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Histological evaluation of uterine tissue and blood factors in short-haired domestic cats before and after administration of Levovist® contrast agent

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Abstract One of the most effective methods to examine the reproductive system in female cats is using ultrasound devices. When it comes to domestic short-haired female cats, which have tiny uterine size and uterine horns, this evaluation can be challenging. In this study, we evaluated the histology of uterine tissue and blood factors in domestic short-haired cats before and after administering the Levovist contrast agent. The study examined 12 domestic short-haired cats of varying ages and weights that were brought to two veterinary centers in Tehran. Blood samples were taken from the cats via the cephalic vein, both before and after injecting the contrast agent into the uterus. Then, the complete blood count was done. Additionally, following tissue sampling, the uteruses of both treated and control cats (at the beginning and end of the treatment period) were examined using hematoxylin and eosin histology. Before and after the injection of Levovist contrast material, the complete blood counts of the cats remained within the normal range. Following the contrast material injection, the myometrium and endometrium became recognizable and observable. In all the examined cats, the uterine tissue exhibited normal and healthy conditions. The examination of blood and histological factors in the uterine tissue and uterine horns before and after the administration of the Levovist contrast agent did not reveal significant changes. The results indicated that the Levovist contrast agent is a safe drug for examining the uterus and uterine horns.

Introduction

Ultrasound, also known as sonography or ultrasonography, is a non-invasive diagnostic imaging test. This technique employs high-frequency sound waves to generate real-time images or videos of internal organs within the body, including blood vessels. The resulting image obtained from this test is called a sonogram. Ultrasound allows doctors to observe soft tissues inside the body without incisions [1,2]. In veterinary centers, ultrasonography is extensively employed as a paraclinical diagnostic technique. Diagnosing pregnancy and fetal age in animals is one of the most important goals of using ultrasonography in veterinary medicine, which provides important information about the fetus and mother to the veterinarian. Therefore, having sufficient knowledge and experience regarding the anatomy of the reproductive system in small animals and how to perform sonography of reproductive organs is essential for a veterinarian [3]. Given the small size of the uterus and fallopian tubes in cats, ultrasonography has limitations in their examination, so one of the solutions for proper examination is the use of

contrast material in examining the uterus and fallopian tubes and their structural disorders in cats. Contrast-enhanced ultrasonography plays a crucial role in imaging the mediastinum and abdominal organs. Since the introduction of contrast agents in the United States for the examination of the gastrointestinal tract and abdominal organs, efforts have been made to apply contrast ultrasonography techniques in other countries as well [4]. The contrast material, also known as a contrast agent, allows radiologists to examine the internal structure of interest with greater detail and clarity. Imaging using a contrast agent provides enhanced scans of organs, ligaments, tendons, blood vessels, bones, or nerves [5, 6]. Unlike radiopharmaceuticals, this material interacts with ultrasound waves by either absorbing or altering them when exposed to external sources. The latter are drugs labeled with one or more types of isotopes and are self-radiating. This contrast material is employed to enhance the visibility of tissues not discernible in ultrasonography [7]. Numerous studies have been published on contrast ultrasonography in diagnosing and examining the characteristics of focal lesions in gastric lymph nodes, liver, lymph glands, and subepithelial tumors. It is also used in the examination of the cardiovascular system and in understanding the principles of tissue description. Furthermore, the ease of use of this contrast material, short half-life, high-quality images produced, and lack of tissue irritation are additional advantages of this material compared to other contrast [8, 9].

There are various types of contrast agents: In diagnostic imaging based on X-ray radiation (radiology and CT scan), iodine and barium sulfate-based compounds are used. Iodine compounds, with their short half-life and lack of residue, can be injected into veins, arteries, spinal discs, cerebrospinal fluid, and other body cavities to examine both structure and function. Barium sulfate, the most common and cheapest contrast material, is often available in powder form mixed with water before use, solution, and tablet. It is usually administered orally and sometimes rectally. However, if it enters the blood vessels, adjacent spaces, peritoneum, or heart and brain ventricles, it can cause symptoms such as infection, shock, and even death. In sonography, contrast material is primarily used in the form of salt (saline) and gas (like air). These two, as a contrast agent, are used in the form of microbubbles and microspheres. Although these compounds are usually useful for sonography, especially for kidney and heart sonography, they do not create a suitable contrast and can sometimes cause sensitivity in patients. Therefore, Levovist®contrast material, with its high contrast and high-quality images and no allergic reactions, is superior to saline and air. In this study, a histological evaluation of uterine tissue and blood factors in short-haired domestic cats was performed before and after the administration of Levovist® contrast material.

Materials and Methods

Experimental animals and ethical aspects

All animal care and experimental stages followed the guidelines established by the Ethics and Animal Care Committee of the Islamic Azad University, Tehran Science and Research Branch, under the ethics code IR.IAU.SRB.REC.1401.432.

This scientific research study spanned two months. During this time, 12 short-haired domestic female cats of varying ages and weights were examined. These cats had been referred to the Central Veterinary Hospital of Tehran and the East Veterinary Hospital in Tehran, Iran. Each cat was individually housed in special animal cages with unrestricted access to water and food for one week. An anti-worm medication (Zipyran Plus, Spain) was administered to the animals, and their health status was subsequently confirmed by a specialist. Additionally, light/dark cycles of 12/12 hours were maintained for all cats, along with a constant temperature of 22±2 ℃ and a humidity level of 55%.

Preparation and injection of Levovist® contrast material

Levovist® contrast material, which contains 99.9% galactose and 0.1% palmitic acid, was

procured (Schering AG –Germany). This material was initially in white granules or powder form contained in a vial. It was then dissolved in sterile water to create a milky white suspension. Subsequently, the Levovist® contrast material was injected into the uterus of short-haired domestic cats using a Foley catheter.

Blood sampling

Once the appropriate conditions were prepared, blood was collected from the cephalic vein of short-haired domestic cats.

Complete blood count

A blood test was conducted on short-haired domestic cats before and after the administration of Levovist® contrast material. The veterinary cell counter (Exigo, Sweden) was used to evaluate the levels of various blood components, including white blood cells, lymphocytes, monocytes, neutrophils, eosinophils, mature neutrophils, young neutrophils, basophils, hematocrit, red blood cells, hemoglobin, the mean volume of red blood cells, mean concentration of hemoglobin in red blood cells, nucleated red blood cells, and platelets.

Histology of uterine tissue

The histology of the uterus involves studying diseases and disorders that affect the uterus, a crucial organ in the female reproductive system. Common uterine histological examinations include the investigation of benign diseases such as fibroids and endometriosis, as well as malignant diseases like endometrial cancer and cervical cancer. Histological diagnostic methods play an important role in identifying these diseases. An ideal microscopic sample is one where the tissue present on the slide is preserved as it was in the body using chemical and molecular materials. In this study, the health indicators of the uterus included clinical examinations, absence of vaginal discharge, normal sonographic presence of both uterine horns, and normal order and number of uterine layers in sonography. A histological examination of the uterus was performed before and after the injection of Levovist® contrast material. For sampling, the animals underwent surgery under anesthesia at the beginning and end of the study period. Tissue samples were taken from the uterus, after which the operated area was sutured again. Post-surgery, comprehensive care was provided during recovery. Uterine tissue samples from the cats, taken before and after the injection of Levovist® contrast material, were prepared using the standard method of paraffin blocks. Subsequently, sections with a thickness of 5µm were prepared and stained with hematoxylin and eosin.

Statistical analysis

The data gathered were analyzed using SPSS version 21. For the analysis of quantitative data, we employed a one-way ANOVA statistical test. The threshold for statistical significance in this study was established at a p-value of less than 0.05 ($p<0.05$) [2].

Results

Complete blood count before and after the injection of contrast material

The results of the complete blood count obtained before and after the injection of contrast material are reported in Tables 1 and 2, respectively. Upon evaluating the reported values, the level of white blood cells, lymphocytes, monocytes, neutrophils, eosinophils, mature neutrophils, young neutrophils, basophils, hematocrit, red blood cells, hemoglobin, the mean volume of red blood cells, mean concentration of hemoglobin in red blood cells, nucleated red blood cells, and platelets in short-haired domestic cats were within the normal range. According to the statistical analysis of the results, the levels of white blood cells, lymphocytes, monocytes, hematocrit, red blood cells, and hemoglobin in cats treated with Levovist® contrast material did not show any statistically significant increase or decrease ($p<0.0001$) (**Fig. 1**) when compared to untreated cats. The only exception was the relative amount of neutrophils, which increased

after the injection of contrast material. However, the absolute amount of neutrophils remained within the normal range. All blood factors remained within the normal range before and after the injection of contrast material. Here, despite the absolute neutrophil count remaining within the normal range, the relative amount of neutrophils increased. Following the injection of contrast material, the neutrophil count was 79%, which falls outside the normal range of 35% to 75%. By calculating the absolute amount of neutrophils from the formula provided below and according to Table 2, it was found that the amount of neutrophils is also within the normal range:

$$\frac{\text{Neutrophil (79\%)}}{\text{Lymphocyte (100\%)}} = \frac{\text{X}}{\frac{5190}{\mu\text{l}}} \quad \text{X} = \frac{5190 \times 79}{100} \quad \text{X} = 4108/\mu\text{l}$$

The normal range for the absolute count of neutrophils in cats is 3000 to 12000 per µL.

Histological examination of the uterus before and after the injection of Levovist® contrast material

The findings revealed that subsequent to the administration of the contrast agent, a clear distinction and visibility of the myometrium and endometrium were observed, in contrast to the pre-injection state. The uterine tissue of the cats under examination was in a normal and healthy state (Fig. 2 and 3).

The uterine tissue comprises the following three layers: Endometrium; a superficial layer of cells that has a basalis layer and a layer associated with the proliferative phase of the endometrium. Myometrium; consists of smooth muscle bundles located beneath the endometrial layer, enveloping the central endometrial cavity of the uterus. Perimetrium; which is the outer serous layer of the uterus. The serous layer secretes a lubricating fluid that aids in reducing friction. The perimetrium also forms part of the peritoneum that covers certain pelvic organs. Typically, the histological indices of a healthy uterus include the evaluation of various cellular and tissue components. In a healthy uterus, histological analysis reveals normal endometrial tissue and the absence of abnormal growths such as tumors or hyperplasia. Histological characteristics of a healthy uterus include a well-organized endometrial layer, the absence of abnormal cellular changes, and the lack of significant fibrotic or destructive areas. Furthermore, the presence of a normal myometrium with distinct smooth muscle tissue, and the absence of necrosis or cellular changes, signifies the health of the uterine tissue.

Discussion

In plane radiography, the healthy uterus is not visible except in pathological cases such as hydrometra, pyometra, and pregnancy. However, due to the widespread use of ultrasonography devices and their ability to visualize female reproductive structures, they are increasingly employed for diagnosing female reproductive system diseases. One challenge in sonography is evaluating and locating the female reproductive organs. In sonography, contrast agents are employed to improve visibility. Sonography with contrast agents has received much attention in recent years. Levovist contrast agent, used in this study on female short-haired domestic cats, facilitated easier visualization of different layers and lumens within the female reproductive system. Importantly, this method is non-invasive and low-risk, causing no harm or stress to the patient's health or histology of uterine tissue and blood factors. It can be valuable for diagnosing conditions such as cysts, masses, fibroid tumors, and various uterine layer diseases.

Olinger, Liu, Quartuccio, Russo, Bigliardi, and Hassan researched the effects of using contrast media during ultrasound examinations. Their findings indicated that contrast-enhanced ultrasound (CEUS) significantly improved the clarity of sonographic images and aided in diagnosing diseases. Olinger (2024) noted that CEUS could greatly enhance imaging for gynecological conditions, with its applications in the female pelvis expected to evolve further. Liu and his team (2023) suggested that CEUS might be useful in predicting endometrial damage in women of reproductive age.

Quartuccio (2020) highlighted CEUS's potential for improving non-invasive assessments

in conditions like CEH-pyometra and CEH-mucometra. In 2009, Russo utilized SonoVue, a second-generation contrast agent, to investigate blood flow in normal prostates of five anesthetized dogs, observing a notable increase in Doppler signals post-injection. A similar study by Bigliardi and Ferrari (2010) using Levovist also showed enhanced visualization of prostatic blood flow [10 – 14]. Hassan et al. (2010) evaluated three different contrast agents—Levovist, Sonazoid, and SonoVue—focusing on their sonochemical and biological impacts at varying concentrations, providing insights into microbubble interactions with biological systems and potential future applications. Other researchers, including Schmiedl, Dorn, and Atsuo Takada, have examined the physiological effects of contrast media on the body. Schmidle et al. (1998) studied Levovist®'s impact on fetal circulation, measuring various blood flow parameters before and after contrast administration. They found no significant hemodynamic effects on the fetus [15, 16].

Table 1. Blood test results obtained before injection of contrast agent

Test type	Result	Normal range	Unit	Test type	Result	Normal range	Unit
White blood cells	7.4	5.19-50.5	10^3/µL	**Hematocrit**	43.9	24-45	%
Lymphocyte	23	20-55	%	**Red blood cells**	10	5-10	10^3/µL
Monocyte	3	1-4	%	**Hemoglobin**	13.7	8-15	g/dL
Neutrophil	70	35-75	%	**Mean volume of RBC**	42.9	39-55	fL
Eosinophil	1	2-12	%	**MCH**	13.2	31-37	g/dL
Mature neutrophil	-	35-80	%	**Nucleated RBC**	-	-	%
Young neutrophil	3	0-31	%	**Platelet**	379	150-550	10^3/µL
Basophil	-	0-1	%				

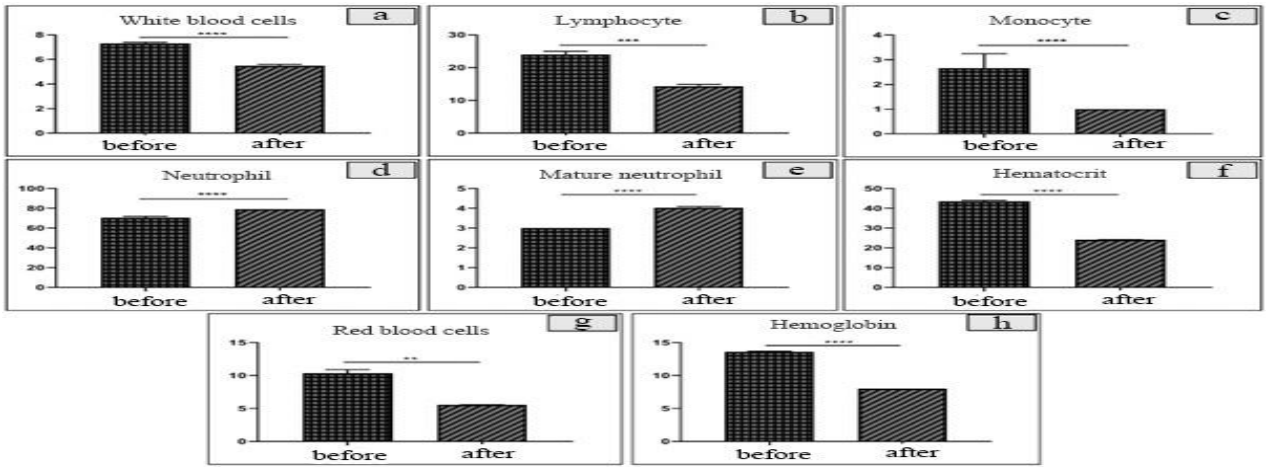

Fig 1. A comparison of blood parameters was conducted in short-haired domestic cats before and after the injection of contrast agent. The parameters, expressed as mean and standard deviation (Mean±SD), included: (a) White blood cells, (b) Lymphocytes, © Monocytes, (d) Neutrophils, (e) Mature neutrophils, (f) Hematocrit, (g) Red blood cells, and (h) Hemoglobin levels in uterine tissue

**** p<0.0001, *** p<0.001, and ** p<0.01

Table 2. Blood test results obtained after injection of contrast agent

Test type	Result	Normal range	Unit	Test type	Result	Normal range	Unit
White blood cells	5.5	5.19-50.5	10^3/µL	**Hematocrit**	24.1	24-45	%
Lymphocyte	15	20-55	%	**Red blood cells**	5.57	5-10	10^3/µL
Monocyte	1	1-4	%	**Hemoglobin**	8	8-15	g/dL
Neutrophil	79	35-75	%	**Mean volume of RBC**	43.3	39-55	fL
Eosinophil	1	2-12	%	**MCH**	31	31-37	g/dL
Mature neutrophil	-	35-80	%	**Nucleated RBC**	-	-	%
Young neutrophil	4	0-31	%	**Platelet**	238	150-550	10^3/µL
Basophil	-	0-1	%				

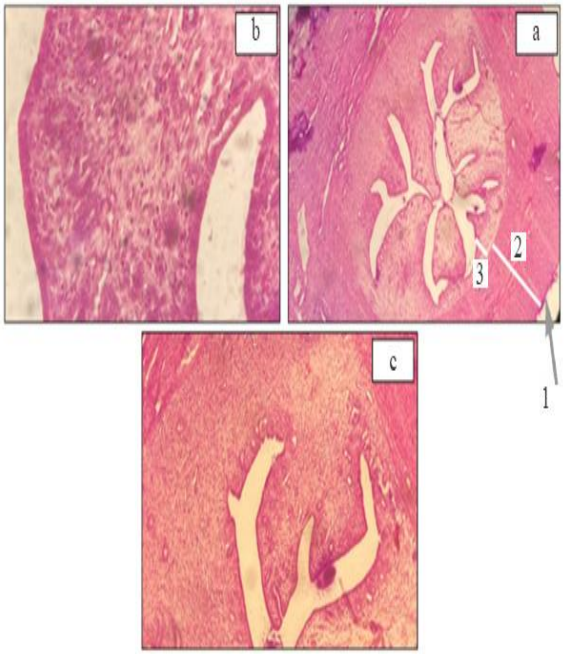

Fig 2. Histopathological examination of uterine tissue, before the injection of contrast agent through Hematoxylin & Eosin staining. Numbered items: 1) Perimetrium, 2) Myometrium, 3) Endometrium. (a) Magnification ×4, (b) Magnification ×40, (c) Magnification ×10.

Dorn et al. (2004) reported increased blood flow parameters under the endometrium post-Levovist® administration, although no correlation with pregnancy rates was established. Atsuo Takada et al. (2012) investigated Levovist®'s effects on liver cells using electron microscopy, concluding that it did not cause significant liver damage [17, 18].

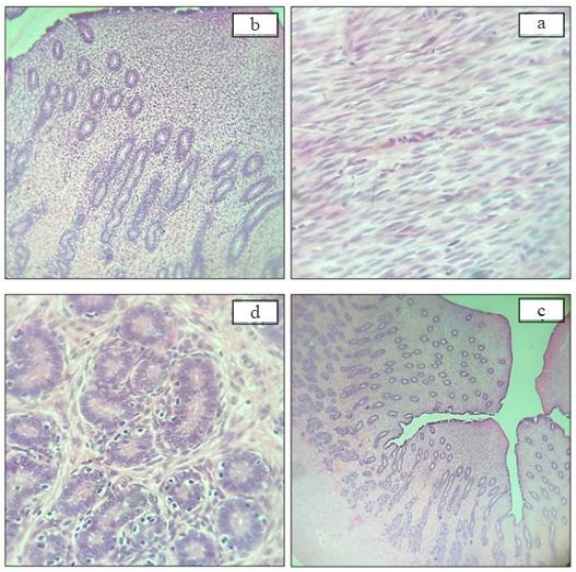

Fig. 3 Histopathological examination of uterine tissue, after the injection of contrast agent through Hematoxylin & Eosin staining. (a) Histopathological image of the uterine myometrium (Magnification ×40), (b) Histopathological image of the uterine endometrium and myometrium (Magnification ×40), (c) Histopathological image of the uterine endometrium and myometrium (Magnification ×10), and (d) Histopathological image of the uterine endometrium (Magnification ×40).

In our study, we examined the effects of the contrast agent Levovist® on the health and changes in the uterus of domestic short-haired cats. The results from the complete blood count (including white blood cell level, lymphocyte, monocyte, neutrophil, eosinophil, mature neutrophil, young neutrophil, basophil,

hematocrit, red blood cell, hemoglobin, average volume of red blood cells, globular hemoglobin, average concentration of hemoglobin in red blood cells, nucleated red blood cells, and blood platelet) and histology of uterine tissue and uterine branches with eosin and hematoxylin staining, showed that the Levovist® contrast agent is non-toxic and has no negative effects on animals. Stern et al. (2000) compared the effectiveness of Levovist in hysterosonosalpingography for assessing tubal patency against chromopertubation, finding that Levovist® provided a cost-effective and accessible diagnostic method. Ferretti et al. (2005) evaluated uterine radiography and sonography post-natural childbirth in cats, revealing that these imaging techniques could facilitate further studies, although identifying the uterus in non-pregnant cats proved challenging [19, 20]. Woodland et al. (2014) compared digital radiography, ultrasonography, and positive contrast vaginourethrography to assess fertility in female cats, concluding that sonography is a sensitive diagnostic tool for this purpose [21]. Iijima et al. (2006) explored Levovist® in laboratory and in vivo settings, finding that it effectively highlighted metastatic liver cancer, likely due to Kupffer cell phagocytosis. Their studies demonstrated that Levovist® microbubbles were phagocytosed by these cells, contributing to the liver-specific imaging obtained after intravenous injection [22]. In 2023, Wilson et al. proposed that CEUS with phosphatidylserine microbubbles could be used to monitor placental inflammation. Their tests on rhesus macaques showed that this method effectively tracked placental health and identified pregnancies at risk for vascular issues (23).

Conclusion

In our study, the analysis of blood factors and histological tissue from the uterus and uterine branches, conducted before and after the administration of the Levovist® contrast agent, revealed no significant changes. These findings suggest that the use of Levovist® as a contrast agent for examining the uterus and uterine tubes is safe. In accordance with the studies conducted by other researchers in previous years, some of which were referenced earlier, we have also demonstrated that the use of the Levovist® contrast agent for examining the uterus and uterine tubes is safe. Therefore, this method can be employed in clinical studies related to the uterus, pregnancy, and the health of the reproductive system in small animals.

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Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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