# Determination of Carotenoids in the Ovaries of Healthy Cats and Cats with Leiomyosarcom

**Ekaterina I. Selifonova[1*], Andrey M. Zakharevich[1], Iuliia S. Kruss[1], Andrey S. Rykhlov[2], Alexey A. Chernov[3], Tatyana Yu. Rusanova[1], and Valery V. Tuchin[1,4,5]**

[1] Saratov State University, 83 Astrakhanskaya str., Saratov 410012, Russian Federation

[2] Saratov State University of Genetics, Biotechnology and Engineering named after N.I. Vavilov, 4 Pyotr Stolypin av., Saratov 410012, Russian Federation

[3] V.I. Razumovsky Saratov State Medical University, 112 Bolshaya Kazachya str., Saratov 410012, Russian Federation

[4] Institute of Precision Mechanics and Control, FRC "Saratov Scientific Centre of the RAS", 24 Rabochaya str., Saratov 410028, Russian Federation

[5] Tomsk State University, 36 Lenin av., Tomsk 634050, Russian Federation

*e-mail: selif-ei@yandex.ru

**Abstract.** The mortality rate of ovarian cancer remains the greatest among other oncological diseases of genital system worldwide. Using carotenoids as a preventive measure against ovarian cancer and a means to improve the state of genital system in general is a popular trend when determining the nutritive factors. In our study we have determined the total content of carotenoids in healthy feline ovaries and ovaries of cats diagnosed with leiomyosarcoma. We have found that total content of carotenoids varies between left and right ovaries of the same healthy cat. However, cats diagnosed with leiomyosarcoma had significantly lower carotenoid content in both left and right ovaries. As carotenoids can be used as chemotherapeutic agents, the study of their content in ovaries is a relevant problem that requires further study. Determination of the impact of carotenoids on oncological processes may find practical use in public healthcare.

**Keywords:** ovary tissues; leiomyosarcoma; oncology; carotenoids; spectroscopy.

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## 1 Introduction

Ovarian cancer has the highest lethality rate out of all gynecological cancers as it usually is not detected until it has already progressed to its later stages, it tends to relapse and it still has a very narrow range of treatment options [1]. Some tumors that form from smooth muscle fibers of uterus, such as leiomyosarcoma, affect up to 70% of all women during their lifetime and their treatment requires significant financial expenditures [2, 3]. Currently, there is still relatively few information regarding the etiology of ovarian cancer. Certain nutritive factors and lifestyle choice may play a role in formation of ovarian cancer and can be used as a preventive measure to decrease the lethality of this disease. The role of carotenoids in prevention of ovarian cancer and other forms of genital system cancers is especially interesting. Carotenoids and retinyl ethers are sources of vitamin A and its natural derivatives in human body; they regulate replication and differentiation of cells in human endometrium. Carotenoids (a series of more than 800 of structurally different molecules) act as chemopreventive agents regardless of whether they transform into vitamin A, therefore, they can be potentially used as efficient replacement to existing chemotherapeutic agents used to treat ovarian cancer [4]. More than 50 types of carotenoids are present in common food products including a group of approximately 20 substances that, after consumed with food, can be found in human blood (plasma or serum). Primary components of that group (more than 90%) include β-carotene, α-carotene, lycopene, β-cryptoxanthin, lutein and zeaxanthin [5]. Currently, there are active discussions on functional activity of carotenoids related to their photochemical, structural, antioxidant, light absorbing and

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photoprotective properties [6]. There are studies dedicated to finding the correlations between the presence of carotenoids in blood serum and occurrence of breast cancer. It has been found that total content of circulating carotenoids, α-carotene, β-carotene, β-cryptoxanthin, lycopene and lutein may correlate with reduced risk of development of breast cancer, however, the reliability of provided evidence is estimated as low or very low [7, 8]. However, Czeczuga-Semeniuk and Wołczyński established that carotenoids may trigger the growth of leiomyoma and are involved in differentiation of endometrium adenocarcinoma [9]. Studies of carotenoid content in normal tissues of uterus and in samples of various tumors taken from human uterus showed that the highest total content of carotenoids were observed for endometrium adenocarcinoma group, thus, confirming certain enzyme defects in carotenoid metabolism or some kind of their metabolic modification as a result of neoplastic process [9]. Fourteen different carotenoids were identified in the sample of tissues taken from human ovaries. Total content of carotenoids was relatively low for the whole group of oncological samples, while the average content of provitamin A was comparable to that observed for samples of normal ovary tissues. It was only higher for group with endometriosis [10]. Other studies of tissues of malignant tumors in uterus reported statistically higher total content of carotenoids compared to samples of normal uterus tissues and samples of benign tumors taken from uterus. The observed discrepancies in the frequency of extraction of several carotenoids do not allow to reach a solid conclusion and require further studies [11]. Greater consumption of carotenoids may reduce epithelial ovarian cancer [12]. Other studies conducted in North America and Europe state that consumption of primary carotenoids by adults does not play any significant role in incidence of ovarian cancer [13]. Thus, currently there are no certain recommendations on carotenoid consumption as there is evidence of both positive and negative effects of carotenoid consumption on human health that require further studies [14]. It is known that cats can digest β-carotene rather well. Cats whose diet included increased β-carotene content had greater concentrations of β-carotene in blood plasma, lutein and endometrium tissues and estradiol, thus, support optimal functioning of ovaries and providing the best medium for embryos to survive and develop in the uterus [15]. Gunyeli et al. recommend astaxanthin, a carotenoid, as a potential nutrient that can be used to protect urogenital tissues from damage caused by chemotherapeutic and cytotoxic agents such as methotrexate [16].

Carotenoids, being powerful antioxidants, can interact with reactive oxygen species through oxidation, reduction, hydrogen extraction or addition reactions, reducing oxidative damage to tissue cells [17]. They may also reduce the risk of cancer by suppressing cell proliferation, inducing apoptosis, influencing intercellular communication, and strengthening immune function [18]. Most studies show that adequate consumption of fruits and vegetables containing carotenoids and other antioxidants significantly reduces the risk of cancer [19]. However, not all carotenoids are able to reduce the risk of cancer development and there are still controversies and discussions on this topic [20]. Recent studies have shown that carotenoids play a dual role, acting as both antioxidants and prooxidants, making it difficult to determine their role in cancer development [21]. Some studies suggest that conflicting results may be due to differences in phytochemical intake, regional differences, dietary and lifestyle factors, age and hereditary factors, limitations in sample size, and data collection methods. Further high-quality and well-designed experimental studies are needed to examine the relationship between carotenoids and cancer incidence [18].

Animal models are often used for research, in particular: ferrets [22], pigs [23], rats [24], cats [25], etc. The purpose of this study was to establish the content of carotenoids in normal feline ovaries and ovaries of cats diagnosed with leiomyosarcoma.

## 2 Methods and Materials

The *ex vivo* studies involved examination of ovaries of non-purebred cats aged 1.5 to 9 years obtained from veterinary clinic performing ovariectomy and ovariohysterectomy. We studied both healthy feline ovaries and ovaries of cats diagnosed with leiomyosarcoma. To confirm the diagnosis, we performed histologic examination of samples within 48 h after ovary extirpation using hematoxylin-eosin staining. Surface morphology and elementary composition of tissue samples was studied using MIRA 2 LMU scanning electron microscope (SEM) (TESCAN ORSAY HOLDING, Czechia) operating in secondary electron registration mode (with acceleration voltage of 30 kV) equipped with INCA Energy 350, a system of energy dispersive X-ray analysis (EDAX) (Oxford Instruments Analytical, Great Britain). INCA Energy microanalysis system allows quantitative determination of chemical elemental composition of sample within selected area. Surface images of samples were produced by fixing the samples on a special carbon substrate (carbon band) and tomographic scanning was performed after the samples were coated with gold. The surface layer of tissues with depth of effective signal generation being 1 μm. The scanning was performed using acceleration voltage of 20 kV in vacuum at pressure of approximately $10^{-2}$ Pa. For SEM analysis we prepared $(0.90 \pm 0.09)$ mm wide sections of ovary tissues that were placed between glass slides and dried at normal atmospheric pressure and room temperature (23 °C) for 10 days.

To determine the total content of carotenoids, ovaries were cut into pieces no greater than 1 mm in size and then dried in ULAB UT-4610 drying cabinet at 40 °C for 3 h. 0.1 g of dried up sample weight was weighted on GR-200 (A&D Companyassay, Japan) balance (extra-fine fit as classified by GOST (State Standard) 24104-20010. Weighted samples were placed into glass beakers, 5 ml of 95% ethanol was poured into

the beakers and the resulting biomass was stirred for 15 min at 40 °C. The content of beaker was transferred to test tubes and centrifuged at 5000 rpm in Zenroe centrifuge (Cence, China) for 10 min. Supernatant was transferred to quartz cuvette with path length of 1 cm; absorption spectra were registered relative to compensation solution (95% ethanol) using Shimadzu UV-2550(PC) double-beam spectrophotometer (Shimadzu, Japan). Collection, processing and output of analysis results was performed by UVProbe for Windows 2.30 software package for PC. Spectral range: 200–500 nm; maximum permissible values of absolute accuracy of wavelength (λ) ± 0.3 nm.

Total quantitative content of carotenoids (in mg/100 g calculated to β-carotene) at the 450 nm wavelength was calculated as follows [26]:

$$C = \frac{A \cdot 5 \cdot 10}{m \cdot 2500} \cdot 100\ \%, \qquad (1)$$

where $A$ is the absorbance of tested sample; $m$ is the weight of sample, mg; 5 is the volume of ethanol, ml; 10 is the weight of β-carotene per 1 ml of 1% ethanol solution, mg; 2500 is the absorption coefficient of β-carotene in ethanol at 450 nm.

Statistical analysis of data was performed by comparing various samples using Mann-Whitney $U$-test. For that, we distributed the samples in ascending order for ovary 1 and ovary 2 for each diagnosed group. The resulting experimental value ($U_{exp}$) was compared to critical (tabular) value ($U_{cr}$).

$$U_{exp} = n_1 \cdot n_2 + \frac{n_1 \cdot (n_1+1)}{2} - \sum r_1, \qquad (2)$$

where $U_{exp}$ is calculated value of Mann-Whitney $U$ parameter; $n_1$ and $n_2$ are the sizes of firth and second samples, respectively; $r_1$ is the rank sum for the first (greater) sample. Initially, we assumed that the discrepancies between two samples were insignificant, while the following inequality was valid: $U_{cr} < U_{exp}$ (with $p = 0.05$). If $U_{cr} > U_{exp}$, then the discrepancy between samples is statistically significant.

## 3 Results

### *3.1 Histological Examination*

Histological examination has revealed general characteristics of the structure of healthy ovary. The ovary's exterior is covered with a single-layer cubical epithelium followed by subepithelial white tissue. The ovary's interior consists of cortical and medullary substances that are not distinctly separated from each other. Cortical substance comprises the majority of ovary and contained follicles. The medullary substance comprises the smaller part of ovary's interior and contains many blood vessels (Fig. 1).

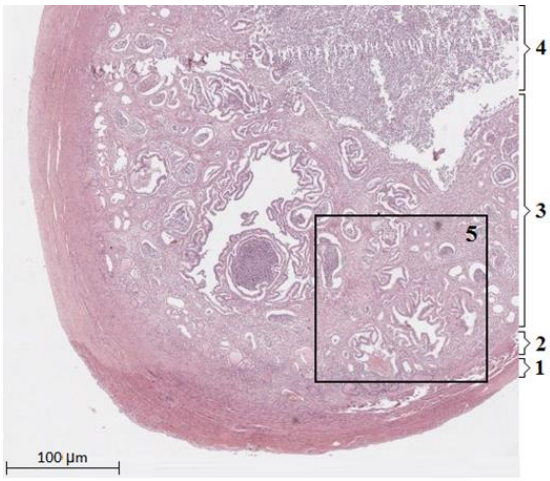
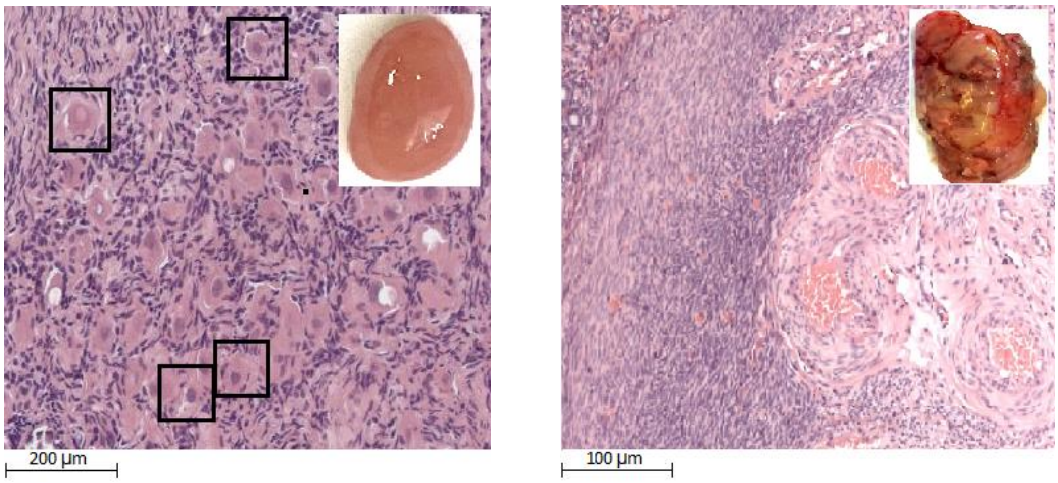



Fig. 1 Histological examination of feline ovary: 1 – single-layer cubical epithelium; 2 – subepithelial albugineous layer; 3 – ovary's cortical substance; 4 – ovary's medullary substance; 5 – area chosen for SEM examination.




(a) (b)

Fig. 2 Images of histological sections of (a) healthy feline ovaries and (b) feline ovaries with leiomyosarcoma.

The images of histological sections of healthy and cancerous feline ovaries are provided in Fig. 2. Fig. 2a shows the histological examination of healthy ovaries. The histological structure of ovary is intact. The cortical substance is well-developed, the ratio of stroma to parenchyma is close to 1:1.5; the theca has a typical appearance. There are many visible follicles at various stages of maturation. There is no evidence of atypical development, necrosis, invasive growth, mitotic activity, inflammation, bacterial or fungal colonies. The histological pattern corresponds to ovary in follicular growth phase.

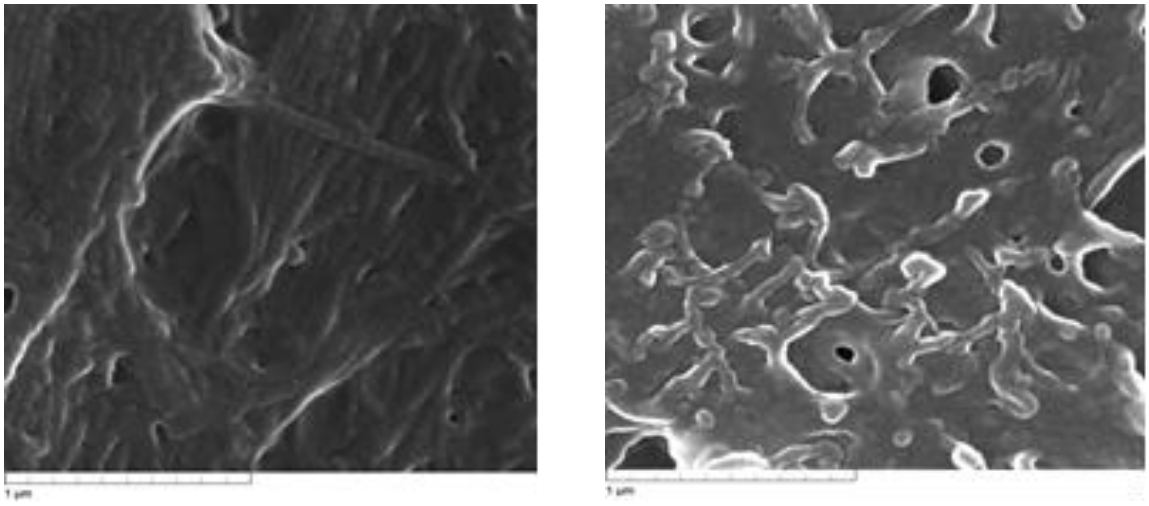
Ovaries with leiomyosarcoma is presented in Fig. 2(b). The examination revealed prominent atypical tissue changes with autolytic tumor fragments. Atypical fusiform tumor cells have high nucleocytoplasmic ratio with 1−2 eosinophilic nucleoli visible at magnification of 400; tumor necrosis fields with cell shadows are also visible. The degree of nuclear atypia is high, tumor cells form solid fields. Stroma is rather poorly developed; vascularization is badly visualized. Lymphovascular invasion is not determined in the sample of studied materials. Mitotic activity shows 20 mitoses per 10 fields of view at magnification of 400× (2.5 mm$^2$). Morphological pattern is mostly typical for leiomyosarcoma.

## *3.2 SEM Scanning of Ovary Tissue Sections*

Dried sections of studied samples were studied using scanning electron microscopy (SEM images are shown in Fig. 3).

The extracellular matrix is formed by elastic collagen fibers whose packing density varied between studied samples. In healthy ovaries collagen fibers have approximately the same diameter without any breaks and are densely packed (Fig. 3(a)). The sample of ovary with leiomyosarcoma contains a specific intertwining of collagen fibers that form several regular compacted fusiform foci (Fig. 3(b)).

Elemental composition of studied samples found via energy dispersive analysis is provided in Table 1. The weight ratio of elements was determined for five points within four selected areas of SEM images of each of the studied samples and averaged. As we can see, the ratios of *N* and *O* in affected tissues is greater, while the ratio of *C* is lower.



(a) (b)

Fig. 3 SEM images (magnification of 100 kx) of sections of cat's ovary tissue samples: (a) healthy ovary, (b) ovary with leiomyosarcoma.

Table 1 Elemental composition of studied samples.

| Studied sample | Element content, wt. % | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***C*** | ***N*** | ***O*** | ***Na*** | ***Mg*** | ***Al*** | ***P*** | ***S*** | ***Cl*** | ***K*** |
| Healthy ovary | 66.9 | 11.5 | 16.8 | 0.62 | − | − | 0.64 | 0.44 | 1.32 | 0.87 |
| Leiomyosarcoma | 52.9 | 18.5 | 22.7 | 0.72 | 0.09 | 0.12 | 0.21 | 0.85 | 1.63 | 1.62 |

Table 2 Parameters of soft tissue evaporation by continuous $CO_2$ laser radiation.

| | $O_1$ | $O_2$ | $O_1$ | $O_2$ | $O_1$ | $O_2$ | $O_1$ | $O_2$ | $O_1$ | $O_2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| **Sample** | **Sample 1** | | **Sample 2** | | **Sample 3** | | **Sample 4** | | **Sample 5** | |
| Healthy ovaries | 1.25 | 0.85 | 1.95 | 0.64 | 1.12 | 0.34 | 1.54 | 0.42 | 2.14 | 0.73 |
| Discrepancy between $O_1$ and $O_2$ | 0.40 | | 1.31 | | 0.78 | | 1.12 | | 1.41 | |
| **Sample** | **Sample 6** | | **Sample 7** | | **Sample 8** | | **Sample 9** | | **Sample 10** | |
| Ovary with leiomyosarcoma | 0.08 | 0.03 | 0.04 | 0.00 | 0.36 | 0.29 | 0.35 | 0.24 | 0.28 | 0.11 |
| Discrepancy between $O_1$ and $O_2$ | 0.05 | | 0.04 | | 0.28 | | 0.11 | | 0.17 | |

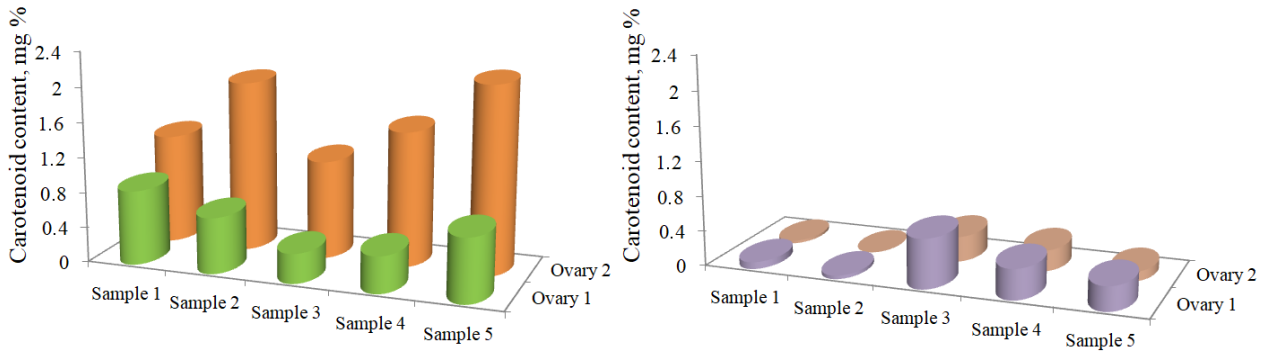



Fig. 4 Total carotenoid content (calculated to *β*-carotene) in the samples of feline ovaries: (a) healthy ovary; (b) ovary with leiomyosarcoma.

### *3.3 Spectrophotometric Studies*

Total carotenoid content calculated to β-carotene has been determined after extraction of these carotenoids from tissues by 95% ethanol solution. The results of spectrophotometric examination are provided in Table 2. We have studied five healthy animals (samples 1−5) and five animals with the same diagnosis (samples 6−10), and for reference we also have studied both ovaries for each cat ($O_1$ and $O_2$).

The highest content of carotenoids was observed in healthy ovaries and equaled 2.14 mg% with discrepancies between the pair of ovaries varying between 0.4 and 1.41 mg%. Total carotenoid content in ovaries with leiomyosarcoma ranged from 0 to 0.57 mg% with discrepancies between the pair of ovaries varying between 0.04 and 0.28 mg% (Fig. 4).

Mann-Whitney *U*-test was used to compare Sample 1 (values obtained for ovary 1, $n = 5$) and Sample 2 (values obtained for ovary 2, $n = 5$) for healthy ovaries and ovaries with leiomyosarcoma. The critical (tabular) values of $U$ are 4 (if $p = 0.05$) and 1 (if $p = 0.01$). For experimental data, the found values of $U$ were 0 and 7 for healthy ovaries and ovaries with leiomyosarcoma, respectively. Therefore, we can see that healthy ovaries significantly differ from each other ($U_{exp} < U_{cr}$). In the case of ovaries with leiomyosarcoma, $U_{exp} > U_{cr}$, thus, there is no significant difference between the ovaries.

The same approach was used to compare samples with different diagnoses, i.e. healthy feline ovaries with ovaries of cats diagnosed with leiomyosarcoma. To do that, we combined results for both cancerous ovaries into a separate sample ($n = 10$). The experimental value of $U$ according to Mann-Whitney test equaled 2. Critical (tabular) values of U are 27 (if $p = 0.05$) and 16 (if $p = 0.01$). Thus, we can see that tested samples significantly differed from each other at both levels of significance.

According to our studies, left and right ovaries of a healthy cats have different total carotenoid content. For examples, total carotenoid content in left ovary ranged from 1.12 to 2.14 mg%. This results in significant discrepancies in total carotenoid content (calculated to β-carotene) between left and right ovaries of one animal that may vary from 0.40 to 1.41 mg%.

Total carotenoid content in left ovaries of cats diagnosed with leiomyosarcoma ranged from 0.04 to 0.57 mg%, while in right ovaries it ranged from 0.00 to 0.29 mg%. The discrepancies in content between left and right ovaries ranged from 0.04 to 0.28 mg%.

Looking ahead, it was interesting to establish a possible correlation between carotenoid levels in the skin and internal organs, including ovarian tissue. If such a correlation were found, it would be possible to monitor carotenoid levels in ovarian tissue in both normal and pathological conditions. Currently, noninvasive and rapid

methods for determining carotenoid levels in living tissues *in vivo* are well developed and are used to monitor changes in their concentration in response to food intake or dietary supplements containing carotenoids. Reflectance spectroscopy is an accessible method used for the rapid, noninvasive, quantitative determination of carotenoids in living human skin [27]. The analyzed skin area is illuminated with white light in the range of 350 to 850 nm, and a compression method is used to reduce the effect of oxyhemoglobin on the reflectance spectra. Detection of carotenoids in skin *in vivo* is also possible using a simple spectroscopic optical setup developed by the authors [28] with a fiber probe without lenses, mirrors, or aperture, which reduces development costs. High correlation between resonance Raman spectroscopy and reflectance spectroscopy data was achieved in the noninvasive determination of carotenoids in human skin *in vivo* and cow udder *in vitro*. [29]. Thus, the introduction of noninvasive measurements of carotenoids in living tissues into clinical practice may improve dietary recommendations for disease prevention.

To improve the accuracy of determining the spectral absorption of beta-carotene within tissue using reflectance spectroscopy, it is possible to simultaneously use compression and immersion tissue optical clearing [30]. Studies on tissue phantoms have shown that the combination of analytical and experimental optical clearing methods expands the capabilities of diffuse reflectance spectroscopy for precise extracting the absorption coefficient of beta-carotene as one of the chromophores within tissue.

## 4 Conclusion

We found that total carotenoid content in healthy feline ovaries differs between left and right ovary of the same animal. However, both left and right ovaries of cats diagnosed with leiomyosarcoma contained significantly lower quantities of carotenoids. As carotenoids can be used as chemotherapeutic agents, the study of their total content in ovaries is relevant and requires further studies.

## Author Contribution

T. Yu. Rusanova and V. V. Tuchin designed the concept of study; A. A. Chernov, E. I. Selifonova and A. M. Zakharevich conducted the experimental study; A. S. Rykhlov gathered clinical data and results of study and conducted histological examination of samples in a specialized laboratory; T. Yu. Rusanova, A. A. Chernov, E. I. Selifonova, A. M. Zakharevich, A. S. Rykhlov and V. V. Tuchin discussed the results of the study; A. A. Chernov, Yu. S. Kruss prepared the first draft of the article and performed statistical analysis of data; T. Yu. Rusanova and V. V. Tuchin edited the final draft of the article.

## Disclosures

All authors declare that there is no conflict of interest in this paper.

## Ethical Practices

This article does not describe any studies involving people or animals as research subjects.

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