



The Role of Neutrophils and NETosis in Local Immunity of Feline Inflammatory Aural Polyps

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ABSTRACT

Feline inflammatory aural polyps are abnormal growths that can occur in the ear canals of cats, particularly in the middle ear. These polyps are frequently linked to persistent inflammation and can result in a range of ear-related complicated pathologies. The etiology is multifactorial. The purpose of the research was to study the cytology of an inflammatory polyp in a cat and to study the role of neutrophils and their mechanisms on the formation of extracellular protective traps by neutrophils (NETs). A 4-year-old, female spayed, Scottish fold cat, weighing 3.5 kg sent to a veterinary clinic (Mirra-Vet, Kyiv, Ukraine). Clinical, otoscopic methods, and laboratory methods of cytological diagnostics were used for the research. At the onset of the clinical investigation, exudate discharge from the ear and a painful response were observed. Upon detailed otoscopy, a polyp in the ear canal was diagnosed. An increase in the number of leukocytes ($23.2 \times 10^9/L$), their absolute content, and an increase in the percentage of neutrophils (48.2 %) in the leukogram. Assessing the capacity of neutrophils to generate NETs (Neutrophil Extracellular Traps) was determined after samples were collected using a cytologic brush. Cytological analysis of samples from the inter-tragic incisure area highlighted a significant presence of neutrophils, forming extracellular protective traps. The results revealed free NETs in separate areas of the slides. The findings indicated the formation of cooperative groups among neutrophils, other phagocytes, and epithelial cells, along with slender nuclear streaks. During the treatment (Otoflox, 2 drops per ear), the inflammatory reaction disappears, polyp size decreases, exudative reactions decrease, and neutrophil activity decreases. After 3 days of treatment, the animal's condition improved. The ear was clean without sulfur and lesions. The complete treatment course spanned 7 days. During the treatment, the inflammatory reaction disappeared, polyp size decreased, exudative reactions decreased, and neutrophil activity decreased. Experimental studies have shown that during the inflammatory reaction in the ear, protective mechanisms of local immune defense are activated. Activated neutrophils perform their function through phagocytosis and the formation of NETs. These studies contribute to supplementing the data on the immunopathological mechanisms of feline inflammatory polyps.

Keywords: Ear polyps, Feline inflammatory, Local immunity, Neutrophils

INTRODUCTION

Feline inflammatory aural polyps are epithelial formations with signs of inflammation that can be localized in the external ear canal, the tympanic cavity, or on the mucous membrane of the nasopharynx (Greci et al., 2014; Momota et al., 2016; Hoppers et al., 2020). Feline inflammatory aural polyps can affect 7 to 12% of cats, breed predisposition has also been identified in Siamese and Himalayan cat breeds. Bilateral ear polyps in cats may account for up to 24% (Greci and Mortellaro, 2016; Hoppers et al., 2020). They typically occur unilaterally, with etiological factors encompassing both genetic and acquired aspects (Sula et al., 2014; Mutua and Gershwin, 2021).

Bacteria, viruses, and pathogenic fungi play an important role in the onset and development of feline inflammatory aural polyps (Hariharan et al., 2011; Mascarenhas et al., 2019). Recurrence is common in feline inflammatory ear polyps. Researchers report the prevalence of this condition among cats (McAulliffe et al., 2020). In dogs, inflammatory polyps statistically occur less frequently, and they are mostly localized on the nasal mucous membrane (Bizikova and Burrows, 2019). Currently, there are several diagnostic and treatment methods developed for animals with feline inflammatory aural polyps (Ginel et al., 2002; Moore et al., 2019). However, the study of local immunity is of particular interest among scientists (Wainberg et al., 2019; Tyler et al., 2020).

Inflammation is a fundamental and complex biological response that serves as a protective mechanism against infections and tissue damage (Mutua and Gershwin, 2021). Within the diverse elements of the immune system, neutrophils stand out as crucial participants in coordinating the inflammatory process (Zhelavskiy et al., 2020). In the dynamic landscape of inflammation, neutrophils play intricate roles, utilizing their unique extracellular protective traps to contribute to the complex immune response (Adrover et al., 2020; Qi et al., 2021).

CASE REPORT
Received: January 17, 2024
Revised: February 20, 2024
Accepted: March 04, 2024
Published: March 25, 2024

Neutrophils, a subtype of white blood cells, act as the initial responders to areas of infection or injury (Zhang *et al.*, 2021). Their primary mission is to neutralize invading pathogens and facilitate tissue repair. Equipped with a diverse array of weapons, including enzymes, antimicrobial peptides, and reactive oxygen species, neutrophils are highly effective in combating microbial threats (Adrover *et al.*, 2020; Demkow, 2021). One of the initial steps in the inflammatory response involves chemotaxis, where neutrophils are attracted to the site of infection or tissue damage by chemical signals (Qi *et al.*, 2021; Zhang *et al.*, 2021). These signals guide the neutrophils through the bloodstream, allowing them to quickly reach the affected area. This recruitment process is crucial for the timely deployment of neutrophils to contain and eliminate potential threats (Dömer *et al.*, 2020).

The role of phagocytes, and their defense mechanisms in the development of acute and chronic otitis, remains insufficiently understood (Ginel *et al.*, 2002; Moore *et al.*, 2019). Further research is needed on the immunopathological mechanisms of feline inflammatory aural polyps and the utilization of effective treatment modalities (Bollez *et al.*, 2018). Neutrophils can induce oxidative stress in various tissues of the animal body (Adrover *et al.*, 2020; Qi *et al.*, 2021). The build-up of free radicals contributes to the breakdown of cells and tissues. Moreover, it can serve as a stimulus for initiating apoptosis in immune cells. These disruptions are accompanied by alterations in cellular homeostasis (Adrover *et al.*, 2020; Qi *et al.*, 2021). Therefore, a detailed understanding of the role of neutrophils in the formation of local immunity is of paramount importance in clinical immunology and pathology. A thorough investigation of immunopathogenic mechanisms will allow researchers to gain new insights into the development of this pathology in animals. Novel approaches will find a place in developing effective methods and means for treating feline inflammatory polyps (Ginel *et al.*, 2002; Tater *et al.*, 2003; Greci and Mortellaro, 2016; Zhelavskiy *et al.*, 2020). The purpose of this study was to survey of an inflammatory polyp cytology in a cat and to study the role of neutrophils and their mechanisms on the formation of extracellular protective traps by neutrophils (NETs).

MATERIALS AND METHODS

Ethical approval

The cats were in the possession of private individuals, and written consent was acquired from these owners. The clinical investigations were conducted in accordance with the Law of Ukraine "On Protection of Animals from Cruel Treatment" (21/02/2006, 3447-IV), and adhered to the guidelines set by the European Commission regarding the treatment of vertebrates, ensuring protection against thirst, hunger, malnutrition, discomfort, fear, pain, and suffering.

Clinical signs

A 4-year-old, spayed female Scottish Fold cat, weighing 3.5 kg, experiencing skin problems on the inner surface of the right ear, reached out to the veterinary clinic (Mirra-Vet, Kyiv, Ukraine). During the clinical examination, the discharge of fluid from the right ear with an unpleasant odor was diagnosed. During the clinical examination, body temperature was 38.7°C, heart rate was 177 bpm, and respiratory rate was 28 breaths per minute. The cat had depression and a decrease in appetite. The animal was depressed, during palpation of the auricle it had a painful reaction.

During a detailed clinical examination and otoscopy (Gima, Italy), inflammation and the presence of a polyp were diagnosed in the right ear canal. During the clinical examination, redness, epithelial proliferation in the recess of the polyp (diameter 3 mm) in the area of intertragic incisure, and accumulation of gray-colored, thick exudate with an unpleasant odor were found in the ear. Samples were taken from the ear using sterile swabs for antibiotic susceptibility testing and cytological examination.

Blood collection and analyses

At the beginning of the study, a blood sample (50.0 µL) was taken. The blood sample was taken at 9:15 AM from the cephalic vein. During the hematological analysis, various parameters were evaluated, including erythrocyte count ($\times 10^{12}/L$), leukocyte count ($\times 10^9/L$), leucogram values ($\times 10^9/L$, %), concentrations of hemoglobin ($\mu\text{mol}/L$), hematocrit (L/L), Mean Corpuscular Volume (MCV, fl), mean corpuscular hemoglobin (MCH, fmol), mean corpuscular hemoglobin concentration (MCHC, mmol/L), and thrombocyte count ($\times 10^9/L$). The Abaxis Vetscan HM5 Hematology Analyzer (USA) was employed by the laboratory for the analysis.

Cytology study

Assessing the capacity of neutrophils to generate Neutrophil Extracellular Traps (NETs). The diagnostic specimens were collected using a cytologic brush premoistened with 1/15 mol/l phosphate buffer solution NeoGalin18 (15 M $\text{NaH}_2\text{PO}_4 + 2\text{H}_2\text{O}$ [11.8 g] + KH_2PO_4 [68.0 g] + $\text{C}_6\text{H}_{12}\text{O}_6$ [10.0 g], pH 7.2). Following this, a smear was created and left to air-dry at room temperature (20 °C). The microslide was then fixed with methanol (90°) and stained using a 1%

phosphate-buffered saline solution (pH 7.2) for 2–3 minutes. Following this, the microslide was rinsed with phosphate buffer and stained with May-Grünwald dye-fixative eosin methylene blue (Sigma-Aldrich®, USA). Neutrophils from NETs were microscopically evaluated at x2500-3000 magnification (Zhelavskiy, 2021).

Treatment

For treatment, the drug Otoflox (LLC BIOTESTLAB, Ukraine) was prescribed, 2 drops per ear. 1 ml of Otoflox contains the following components including ivermectin 1 mg, clotrimazole 10 mg, florfenicol 2.5 mg, betamethasone (in the form of uniformidipropionate) 1 mg, as well as auxiliary substances (dimethyl sulfoxide, ethanol, polyethylene glycol, propylene glycol). The full course of treatment was 7 days.

RESULTS

Upon clinical examination, it was established that the cat periodically shakes its right ear and scratches its paw. During the examination of the external ear, a gray thick exudate with an unpleasant odor was established, which was most localized in the area. After cleaning with sterile cotton swabs, an epithelial thickening in the form of a polyp was revealed. The polyp had a diameter of 3 mm and a thickness of 4 mm, and the color was red (Figure 1).

Since the beginning of the treatment, positive dynamics have been observed. By the third day, there was a reduction in exudative reaction and hyperemia, the size of the polyp was decreasing, pain reactions disappeared, and body temperature normalized. Complete resolution of inflammatory signs was noted on the 7th day of treatment.

Based on the microbiological study, *Staphylococcus aureus* ($\times 10^8$ colony-forming units CFU/ml), *Pantoea* spp. ($\times 10^8$ CFU/ml), *Enterococcus faecium* ($\times 10^7$ CFU/ml) microbes were identified. The highest sensitivity of microbes was established to the antibiotics (Ukrmediasnab, Ukraine), including enrofloxacin (concentration 30 mcg, 34 mm radial growth inhibition on agar medium), tetracycline (concentration 30 mcg, 31 mm), and doxycycline (concentration 30 mcg, 28 mm). When touched, the animal had a painful reaction. An increase in the number of leukocytes, their absolute content, and an increase in the percentage of neutrophils in the leukogram was established during the morphological examination of the blood (Table 1).

Cytological examination of samples from the area of intertragic incisure revealed a significant number of neutrophils. Neutrophils formed extracellular protective traps in which microorganisms were fixed. Free and unfixed NETs were diagnosed in separate areas of the slides. It was found that neutrophils formed cooperative groups with other phagocytes, epithelial cells, and slender nuclear streaks (Figure 2). During the treatment (Otoflox, 2 drops per ear), the inflammatory reaction disappears, polyp size decreases, exudative reactions decrease, and neutrophil activity decreases (Figure 3).

Table 1. Hematological parameters with Feline inflammatory aural polyps for 4-year-old, spayed female Scottish Fold cat

Parameter	Units	Measured values	Reference ranges*
Erythrocytes	$\times 10^{12}/L$	5.0-10.0	8.3
Leukocytes	$\times 10^9/L$	5.5-19	23.2
Neutrophil	%	35.0-37.0	48.2
	$\times 10^9/L$	2.5-14.0	11.1
Lymphocyte	%	20.0-50.0	45.5
	$\times 10^9/L$	1.5-7.0	10.5
Eosinophils	%	2.0-12.0	4.0
	$\times 10^9/L$	0-1.0	0.9
Monocytes	%	1.0-4.0	2.3
	$\times 10^9/L$	0-1.50	0.5
Basophils	%	0-0.05	0
	$\times 10^9/L$	0-1.0	0
Hemoglobin	G/L	80.0-150.0	92.4
Hematocrit	L/L	0.24-0.45	0.52
MCV	fl	12-17	14.3
MCH	fmol	0.78-1.08	15.1
MCHC	mmol/L	18.62-22.34	21.4
Thrombocyte	$\times 10^9/L$	300-600	537.0

MCV: Mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; *Harvey (2012).

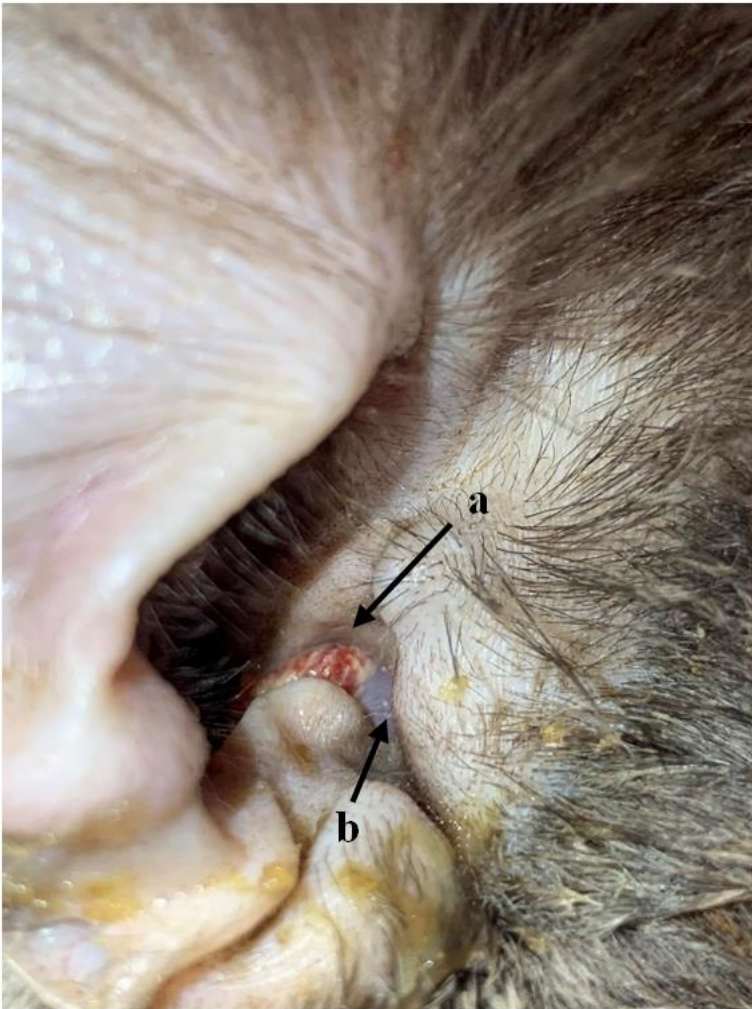


Figure 1. Clinical characteristics of feline inflammatory aural polyps of a 4-year-old, female spayed, Scottish fold cat (right ear). **a:** Visualization of a polyp. **b:** Inflammatory exudate in area *intertragic incisure*

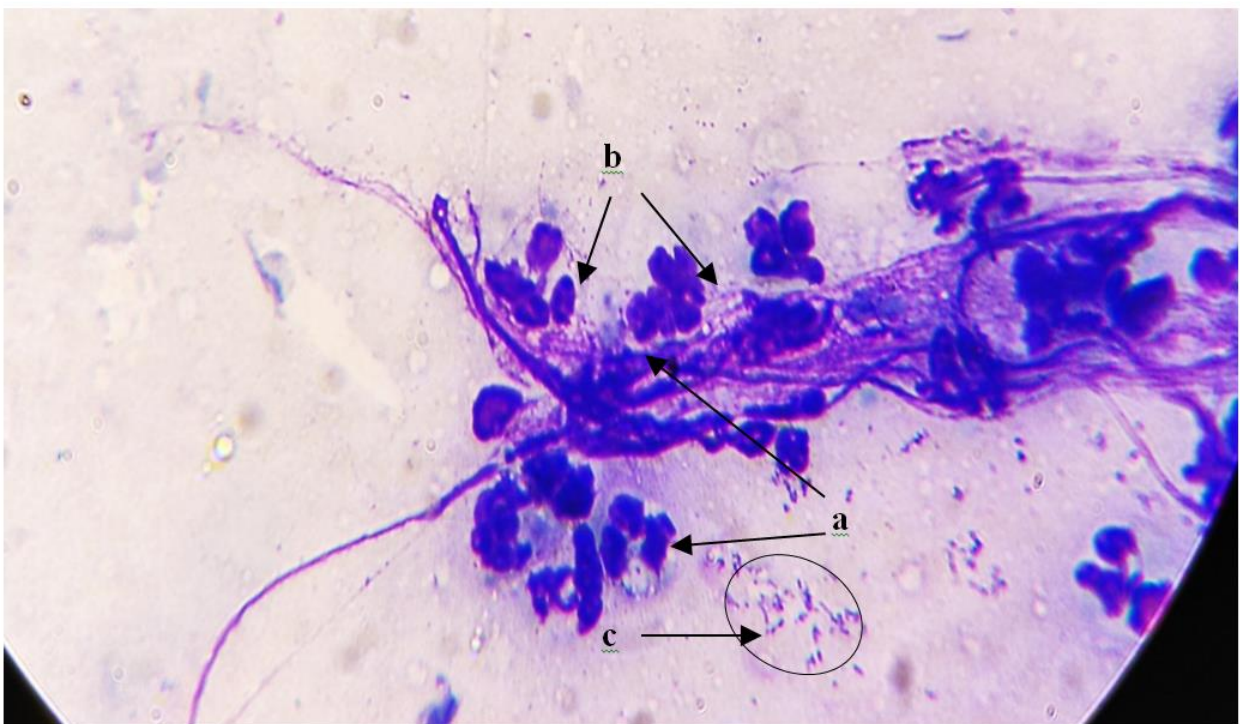


Figure 2. Cytology diagnostic of feline inflammatory aural polyps of a 4-year-old, female spayed, Scottish fold cat. **a:** visualization neutrophils. **b:** formed extracellular protective traps, in which microorganisms were fixed (**c**). Magnification x2000, stained with May-Grünwald

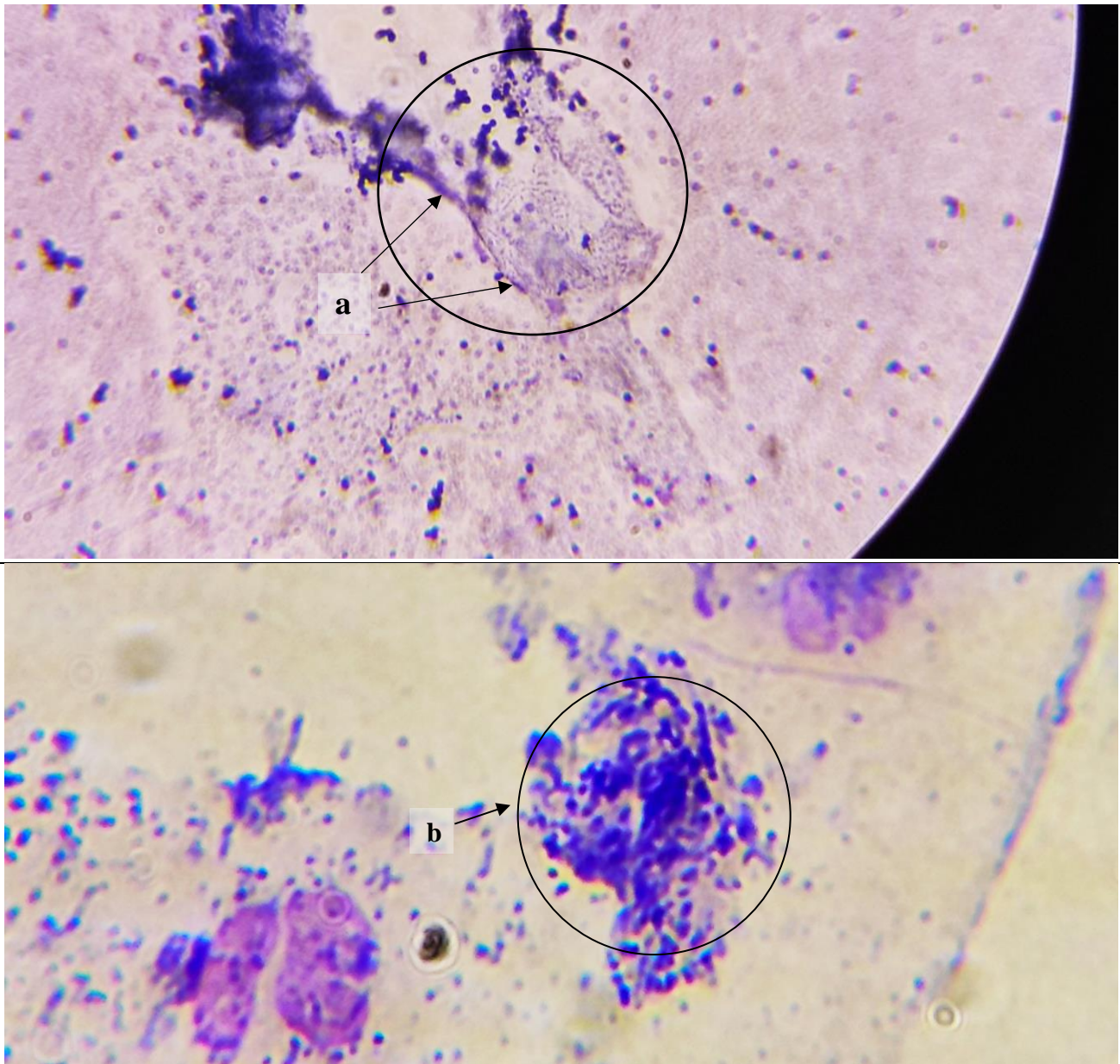


Figure 3. Cytology diagnostic of feline inflammatory aural polyps of a 4-year-old, female spayed, Scottish fold cat. **a:** Visualization unfixed NETs (magnification $\times 2500$); and NETs which microorganisms were fixed (**b**), magnification $\times 3000$), stained with May-Grünwald

DISCUSSION

Feline inflammatory aural polyps are abnormal growths in the ear canal of cats, often associated with chronic inflammation (Ginel et al., 2002; Tater et al., 2003). Feline inflammatory aural polyps may exhibit symptoms such as head shaking, scratching of the affected ear, and the presence of gray-colored, thick exudate with an unpleasant odor. Clinical examination often reveals the polyps, which may vary in size and color. Studies have demonstrated a breed predisposition in certain cat breeds, notably Siamese and Himalayan (Greci et al., 2014; Greci and Mortellaro, 2016; Mutua and Gershwin, 2021).

While local immunity plays a role in various aspects of the body's defense against infections and inflammation, specific information about the role of local immunity in feline inflammatory ear polyps may be limited (Adrover et al., 2020; Zhelavskiy, 2021). A remarkable aspect of neutrophil function in inflammation is the release of extracellular traps, known as NETs. In response to certain stimuli, such as bacterial or fungal infections, neutrophils undergo a unique form of cell death called NETosis (Hidalgo et al., 2021). During NETosis, the neutrophil releases its chromatin, adorned with antimicrobial proteins and enzymes, creating a web-like structure that entraps and neutralizes pathogens extracellularly (Vidémont and Pin, 2010). NETs not only immobilize microbes but also contribute to the amplification of the immune response by signaling other immune cells (Hidalgo et al., 2021; Zhelavskiy et al., 2023).

While extracellular traps play a vital role in microbial defense, their release can have both beneficial and detrimental consequences in the context of inflammation. On the positive side, NETs aid in pathogen containment and stimulate the immune system. However, excessive NETs formation or impaired clearance can lead to tissue damage and contribute to the pathogenesis of various inflammatory disorders (Dömer *et al.*, 2021).

Neutrophils, having completed their tasks, undergo apoptosis, a programmed cell death. This controlled death allows for the removal of neutrophils by phagocytic cells, facilitating the resolution of inflammation. Failure in the timely removal of neutrophils may lead to chronic inflammation, emphasizing the importance of a balanced and regulated immune response. Neutrophils, with their versatile weaponry and unique ability to form extracellular traps, emerge as pivotal contributors to the inflammatory process (Hariharan *et al.*, 2011; Greci *et al.*, 2014; Hidalgo *et al.*, 2021). Understanding the delicate balance between the protective and potentially harmful aspects of neutrophil function provides insights into the development of therapeutic strategies for inflammatory disorders. From chemotaxis to NETs formation, neutrophils exemplify the complexity of the immune system's response, underscoring their indispensable role in maintaining the delicate equilibrium between protection and pathology in inflammation (Moore *et al.*, 2019).

Inflammatory polyps in felines pose a complex challenge for both veterinarians and cat owners. Understanding the underlying factors contributing to their development, recognizing the clinical signs, and applying a comprehensive diagnostic and treatment strategy are critical to ensuring the well-being of affected felines (Sauvé, 2019). Ongoing studies of etiology, study of immune mechanisms, the role of neutrophils, and phagocytosis will deepen knowledge of pathogenesis. The study of immunopathological mechanisms in the pathogenesis of feline inflammatory aural polyps will serve as a foundation for the development of informative diagnostic tools and effective treatment methods (de Bont *et al.*, 2019). Considering that feline inflammatory ear polyps may have recurrences, immunomodulatory therapy should be applied. It is important to emphasize that the destruction of epithelial cells, neutrophil apoptosis, extracellular trap migration, the release of inflammatory mediators, and apoptosis promoters require new approaches in treating this pathology (Hidalgo *et al.*, 2021; Zhelavskiy *et al.*, 2023).

An intriguing facet of neutrophil activity in inflammation is the generation of extracellular traps, referred to as NETs. When exposed to specific triggers like bacterial or fungal infections, neutrophils undergo a distinctive type of cell death termed NETosis. In the course of NETosis, the neutrophil releases its chromatin, adorned with antimicrobial proteins and enzymes, forming a mesh-like structure that captures and neutralizes pathogens outside the cell. NETs not only immobilize microorganisms but also play a role in enhancing the immune response by signaling to other immune cells (Moore *et al.*, 2019; Hidalgo *et al.*, 2021).

CONCLUSION

Feline inflammatory aural polyps can occur against the background of recurrent inflammation. Experimental studies have shown that during the inflammatory reaction in the ear, protective mechanisms of local immune defense are activated. Activated neutrophils perform their function through phagocytosis and the formation of NETs. These studies contribute to supplementing the data on the immunopathological mechanisms of feline inflammatory polyps. Further studies on the mechanisms of phagocytosis will enable a deeper understanding of the role of immune cells in the system of cellular homeostasis and dysfunctions of local immunity.

DECLARATIONS

Availability of data and materials

Data from the study can be provided upon a reasonable request.

Funding

The present study had no financial support.

Authors' contributions

Mykola Zhelavskiy conceptualized the presented idea, validated the medical history, contributed to data collection, and conducted the experiment. Mykola Maryniuk designed the study and conducted clinical research. Maryna Drobot performed laboratory research. The final version of the manuscript was reviewed and approved by all authors.

Competing interests

The authors state that they do not have any competing interests.

Ethical considerations

The authors have diligently examined various ethical considerations, including but not limited to aspects such as plagiarism, obtaining consent for publication, preventing misconduct, avoiding data fabrication and/or falsification, ensuring against double publication and/or submission, and eliminating redundancy.

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