and rodent findings, rodents provide a good model for further research on the sex-dependent effects of SSRIs and other antidepressants.

Key words: depression and anxiety; SSRI antidepressants; fluoxetine; sex differences; animal models

Introduction

Decreased serotonergic activity has been implicated in depressive and anxiety disorders, and antidepressants directly increase the long-term activity of the serotonin system (1). Selective serotonin reuptake inhibitors (SSRIs) are commonly prescribed antidepressants in the treatment of depressive and some anxiety disorders (2). This predominance is due in part to their limited side-effects and high selectiveness to serotonin transporter inhibitor, in comparison to tricyclic antidepressants (3). Fluoxetine was the first of SSRIs and is the most studied antidepressant (4), mostly in men and male animal models. Results obtained in men have been often uncritically generalized to women, therefore exact response to SSRIs in women is still not well known. A growing amount of data shows that differences in pharmacokinetics, pharmacodynamics, and physiology exist between women and men and that they contribute to the occurrence of sex-gender differences in drugs response (reviewed in 5).

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Depressive disorders

Depression is a heterogeneous, multifaceted disorder with symptoms manifested at the psychological, behavioral and physiological levels (6). There are three frequent types of depressive disorders that vary in severity of symptoms and persistence: *major depression* (also called *unipolar depression*) where symptoms interfere with the ability to eat, sleep, work and enjoy life and last chronically for at least 2 weeks; *dysthymia* which is a long-term or chronic disease lasting for at least 2 years and is characterized by less severe, non-disabling symptoms; and *bipolar disorder*, which is characterized by wide mood swings ranging from deep lows to manic highs (1, 7, 8). Both major depression and dysthymia occur in the absence (*primary depression*) or presence (*secondary depression*) of the other psychological or physical problems beside the reduced mood, low self-esteem, feelings of worthlessness, general fatigue, feelings of guilt, disturbances in sleep, sex drive and food intake, anger, absence of pleasure and agitated or retarded motor symptoms (6). Depressive disorders are the fourth leading cause of disease burden worldwide (9, 10). Epidemiological and clinical studies have consistently observed significant sex-specific differences among patients with depression, with women outnumbering men at least 2:1 (11, 12) and this sex difference becomes evident after the onset of puberty (13). While in recent years a number of hormonal systems have been demonstrated to be associated with depression (i.e., appetite-regulatory, thyroid and growth hormones; reviewed in (7), evidence overwhelmingly supports the involvement of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes in the development of mood dysregulation (7, 14). Co-morbidity with HPA-HPG-axis dysregulation is not surprising, as depression is a disorder that involves hypothalamic nuclei (paraventricular and ventromedial), central amygdala, hippocampus, subgenual anterior cingulate cortex, and medial and orbitofrontal cortex, regions that have dense expression of glucocorticoid and sex steroid hormone receptors (reviewed in (1, 14).

Anxiety disorders

Anxiety disorders can be described in terms of the situation, object or thoughts which provoke anxiety, the specific expression of anxiety in terms of autonomic, and cognitive or motoric features, as well as the specific behaviors used to cope with the provoked anxiety (6). Anxiety reactions can vary in intensity, frequency, persistence, trigger situations, severity and consequences and other qualifying features (15). DMS-5® specifies over 12 different anxiety disorders (6), classified in five types: phobias, panic disorder, obsessive-compulsive disorder, post-traumatic stress disorder, and generalized anxiety disorder. Anxiety is reported to be the most prevalent disorder among all psychiatric diseases (16). Data from epidemiological studies have consistently shown that anxiety disorders are at least twice as common in women as in men (11, 12). Anxiety is also a common symptom of depression. Many individuals with major depression disorder experience severe anxiety and many individuals with anxiety disorders develop major depression disorder (3), what is not surprising as it is known that neural circuits thought to regulate both conditions do overlap (17). Corticotropin-releasing hormone (CRH), a strong anxiogenic neuropeptide, and its receptors are localized within the serotonergic raphe nuclei suggesting that interactions between the CRH system and serotonin may play a role in fear and anxiety (reviewed in 18).

SSRI antidepressants

In the treatment of depression, different antidepressant such as selective serotonin reuptake inhibitors (SSRIs), tricyclics (TCAs), and monoamine oxidase inhibitors (MAOIs) are used. Today, the most widely prescribed antidepressants with a minimum of side effects are SSRIs (3). SSRI antidepressants are also effective in treating some anxiety disorders (2).

Serotonin (5-HT) is produced by serotonergic cell bodies in the raphe nuclei, which form a cluster of nuclei in the brain stem (3) and send their axons to many brain regions throughout the brain and affect multiple central processes, including emotion, learning and memory, feeding, sleep, sexual and other social behaviors and sensory perception (19). Serotonin at the synapses may undergo several different molecular processes after release into synaptic cleft, one of them is reuptake by a presynaptic serotonin transporter channel (5-HTT or SERT). The targets

of SSRIs are 5-HTTs, which are located at the plasma membrane of serotonergic neurons, and are responsible for 5-HT reuptake (3). SSRIs inhibit the 5-HTT, resulting in increased extracellular 5-HT levels, and thereby sustained activation of pre- and postsynaptic 5-HT receptors (3, 19). However, the therapeutic action of SSRI antidepressants is dependent on long-term administration, suggesting that adaptations to the upregulation of 5-HT are required for therapeutic responses (mood improvement) (1).

The mostly prescribed SSRIs are fluoxetine, sertraline, paroxetine, citalopram and escitalopram (3, 19, 20). Fluoxetine was first synthesized in 1971 (21) and the United States Food and Drug Administration (FDA) approved fluoxetine in 1987. In 1988 it was launched on the market under the trade name Prozac as a first SSRI to be marketed in the United States (reviewed in 22).

Sex differences in treatment of depression and anxiety disorders

Women are clearly different from men in clinical appearance and characteristics of many psychiatric illnesses (12, 23), and also in therapy responses. An increasing number of studies have reported differences in the pharmacokinetics and/ or pharmacodynamics of antidepressants between women and men, although the clinical treatment at present is still identical in both sexes (reviewed in 24). Physiological differences in women and men that may affect pharmacokinetics include average body weight, body composition, and the affinity and/or capacity of metabolizing enzymes for the administered drug. Many studies have shown that sex hormones could influence absorption, distribution, metabolism, pharmacodynamics, and adverse effects of many different drugs (reviewed in 5).

Several studies have identified sex differences in fluoxetine treatment with women of reproductive age responding to fluoxetine better than men (25, 26). Estrogens may boost the effects of SSRIs, as postmenopausal women taking estrogens and treated with fluoxetine responded significantly better than women treated with fluoxetine only (27). Some laboratory studies in rodents also suggest that gonadal hormones modulate mood and the response to SSRIs (e.g., 28, 29) with inducing changes in the serotonin systems (30). Antidepressant effects in female rats are reported to be weaker during phases with lower levels of gonadal hormones (metestrus/diestrus) in comparison to females in higher gonadal hormone phases (proestrus/estrus) or to males (31). Gonadal hormone responsible for these differences seems to be estradiol, as orchidectomized male rats treated simultaneously with 17β-estradiol (10 μg/rat) and fluoxetine had much better behavioral response in comparison to males treated with fluoxetine only (29).

Animal models

The ideal animal model for any human clinical condition must fulfill three criteria (16): [1] pharmacological treatments effective in patients should induce comparable effects in the animal model (predictive validity); [2] the responses/ symptoms in patients should be the same in the animal model (face validity); [3] the underlying rationale should be the same in both humans and animal models (construct validity). Meeting all three validity criteria is difficult for an animal model of depressive/ anxiety disorders. Namely, many of the human symptoms of depression/ anxiety like recurring thoughts of death or suicide or excessive thoughts of guilt are impossible to be modeled in laboratory rodents (6). However, the physiological and behavioral responses to aversive stimuli, similar in both humans and animals, are allowing animal models to be used for at least two distinct purposes: as behavioral tests to screen for potential antidepressant/ anxiolytic properties of drugs and as tools to investigate specific pathogenetic aspects of cardinal symptoms of disease (reviewed in 16).

Behavioral data from our laboratory (32) in C57BL/6J mice of both sexes as a potential animal model to study depression/anxiety in comparison to behavioral data of other studies in mice and rats is presented. C57BL/6J male and female mice were originally obtained from Harlan Italy and bred at the University of Ljubljana, Veterinary Faculty, in standard conditions with 12-12 LD cycle (lights on at 3 am and off at 3 pm) and food (phytoestrogen free diet; Harlan Teklad Diet 2016, Harlan, Milan, Italy) and water *ad libitum*. Mice were weaned at 21 days of age and group-housed (3 mice of same sex per cage) in 15 cm high cages with floor area of 37.5 x 22

cm. At 55 days of age mice were divided into four groups with 9 mice per group: Control males and females, Fluoxetine males and females. Fluoxetine (Sigma-Aldrich®) was delivered in drinking water (10 mg/kg/day) as described elsewhere (33). At approximately 70 days of age (or at least 14 days of fluoxetine treatment) the behavior assessment using "stopwatch" software (Center for Behavioral Neuroscience, Atlanta, GA, USA) began with at least 2 days break between each behavioral test in the following order: elevated plus maze (EPM), open field (OF) and forced swim test (FST). Females were tested in the diestrus phase what was checked before each behavior assessment by vaginal smears as described previously (34). All animal experiments were approved by Veterinary Administration of the Republic of Slovenia and were done according to ethical principles, EU directive 2010/63/EU, and NIH guidelines. Statistical analyses were done using NCSS software (NCSS statistical software, Kaysville, UT, USA). To test differences between groups, repeated measures ANOVA was performed with sex and treatment as independent variables, followed by post hoc Fisher LSD test. Differences were considered statistically significant with $p < 0.05$ (32).

Depression-related behavioral assessments

Forced swim stress is one example of acute stressors that was developed as a tool to test the efficacy of antidepressant compounds (35) and is probably the most used tool among all animal models for screening antidepressants in mice and rats (36, 37). The critical response measured is immobility in an inescapable situation, which is believed to measure despair-like behavior (38).

Forced swim test (FST)

The first forced swim test (FST), also termed as behavioral despair test, was developed by Porsolt and coworkers in the rat (35) and subsequently in the mouse (39). In this animal model of depression animals are forced to swim in a tall cylinder and the time spent swimming or climbing (active behavior) versus the time spent floating (passive behavior) is measured. Session durations between 4 and 20 minutes have been used in mice, with 2 to 5 minutes of pre-exposure period (36, 40). If the animals cease all movements (active swimming motions), except those necessary for survival (keeping the head above the water), the behavior is considered to be immobile (floating). This immobile behavior is considered as an index of despair in response to the stressor or as an index of coping with the stressful procedure (41) and is diminished by antidepressant administration (38).

In our lab the FST was performed as described elsewhere (33), with 5 minutes session duration and 2 minutes of pre-exposure period (32).

Sex differences in FST

Studies of sex differences in the FST in rats and mice have shown highly controversial results likely due to several causes such as strain, different behaviors analyzed, exposure to various conditions prior to testing, estrous cycle phase and others (42). Some studies have shown that female Wistar rats in estrus phase are showing lower immobility and higher active behaviors in comparison to males (28, 43) what could be the result of high estrogens levels in females. However, some other studies that did not control for the phase of the estrus cycle showed higher levels of despair (longer immobility periods) during the FST in female rats (Wistar, Sprague-Dawley) in comparison to males (44, 45, 46). The second important difference between these contradictory results is that in the latter studies rats were exposed to at least two other stressors/ behavior tests (open field, light and dark transitions) prior to the exposure to the FST, suggesting that expositions to other stressors might increases the vulnerability of female rats to develop depressive-like behaviors (45).

In another study, chronic fluoxetine treatment (10 mg/kg) reduced immobility and increased active behaviors in male rats (Sabra strain derived from Wistar) only, and had no effects in females (estrous cycle phase was not reported; 47). However, some newer studies have shown that acute or chronic fluoxetine treatment (10 or 20 mg/kg) produced an antidepressant-like effect (reduced immobility) in both male and female rats (Wistar; females tested in estrus phase; 28, 48) and in females this effect was observed at lower doses (5 mg/kg) in comparison to males (10 mg/kg) (28), suggesting that estrus females are more sensitive to the antidepressant-like effects of fluoxetine.

In our laboratory, similar studies were performed with socially housed adult C57BL/6J mice, chronically treated with fluoxetine in drinking water (10 mg/kg for at least 14 days). All females were tested in the diestrus phase what was checked by vaginal smears, taken before each behavior assessment. Although we did observe fluoxetine effect in both male and female mice, no sex difference in immobility/ swimming time in the FST was observed (Figure 1), even after exposure to three other behavioral tests (elevated plus maze, open field, social recognition test) prior to FST (32), suggesting that female rats might be more vulnerable to the acute stress caused by FST than female mice.

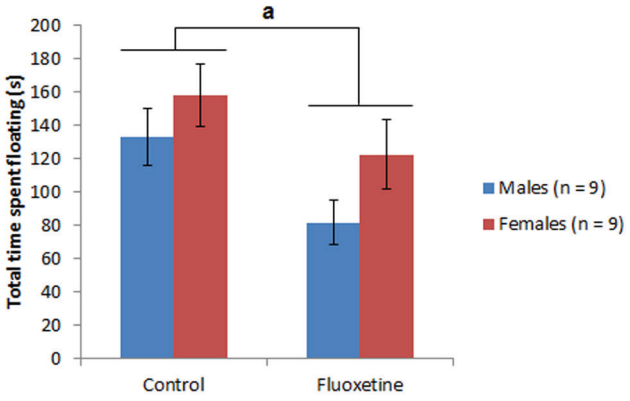

Figure 1: FST in male and female mice did not reveal any significant sex difference in response to fluoxetine or in behavior in FST, although fluoxetine treatment significantly reduced immobile time in both sexes. Data are reported as mean ± SEM; [a] Significant effect of treatment, $p < 0.05$

Anxiety-related behavioral assessments

Anxiety in rodents is defined as a high level of avoidance of novel and unfamiliar environment and increased fear reaction (16). Probably the most widely used test to assess the anxiety is the elevated plus maze (EPM), and less often the open field test (OF). OF test is mostly used to check whether changes in immobility observed in FST are associated with alterations in the motor activity (e.g., 48).

Elevated plus maze (EPM)

Probably the most frequently used test for unconditioned anxiety assessment, widely used in pharmaceutical companies, is the elevated plus maze (EPM), which was first introduced by File and coworkers in rats (49) and later in mice (50). The plus maze, elevated above the ground, consists of four arms arranged in a cross formation: two opposing non-anxiogenic closed arms with walls and other two anxiogenic open arms without walls (40). Rodents tend to avoid elevated, brightly lit areas, and avoidance of the open arms is interpreted as anxiety like behavior (49, 50). The animal is placed in the junction of the open and closed arms, and entries into the each arm and time spent in each arm over a 5-minute test session is scored (40).

In our lab the EPM was performed as described elsewhere (50), with 5 minutes session duration (32).

Sex differences in EPM

Previous reports in male mice are inconsistent, with some studies reporting higher levels of anxiety in C57BL/6J compared to BALB/c mice (51), other reported opposite results (52, 53). A newer study in both sexes showed that C57BL/6J female mice are more anxious, spending less time in open arms, than males, but no sex difference was observed in BALB/c mice when females were tested in the diestrus phase (53). This is in agreement with our study (32) showing that females of C57BL/6J strain are more anxious than males (Figure 2), suggesting that C57BL/6J strain could be a good animal model for studying sex differences in anxiety disorders. In contrast, female rats tested in proestrus phase appear to be less anxious than male rats (54, 55).

Many previous studies of fluoxetine effects were performed only in males and are showing controversial results in behavior responses. Some studies in male rats (mostly used Wistar strain; ~10 mg/kg) of acute fluoxetine administration have shown an anxiolytic (56, 57), anxiogenic (58, 59, 60), or no effect (49, 61). In several studies (55, 57, 59) the chronic treatment (5, 10 or 20 mg/kg) did not affect behavior in EPM of male rats (Wistar-Kyoto, Sprague-Dawley or Wistar). Interestingly, one study reported that chronic fluoxetine treatment (5 mg/kg) decreased the time spent in the open arms (anxiogenic effect) in female rats during proestrous phase (Sprague-Dawley), and the stress exposure even potentiated

this effect (55). Our study with C57BL/6J male and female mice showed no treatment difference, neither in the number of entries nor in the total time spent in the open arms (32), what is in agreement with the study by Kobayashi and coworkers in males (62), suggesting that chronic fluoxetine has neither anxiolytic nor anxiogenic effects in EPM in either sex in C57BL/6J mice (Figure 2) and that fluoxetine treatment does not contribute to the major improvement of anxiety behavior like in humans (63).

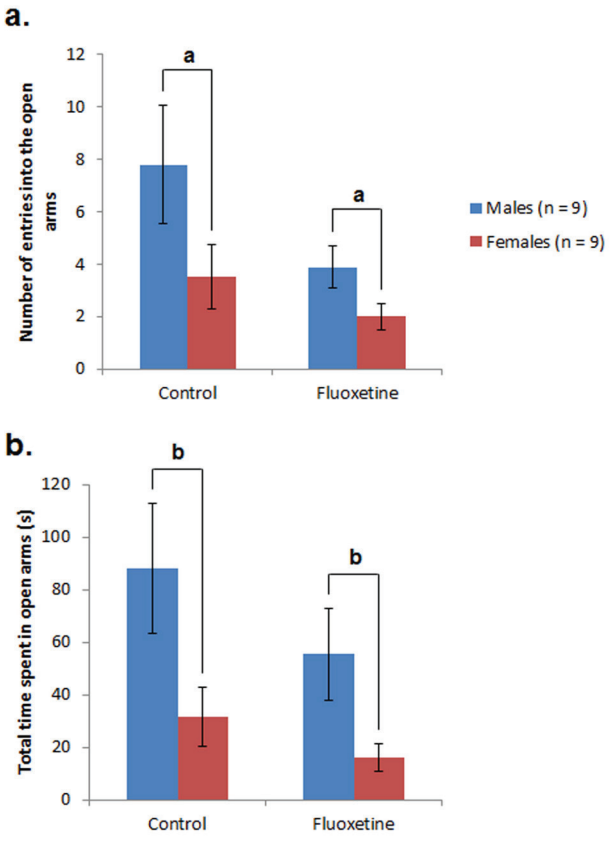

Figure 2: Sex differences are present in C57BL/6J mice (females were tested in the diestrus phase): **(a.)** number of entries into the open arms, **(b.)** total time spent in open arms. No significant effect of fluoxetine treatment in either sex was found ([a] $p < 0.05$, [b] $p < 0.01$). Data are reported as mean ± SEM; [a,b] Significant difference between males and females

Open field (OF)

In 1934 Calvin Hall designed the first open field test to assess "emotionality" in rats (64) and since then different types of open fields have been used. The modern standard open field is a Plexiglas box with square floor area, surrounded by high walls to prevent animals from escaping, and usually equipped with either photocells or videotracking and computer software to assess locomotor parameters. The animal is placed in the center or in the periphery of the area and the behavior assessment can last from 2 min to several hours. Like in EPM the avoidance of exploratory behavior towards the anxiogenic unprotected area (center zone) is the indicator for anxiety or fear-related behavior (16, 40). OF is mostly used for assessing spontaneous motor activity (distance traveled, average speed, duration of (im)mobility and others), which is the most standardized general measure of locomotor function (40), or to exclude the increased immobility in FST due to reduced locomotor ability (48).

In our lab the OF was performed as described elsewhere (62), with 30 minutes session duration (32).

Sex differences in OF

Previous studies in C57BL/6J and BALB/cJ mice have shown that males and females in diestrus phase did not differ in their locomotor or exploratory activity having similar duration of locomotion and spent similar time in the center area of OF (53), what is in agreement with our study (unpublished results) performed in C57BL/6J strain (Figure 3 and 4b).

There are numerous studies of fluoxetine effects on OF activity in mice but far less in rats. Neither acute (2 and 10 mg/kg) nor chronic (10 and 20 mg/kg) treatment in male rats (Wistar) have shown any effect on locomotor and exploratory activity of center area in comparison to controls (48, 61), and study in both sexes by Ghorpade et al. does not mention any sex differences between treated or control rats (48).

In regard to spontaneous motor activity, previous studies in male mice after chronic fluoxetine treatment (mostly 10 mg/kg) have shown differences between strains, with C57BL/6J mice having reduced, and BALB/cJ mice unchanged distance traveled in comparison to untreated males (62, 33). Indeed, C57BL/6J treated males in our study (unpublished results) also traveled shorter distance (Figure 3a), moved slower (Figure 3b) and had longer immobile periods (Figure 3c) in comparison to controls, and there was no sex difference observed (Figure 3).

Our results in females (unpublished results) are in agreement with the study of Marlatt et al. where chronically treated (18 mg/kg) C57BL/6J females also traveled shorter distance than control mice (65). Similar decrease in traveled distance with no sex difference was reported also after acute fluoxetine administration (15 mg/kg) (66).

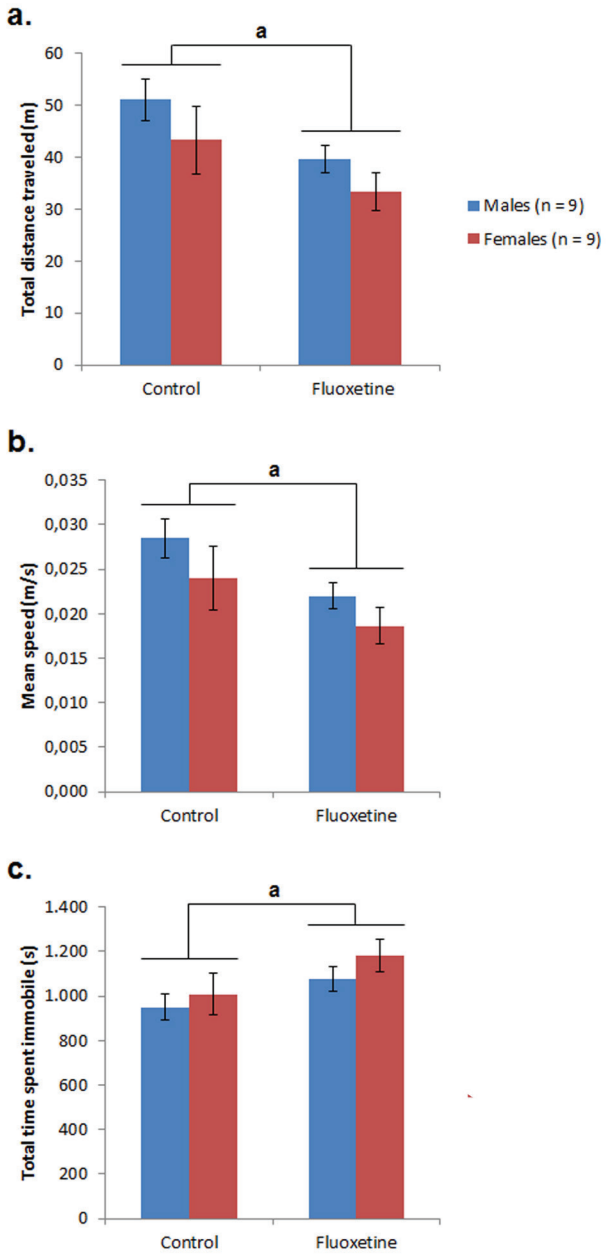

Figure 3: Spontaneous motor activity in OF did not differ between male and female C57BL/6J mice in response to fluoxetine or in behavior in OF, although fluoxetine treatment significantly affected locomotor activity in both sexes: **(a.)** reduced distance traveled, **(b.)** reduced average speed and **(c.)** prolonged time of immobility. Data are reported as mean ± SEM; [a] Significant effect of treatment, $p < 0.05$

In regard to the anxiety like behavior, chronic fluoxetine exposure in previous studies reduced the number of entries or time spent in the center of the OF in C57BL/6J, but not in BALB/cJ males relative to controls (62, 33), and such reduction in time spent in the center zone was revealed also in C57BL/6J females in comparison to controls (65), but there are no previous reports about sex differences in such effects of fluoxetine. However, in contrast to these studies, in our study (unpublished results) there was no effect of fluoxetine treatment on these two parameters in C57BL/6J males and females (Figure 4a and b), although there was a small, but significant sex difference in the number of entries into the central zone that was reduced in females but not in males (Figure 4a).

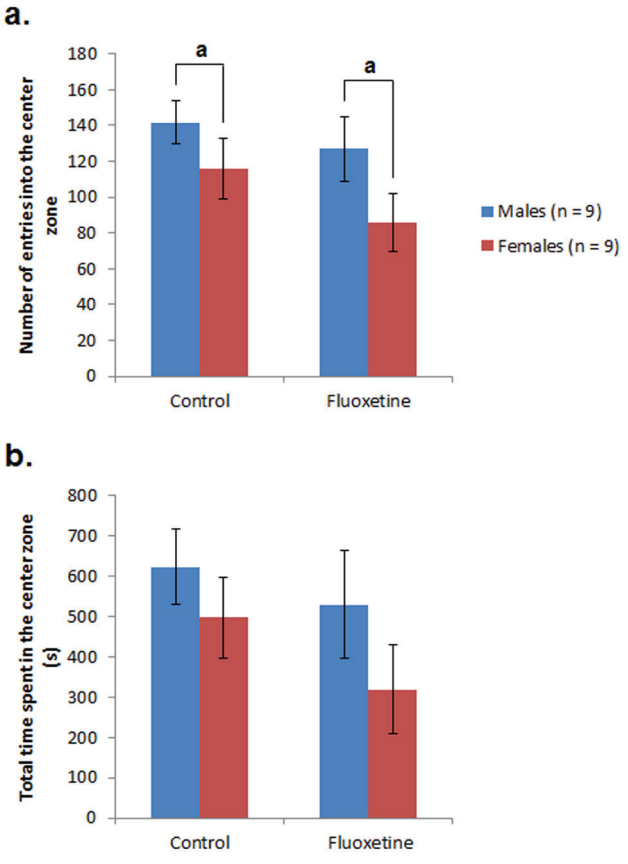

Figure 4: Sex differences in C57BL/6J mice (females were tested in the diestrus phase) were observed in the number of entries into the center zone of OF **(a.)**, but not in the total time spent in the anxiogenic area **(b.)**. No significant effect of fluoxetine treatment in either sex was found. Data are reported as mean ± SEM; [a] Significant difference between males and females, $p < 0.05$

Conclusions

Studies of sex differences in the FST, EPM and OF behavioral tests in mice and rats have yielded controversial results, most likely caused by several factors which are known to influence animal behavior such as species, strain, age, body weight, handling, social isolation or enriched environment, food, various kinds of stress, endocrine manipulations and surgery, schedule and routes of treatment, dosage of the drugs as well as experimental design and others. Consideration of these factors in planning experiments could result in more consistent results. However some common conclusions connected the main findings in the different rodent studies of FST, EPM and OF can be made:

- Proestrus/estrus females are usually less despaired and anxious than males or females in metestrus/diestrus.
- No consistent sex difference in the locomotor or exploratory activity in mice and rats are found.
- Chronic fluoxetine treatment provided more consistent effects than acute treatment.
- Proestrus/estrus females are usually more sensitive to the antidepressant like effects of fluoxetine than males.
- No effect of anxiogenic/anxiolytic treatment is usually found in males and metestrus/diestrus females, but anxiogenic effects of fluoxetine have been described in estrus/proestrus females.
- No sex/treatment difference in locomotor or exploratory activity in rats, but reduced locomotion in treated mice regardless of sex, was found.

Our data (32) with chronic fluoxetine administration in C57BL/6J mice of both sexes revealed that fluoxetine has an antidepressant like effect in FST with decreased immobility time but no effect on latency to float. Males and females did not significantly differ in their despair like performance. In regard to the anxiety like behavior, a chronic fluoxetine treatment had no anxiolytic or anxiogenic effect in the EPM or in the OF, but females behaved more anxiously than males in EPM and OF tests. However, fluoxetine did impair locomotor activity in comparison to control mice of both sexes as tested in OF. Taking together, C57BL/6J mice could be a good animal model for anxiety assessment studies as females were significantly more anxious than males, but not for despair behavior studies as females were equally depressed than males. Chronic fluoxetine treatment might not be a good model to study its effects in anxiety related disorders studies in C57BL/6J mice.

Acknowledgements

We would like to thank Ana Strgar and Ariadna Štorman for performing EPM and OF behavioral assessments.

Ethical statement

Animal experiments from our lab were approved by Veterinary Administration of the Republic of Slovenia and were done according to ethical principles, EU directive, and NIH guidelines.

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RAZLIKE MED SPOLOMA V DELOVANJU ZDRAVILA FLUOKSETIN PRI ŽIVALSKIH MODELIH ZDRAVLJENJA DEPRESIVNIH IN ANKSIOZNIH MOTENJ

J. Kerčmar, G. Majdič

Povzetek: Bolezenski znaki in uspešnost zdravljenja z različnimi zdravili se pri številnih duševnih boleznih med spoloma močno razlikujejo. Depresivne in anskiozne motnje se 2- do 3-krat pogosteje pojavljajo pri ženskah kot pri moških, vseeno pa se večina predkliničnih raziskav in preizkušanj novih zdravil opravi samo pri samcih poskusnih živali. Več raziskav je nakazalo, da je odziv žensk in ženskih živali na selektivne zaviralce prevzema serotonina (SSRI) boljši kot pri moških, kar kaže, da spolni hormoni vplivajo na odzivnost organizma na tovrstna zdravila. Uvedba prvega zdravila iz skupine SSRI, in sicer prozaca v osemdesetih letih prejšnjega stoletja, je bil pomemben napredek pri zdravljenju motenj depresivnosti od odkritja zaviralcev monoaminskih oksidaz in tricikličnih zdravil proti depresiji trideset let prej. Fluoksetin je danes v široki uporabi za zdravljenje depresivnih motenj, pa tudi za zdravljenje motenj anksioznosti. Živalski modeli predstavljajo dober model za proučevanje vpliva zdravil proti depresivnim in anksioznim motnjam in tudi za proučevanje spolnih razlik v delovanju tovrstnih zdravil. V preglednem članku smo predstavili različne živalske modele za proučevanje motenj depresivnosti in anksioznosti, in sicer test prisilnega plavanja, test dvignjenega labirinta in test odprtega polja. Poleg tega je predstavljen vpliv fluoksetina na obnašanje živali v teh testih s poudarkom na razlikah med spoloma. Številne raziskave v preteklosti so pokazale, da so laboratorijski glodavci primeren model za proučevanje tovrstnih motenj , v prihodnosti pa bo treba večji poudarek dati raziskavam razlik med spoloma pri nastanku tovrstnih obolenj in pri njihovem zdravljenju.

Ključne besede: depresivne in anksiozne motnje; zdravila iz skupine SSRI; fluoksetin; spolne razlike; živalski modeli

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Case Report

RETROGRADE JEJUNAL INTUSSUSCEPTION IN ONE YEAR OLD CAT AFTER TREATMENT WITH METOCLOPRAMIDE AND MENBUTONE

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Summary: Intussusception refers to invagination or prolapse of one portion of the intestine into the part of the tract that either precedes or follows it. Young cats may be more likely to have idiopathic intussusception, and older cats with intussusception may be more likely to have primary gastrointestinal tract disease, i.e. neoplasia. An intussusception will result in either a partial or complete intestinal obstruction and this will lead to a variety of clinical signs depending on the chronicity, size and location of the intussusceptions.

In the literature no suggestion has been made to show association between intussusception and prokinetic or choleretic drug.

A one year old male Maine Coon cat was presented with a history of anorexia, depression and inability to defecate for few days. On two consecutive days the cat was treated with metoclopramide, ranitidine, hyoscine butylbromide and menbutone. On the same day of therapy vomiting started and continued for three days before the moribund cat was presented to the clinic. In transverse ultrasonographic view, a target-like mass with multiple concentric hypo- and hyperechoic rings consistent with intussusception was seen in the left mesogastrium.

This paper describes that possible cause of intussusception may be iatrogenic by application of prokinetic metoclopramide and choleretic menbutone given to an obstipated cat.

Key words: cat; jejunal intussusception; surgery; prokinetic; choleretic

Introduction

Intussusception refers to invagination or prolapse of one portion of the intestine into the part of the tract that either precedes or follows it and may occur anywhere in the gastrointestinal tract (1).

Intussusceptions have been reported as sequelae to a number of conditions, including intestinal parasitism, viral-induced enteritis, alimentary foreign bodies (1, 2, 3, 4, 5) intestinal masses (1, 2, 4) recent abdominal or extra-abdominal surgery (1, 2, 4, 5, 6) and nonspecific gastroenteritis (1, 4, 5).

There is a bimodal age distribution of cats presented with intussusception. In older cats, most likely intussusceptions were associated with neoplasia or IBD in some cases. Underlying causes for younger cats are ill defined and may be idiopathic in many cases, but associations with parasitism and, in one case, a linear foreign body have been made (4, 7).

An intussusception will result in either a partial or complete intestinal obstruction and this will lead to a variety of clinical signs depending on the chronicity, size and location of the intussusception (5).

Diagnosis is established using a combination of history, physical examination and diagnostic

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imaging, including survey and contrast radiography and ultrasonography. Ancillary testing such as haematology, biochemistry, urinalysis, and faecal examination is frequently performed to try and identify an underlying cause (7).

Case description

A one year old, 4.9 kg, castrated, indoor, male Maine Coon was presented with a history of anorexia, vomiting, depression and unable to defecate for one week. Micturition was normal. He was regularly vaccinated but dehelmintisation was not carried out. The cat was treated on the other clinic four and three days previously with metoclopramide 1.0 mg/kg (Reglan, Alkaloid, Skopje, Macedonia), ranitidine 4.0 mg/kg (Ranital, Sandoz, Ljubljana, Slovenia), hyoscine butylbromide (Buscopan, Boehringer Ingelheim, Ingelheim, Germany) and menbutone (Genabil, Boehringer Ingelheim, Ingelheim, Germany). After therapy vomiting started and lethargy worsened therefore the cat was presented to our faculty clinic.

On clinical examination the cat was depressed and unresponsive, unable to stand, hypothermic (T=37.0°C), tachycardic (200bpm) with weak pulse, eye bulbs were sunken with scleral injection, capillary refill time was prolonged (> 4 sec) and skin turgor decreased. The cat was assessed 10% dehydrated. On auscultation heart murmur was detected. Lymph nodes were normal. Abdominal palpation was painful. The cat was hospitalised for stabilisation and further diagnostics.

Abnormalities on the complete blood count included leucocytosis with mature neutrophilia and lymphopenia, erythrocytosis, elevated hematocrit and haemoglobin. Blood chemistry abnormalities included decreased activity of ALP (alkaline phosphatase), hyperproteinemia, hyperalbuminemia, hypochloremia, hyponatremia, elevated urea and creatinine (Table 1).

Survey radiography of the cat in right lateral recumbency was performed (Figure 1). In thorax a microcardia was seen. In abdomen there was some air in the stomach and caudal to the liver a mass of soft tissue opacity. Some parts of small intestine were dilated. In transverse and descending colon formed faeces were present. In transverse ultrasonographic view (Figure 2), a target-like mass with multiple concentric hypo- and hyperechoic rings was seen in the left mesogastrium. The small intestine was fluid filled and dilated consistent with obstruction (Figure 3).

For the stabilisation of the cat we used saline (500 ml 0.9% NaCl, B Braun, Melsungen, Germany) in which we added 10 mmol potassium chloride (KCl) (University Medical Centre Ljubljana, Slovenia). It was administered with perfusion pump at rate of 20 ml/h for 7 hours, and then increased to 30 ml/h. A solution of vitamin B complex, electrolytes, amino acids and dextrose for injection (Duphalyte, Fort Dodge Veterinaria S.A., Girona, Spain) at dose of 50 ml was administered slowly intravenously. For analgesia tramadol 1 mg/kg (Tramal 50 mg/ml, Grünenthal GmbH, Germany) subcutaneously was given. Antibiotic therapy was started with cefazoline 20 mg/kg/8h (Cefamezin 1g, Krka, Novo mesto, Slovenia) intravenously.

The next day the cat underwent exploratory surgery, he was premedicated with methadone 0.18 mg/kg (Heptanon, Pliva, Zagreb, Croatia) subcutaneously two hours before intravenous induction with propofol 3.6 mg/kg (Norofol 10 mg/ml, Norbrook, Newry, Ireland). Five minutes before induction with propofol the cat was preoxygenated with oxygen 2 l/min. After induction the cat has vomited a lot of fluid and some of that was aspirated. The cat was intubated with cuffed endotracheal tubus with internal diameter 4.5 mm. Fluid from trachea, bronchi and oral cavity was aspirated and the cat was then connected to the anaesthetic machine via circle breathing system. Anaesthesia was maintained with isoflurane (Forane, Abbott, Berkshire, UK) in 100 % oxygen for 145 minutes. End tidal isoflurane was 1.4 – 1.3%. Lactated Ringer's solution (RL) (Hartmannova raztopina Braun, B Braun, Melsungen, Germany) was given at rate 10 ml/kg/h.

On surgery a ventral midline laparotomy had been performed. We found that the large portion of the jejunum has telescoped into the oral part of intestine (Figure 4). As traction failed to reduce the jejunojejunal intussusception, resection of the affected bowel was deemed necessary, with anastomosis of the healthy tissue. Approximating end-to-end intestinal anastomosis was created with simple interrupted suture pattern with monofilament synthetic absorbable suture material Glycomer 631 (Biosyn 4/0, Syneture, Grimsby, UK) (Figure 5). The large bowel was filled with formed hard dry faeces which were milked out through the anus. Abdominal wall was closed in

three layers with absorbable monofilament suture.

Intravenous fluids RL with KCl supplementation were administered at 30 ml/h on the day of surgery and 20 ml/h next two days, during hospitalisation. Duphalyte solution for injection (50 ml) was administered on the day of surgery and discontinued after. Cefazolin was discontinued and broad-spectrum antibiotic therapy was started with amoxycilin and clavulanic acid 20 mg/kg/12h (Synulox, Pfizer, Roma, Italy) subcutaneously and metronidazole 10 mg/kg/12h (Efloran, Krka, Novo mesto, Slovenia) intravenously. Both antibiotics were discharged for another 12 days postoperatively. Pain was treated with methadone 0.2 mg/kg/12h and fentanyl patch (Durogesic 12 µg/h, Janssen pharmaceutica, Beerse, Belgium). Two days postoperatively meloxicam 0.1 mg/kg/24h (Loxicom 1.5 mg/ml, Norbrook, Newry UK) was started intravenously and continued orally three consecutive days. He was also treated with sucralfate 0.25 g/8h (Venter 1g, Krka, Novo mesto, Slovenia) orally.

Table 1: Laboratory findings from the day of operation to the first postoperative day

	Unit	Reference range	Preoperative day	Day of operation	Postoperative day
WBC	x 10^9 /l	5.5 – 19.5	**26.84**	**22.56**	**40.48**
RBC	x 10^{13} /l	5 - 10	**10.89**	9.74	7.07
HGB	g/l	80 - 150	**171**	**151**	111
HCT	l/l	0.3 – 0.45	**0.49**	0.44	0.33
MCV	fl	39 – 55	45.1	45.6	46.3
MCH	pg	12.5 – 17.5	15.7	15.5	15.7
MCHC	g/l	300 – 360	348	339	339
RDW	%		15.2	14.9	14.6
HDW	g/l		29.1	29.3	27.6
PLT	x 10^9 g/l	300 - 700	360	**144**	**263**
MPV	fl		9.2	8.2	8.4
PDW	%		36.4	36.3	44.5
PCT	l/l		0.003	0.001	0.002
Differential cell count					
NEUT	x 10^9 /l	2.2 – 12.8	**23.84**	**20.19**	**35.42**
LYMP	x 10^9 /l	1.5 – 7	**1.02**	**0.98**	2.41
MONO	x 10^9 /l	0.1 – 0.85	0.55	0.49	0.16
EOS	x 10^9 /l	0.1 – 1.5	**1.57**	0.87	**2.45**
BASO	x 10^9 /l		0.06	0.03	0.04
LUC	x 10^9 /l		0	0	0
Urea	mmol/l	5.3 – 12.1	**76.24**	**26.5**	11.9
Creatinine	mmol/l	70.7 – 159	**328.4**	127.7	112.0
Na	mmol/l	147 – 156	**128.8**	154.1	147.8
K	mmol/l	4.0 – 4.5	4.13	**3.42**	4.12
Cl	mmol/l	117 – 123	**69.0**	**110.6**	**114.3**
ALP	U/l	25 – 93	7.4		
ALT	U/l	6 – 83	80.8		
Total protein	g/l	54.0 – 78.0	90.2		
Albumins	g/l	21.0 – 33.0	46.17		

Legend: WBC – white blood cells, RBC – red blood cells, HGB – haemoglobin, HCT – haematocrit, MCV – mean corpuscular volume, MCH – mean corpuscular haemoglobin, MCHC – mean corpuscular haemoglobin concentration, RDW – red cell distribution width , HDW – hemoglobin distribution width, PLT – platelets, MPV – mean platelet volume, PDW – platelet distribution width, PCT – plateletcrit, NEUT – neutrophils, LYMP – lymphocytes, MONO – monocytes, EOS – eosinophils, BASO – basophils, LUC – large unstained cells, Na – sodium, K – potassium, Cl – chloride, ALP – alkaline phosphatase, ALT – alanine aminotransferase

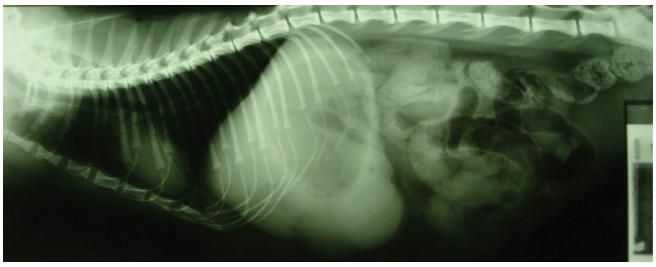

Figure 1: A right lateral thoracic and abdominal radiograph. In ventral abdomen, caudal to the liver, a mass of soft tissue opacity is seen. Some loops of small intestine are dilated. Large intestine is filled with formed faeces

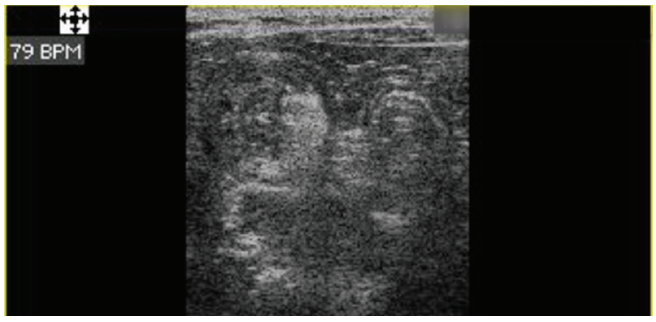

Figure 2: Transverse sonogram of jejunal intussusception. Note the multilayered appearance of the intestinal wall (called also *concentric rings*). Only a small amount of fat is invaginated. There is some gas in the lumen

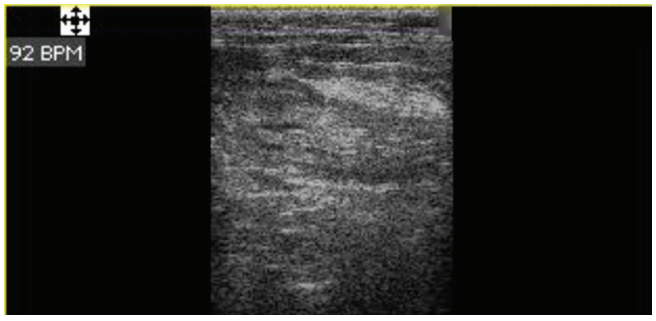

Figure 3: Longitudinal sonogram of jejunal intussusception

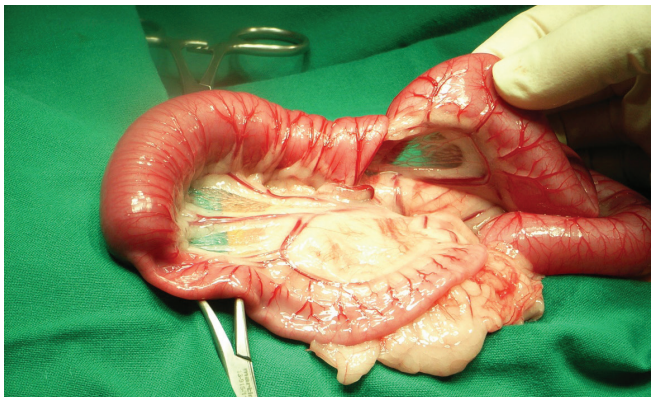

Figure 4: Intra-operative appearance of a retrograde jejunojejunal intussusception of a cat at laparotomy

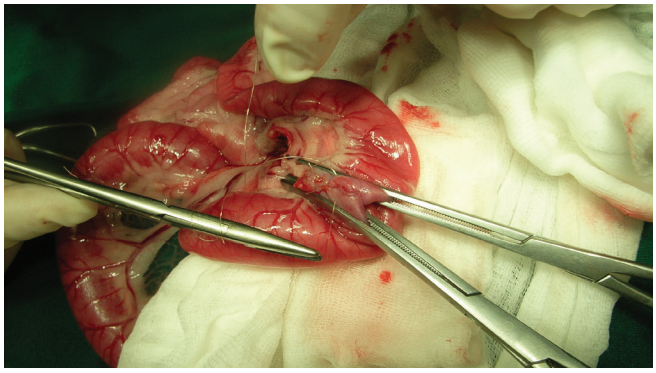

Figure 5: Apposing the wound edges with simple interrupted appositional sutures after resection of affected jejunum

Discussion

Because cats and dogs differ in their anatomy, physiology and behaviour, there may be differences in the clinical presentation of intestinal intussusceptions (7). It usually occurs in the direction of normal peristalsis, these are referred to as direct or normograde intussusceptions (8, 9, 10). In our case intussusception occurred against the direction of normal peristalsis, referred to as indirect or retrograde intussusception.

The most common location of intussusception is ileocolic and jejunojejunal (4, 6, 7, 8, 9, 11, 12). Burkitt et al., 2009, found that 40% intussusceptions in cats were jejunojejunal (4). Levien and Baines, 2011, reported, that two of 18 cats presented with jejunojejunal intussusception were Maine Coon breed aged 17 and two months with no underlying cause detected (11), which was consistent with our case, Maine Coon, 13 months old of jejunojejunal intussusception detected. Unpublished data from our clinic show that two of three cats presented with jejunojejunal intussusception in last year were Maine Coons, suggesting that Main Coons may be overrepresented; however the number of cats was too small to make any conclusions.

The cat in our study had signs of shock consistent with findings in previous study (4). The most consistent clinical signs seen in cats in one study were a palpable abdominal mass and anorexia (7, 12) however other study reported that palpable abdominal mass was evident only in three of nine cats presented (6). In our case abdomen was painful on palpation without resistance. Most common clinical signs in cats were also vomiting, depression, weight loss and dehydration (4, 6, 7) which were all present in our cat. Bellenger and Beck, 1994, suggested that cats with intussusception are less likely to eat and drink and, therefore, there is less ingesta to result in diarrhea (7).

Abdominal radiographs revealed intestinal dilatation in all cats and gastric dilatation in two of 10 cats with intussusceptions (4), and consistent with those findings our cat had intestinal and gastric dilatation. Our final diagnosis based on ultrasonographic examination was jejunojejunal intussusception.

Surgical treatment in our cat involved unsuccessful simple manual reduction, resection and anastomosis of the involved portion of the intestine. Manual reduction of the intussusception was attempted by gentle "milking" of the intussusceptum from within the intussuscipiens. This technique should employ more pressure on the intussuscipient in an effort to reduce the intussusceptum by pushing it out rather than using traction on the intussusceptum (8, 9). However gentle milking failed to reduce the jejunojejunal intussusception, because there was significant venous infarction, edema, and congestion as well as adhesions from fibrin and effusions from the affected bowel. Adhesions between the intussusceptum and intussuscepiens that we observed are reported to be common in cats, but there is no relationship evident between the duration of clinical signs and the presence or absence of adhesion (6). We performed resection and anastomosis of the intussusception, which is reported to lessen the incidence of recurrence when compared with manual reduction (6, 13, 14).

A simple interrupted appositional suture that we used incorporates all tissue layers and gently apposes the wound edges. Interrupted pattern is generally easier to perform, but the simple continuous pattern minimizes mucosal eversion and therefore provides better serosal apposition and primary intestinal healing. Regardless of the suture technique used, proper incorporation of the tough submucosa and reduction of mucosal eversion are vital in performing consistently successful intestinal anastomosis (15). Recurrence of intussusception is not related to intestinal plication performed at the initial surgery (6). In our cat the plication was not performed and intussusception has not recurred. The decrease in incidence of intussusception was hypothesized to be the result of increased smooth muscle tone along the whole gastrointestinal tract together with a decrease in propulsive peristalsis secondary to opioid administration (16, 17).

Although the results of histological examination are not commonly reported in the literature, they may identify an underlying cause and allow diagnosis of associated or concurrent diseases (11). In our case a portion of the affected intestine had been submitted for histology examination. Intussusception was considered idiopathic because the results revealed only inflammatory changes considered secondary to the intussusception. Major limitation of the present case report is the lack of faecal parasite

examination which may have ruled out parasitism as cause of intussusception.

Metoclopramide stimulates motility of the upper gastrointestinal tract; gastric emptying and intestinal transit time can be significantly reduced but has no effect on colon motility. It also increases duodenal and jejunal peristalsis (18), therefore it should not be used when gastrointestinal obstruction is suspected (19), as metoclopramide may cause constipation itself (18). Side effects are uncommon in metoclopramide therapy but occur more often in cats than in dogs (19).

Menbutone is indicated for constipation and intestinal atony in dogs and large animals (20) and should according to manufacturer's instructions never be given to cats. The product contains cresol and cats have limited ability to glucoronidate it (21). Menbutone increases the excretion of the bile, the gastric and the pancreatic juice into the gut by two- to five-fold the normal secretion, stimulates the function of the gastrointestinal tract and increases peristalsis (22).

With regard to the fact that metoclopramide was given to our cat in overdose (1 mg/kg) compared to 0.2 – 0.4 mg/kg, which is recommended dose for cats by Plumb, 2011, and vomiting occurred after overdosed therapy with it we assume that metoclopramide was the cause of vomiting. However, vomiting in a cat may have also appeared due to formation of jejunojejunal intussusception as a result of application of both drugs metoclopamide and menbutone, which increased jejunal peristalsis, since the cat was already unable to defecate for seven days. On the other hand initially intussusception, if already present, might have caused only partial intestinal obstruction, which had progressed to complete obstruction after the prokinetic and choleretic therapy and to worsening of clinical signs, which had already been present for four days. It was in our cat impossible to discern whether the cat was initially presented with intussusception, because the duration of illness in cats varies from 12 hours to three weeks (7). Application of metoclopramide in gastrointestinal obstruction may delay diagnosis (19), in general in jejunojejunal intussusception the duration of signs is longer than in ileocolic intussception (7). However due to acute worsening of clinical signs after the therapy we assume that prokinetic and choleretic therapy caused intussusception. Intussusception should be included in the differential diagnosis of all cats presented with typical signs of intussusception, i.e. anorexia, lethargy and vomiting. We conclude that metoclopramide should be given with caution or not given at all in cats presented with obstipation as it may cause the intussusception or, if already present, partial intestinal obstruction may progress to complete obstruction (23). The prognosis for cats with intussusception appears to be good given appropriate medical and surgical treatment (7).

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RETROGRADNA INVAGINACIJA JEJUNUMA PRI ENO LETO STARI MAČKI PO ZDRAVLJENJU Z METOKLOPRAMIDOM IN MENBUTONOM

Povzetek: Invaginacija je uvihanje ali prolaps enega dela črevesja v drugi del pred ali za njim. Pri mladih mačkah je navadno idiopatska, medtem ko je pri starejših pogostejši vzrok primarno gastro-intestinalno obolenje, t.j. neoplazija. Posledica invaginacije je delna ali popolna obstrukcija, ki se kaže z različnimi kliničnimi znaki, odvisno od trajanja, obsega in lokacije invaginacije. V literaturi nismo zasledili neposredne povezave med invaginacijo in prokinetiki ali holeretiki.

Leto star maček, pasme main coon je bil pripeljan na kliniko z anamnezo, da nima apetita, da je apatičen in že nekaj dni ne more odvajati blata. Dva zaporedna dneva je bil zdravljen z metoklopramidom, ranitidinom, hioscinijevim butilbromidon in menbutonom. Dan po zdravljenju je začel bruhati. Po treh dneh bruhanja je bil moribunden pripeljan na kliniko. Z ultrazvočnim pregledom trebuha smo v levem mezogastriju našli maso z multiplimi koncetričnimi hipo- in hiperehogenimi krogi, ki je značilna za invaginacijo črevesja.

Predstavljamo primer invaginacije črevesja pri obstipirani mački po aplikaciji prokinetika metoklopramida in holeretika menbutona.

Ključne besede: mačka; invaginacija jejunuma; kirurgija; prokinetik; holeretik

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