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ISSN: 0015-5659

e-ISSN: 1644-3284

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DOI: 10.5603/FM.a2019.0097

Article type: ORIGINAL ARTICLES

Submitted: 2019-05-21

Accepted: 2019-08-03

Published online: 2019-08-26

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Different fixative solutions in the detection of mast cells in the canine and feline reproductive organs

Running head: Fixative fluids for mast cells detection

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Abstract

The aim of the study was to evaluate the usability of different fixative fluids in the detection of mast cells in ovaries and uteri of bitches and cats. Samples were fixed in 4% formaldehyde, Carnoy's fluid, Mota's basic lead acetate and isotonic formaldehyde-acetic acid (IFAA). Mast cells were detected by acidified toluidine blue staining and counted for various parts of the ovaries and uteri. In the ovaries of both species, the numbers of MCs were significantly ($P < 0.05$) higher in Carnoy than in formalin. No significant differences were found between Carnoy and Mota (tested only in cats). In the uterus, numbers of MCs were significantly ($P < 0.05$) higher in Carnoy, Mota and IFAA compared to formalin (canine endometrium, feline endometrium and feline myometrium), in Carnoy and Mota compared to formalin (canine myometrium) and in Mota compared to IFAA (feline myometrium). The majority of MCs were formalin-sensitive in the canine and feline uterus, in the canine ovary and in the feline *cortex ovarii*. In the feline *medulla ovarii*, the majority of MCs were formalin-resistant. No formalin-resistant MCs were detected in the feline *tunica albuginea ovarii*. Thus, using Mota's or Carnoy's fluid in the canine or feline female reproductive organs is recommended. This study improves methodology for all studies which clarify the role of MCs in the reproductive organs of the domestic and laboratory animals.

Key words: Carnoy, dog, formol, Mota, ovary, uterus

Introduction

The significant effect of the used fixation technique on the total number of detected mast cells (MCs) has been described [1, 17, 19, 31]. Glycosaminoglycans (responsible for metachromatic staining) contained in granules of certain MCs are soluble in water and therefore require alcohol fixation in combination with other substances for their preservation [31].

MCs can be classified on the basis of their sensitivity to aldehyde fixation [17]. This sensitivity is related to the content of different glycosaminoglycans [3, 18] or even to the amount of histamine [8] in granules of MCs.

Choosing the most suitable fixative fluid is therefore essential for the optimal detection of MCs. The only study of the canine uterus compared only Carnoy's fluid and formalin [17]. The situation in cats is unknown even if these are widespread veterinary patients.

The absence of optimal method of specimen fixation for the detection of MCs significantly limits the possibilities of research of MC function in physiological processes in the reproductive organs or their role in the pathologies. This study compares the number of MCs detected in samples fixed in several fixation fluids and describes the distribution of formalin-sensitive MCs.

As to the methods of MC detection, situation is very complicated in veterinary sciences. Although a tryptase detection is known as a very reliable method for human uterine MCs [13], tryptase-negative MCs were reported in various animal species including a dog [17]. Therefore, classical histochemical staining such as toluidine blue is still widely used for detection of the total number of MCs in animals [e.g. 4, 7, 10, 21, 24, 25, 26, 37, 38]. Moreover, a significantly higher number of MCs was detected by thiazine dyes than by immunohistochemistry in various canine organs including uterus [17]. Because thiazine dyes are still preferred in many veterinary studies, it is necessary to improve the fixation methodology for them. Thus, the aim of this study was to find out which fixative fluids are best suited to use in studies where the total number of MCs is monitored in animals, especially in the reproductive organs. (The topic of the role of MCs in the reproductive organs was recently reviewed [28] and their possible effect on the reproductive processes is studied intensively [e.g. 6, 9, 10, 11, 12, 15, 20, 21, 22, 23, 24, 27, 30, 32]. Thus, it is necessary to know the best detection techniques of MCs in the reproductive system.)

Material and Methods

Animals

The samples were collected from 9 healthy bitches and 12 healthy cats (sexually mature, cycling) that were kept as pets. The animals were brought to the veterinary clinic in order to be spayed. No experiment was performed, only the material from the preventive neutering was used.

Tissue Processing

This study was focused on ovaries (9 canine, 12 feline) and uterine horns (9 canine, 9 feline). The obtained part of the uterine horn was equally divided into 4 fixation fluids. The ovaries were divided only into two fixative fluids (due to their size and problematic division into the equal parts). Each sample of organ was divided into parts and each part was fixed in a different fixation fluid. Three fixative fluids were used in the feline ovaries (Carnoy, Mota and formalin), two fixation fluids were used in the canine ovaries (Carnoy, formalin). Four fixation fluids were used in canine and feline uteri (Carnoy, Mota, isotonic formaldehyde acetic acid and formalin). Ovaries (especially in cats) are very small. Moreover, they are hard to cut into smaller and equal parts. Therefore, they were fixed in a smaller number of fluids than the uteri. All fixation fluids were prepared in the laboratory. Carnoy's fluid was mixed from absolute ethanol, chlorophorm and glacial acetic acid in proportion 6 : 3 : 1 respectively immediately before fixation. Mota's basic lead acetate (BLA) contained 1g basic lead acetate, 50 ml distilled water, 50 ml absolute ethanol and 0.5 ml glacial acetic acid [33,34,35]. Isotonic formaldehyde acetic acid (IFAA) was mixed from 1.5 ml 37% formaldehyde, 98 ml distilled water and 0.5 ml glacial acetic acid [33,34]. Formalin was common neutral buffered 10% formol. All fluids were stored at a room temperature.

Samples in Carnoy's fluid were fixed for 4 - 5 hours and then transferred into absolute ethanol where they were fixed for another 20 hours. This technique provides a higher stability of the samples. In all other fluids, samples were fixed for 24 hours.

After the fixation had been completed, the samples from Mota, formalin or IFAA were dehydrated in graded ethanol series (the ethanol concentration in each subsequent bath was increased by 10 %), acetone, and three baths of xylene. At the end of dehydration process, samples were infiltrated with hot paraffin and embedded in paraffin wax. Then, 3 – 4 µm thin sections were cut in the routine manner. The sections were deparaffinized and stained with

acidified toluidine blue,(0.5% solution in 0.25 M HCl).The staining time was 15 minutes.The toluidine blue powder was T3260; Sigma-Aldrich.

The dehydration in graded ethanol series was omitted in the Carnoy's fluid fixed samples because this fixative was prepared from anhydrous reagents. These samples were only immersed in xylene after the fixation and subsequently processed identically to the other samples.

A single paraffin block was made from each sample of each fixation fluid. Three sections were taken from each block and evaluated under an Olympus BX 51 light microscope at 400× magnification.

In the ovary, MCs were counted separately in the cortical stroma (ovarian follicles and *corpora lutea* were excluded) and in the medulla. In the uterus, MCs were counted separately in the endometrium and myometrium. Results are expressed as the number of MCs per 1 mm².

Statistical analysis

Results were statistically evaluated by the ANOVA method of analysis of variance. The differences between mean values were evaluated (after a previous error analysis and evaluation of the influential values) by the Tukey HSD test in the SAS computer program (SAS Institute, Carry, USA), version 9.4 at the level of significance $P = 0.05$. (Mean, standard deviation and median described in tables are based on section mean per animal.)

Results

Ovary

In all parts of the ovary, the numbers of MCs were significantly ($P < 0.05$) higher in Carnoy than in formalin in both species. No significant differences were found between Carnoy and Mota (tested only in cats), $P > 0.05$. More detailed results are described in Table 1. The distribution of MCs in the canine and feline ovaries can be seen in Fig. 1–6.

Uterus

In the uterus, the numbers of MCs were significantly ($P < 0.05$) higher in Carnoy, Mota and IFAA compared to formalin (canine endometrium, feline endometrium and feline myometrium), in Carnoy and Mota compared to formalin (canine myometrium) and in Mota

compared to IFAA (feline myometrium). Other differences were not significant ($P > 0.05$). More detailed results are described in Table 2. The distribution of MCs in the canine uteri can be seen in Fig. 7–10.

Sensitivity to Formalin

The percentage of formalin-sensitive MCs with respect to the number of MCs detected after the fixation in Carnoy's fluid was determined. No formalin-resistant MCs were detected in the feline *tunica albuginea ovarii*. In other parts of assessed organs, the percentage of formalin-sensitive MCs fluctuates from 37.8 ± 12.9 % in the feline *medulla ovarii* to 90.4 ± 11.4 in the feline *endometrium*. The majority of MCs were formalin-sensitive in the canine and feline uterus, in the canine ovary and in the feline *cortex ovarii*. However, in the feline *medulla ovarii*, the majority of MCs were formalin-resistant. More detailed results are described in Table 3.

Discussion

In all examined organs, significant differences in the total number of MCs between individual animals were found. Kube et al. also described significant differences between individuals in the canine uterus [17]. In our opinion, the reason could have been the use of material from animals in different stages of the estrous cycle. The effect of the phase of the estrous cycle on the number of MCs has been described in both species [7, 9, 10]. Nevertheless, the differences among the individual animals were not so important for this study because the only changed and monitored parameter in each tested organ was the fixation fluid used.

We found that Carnoy's fluid and Mota's BLA were equally applicable in all parts of the canine and feline ovaries, whereas fixation in formalin showed that the number of formalin-sensitive MCs was high in the ovaries of both species so fixation in formalin can not be recommended.

Based on these results, Carnoy's fluid and Mota's BLA are equally applicable in all parts of the canine and feline uterus. IFAA can be reliably used in all parts of the feline uterus and in the canine endometrium but gave lower numbers of detected MCs in the canine myometrium.

The majority of MCs were formalin-sensitive in all evaluated areas of the organs except the feline *medulla ovarii*. Kube et al. also found formalin-sensitive MCs in the canine uterus but the majority of MCs were formalin-resistant [17]. Changes in the percentage of formalin-sensitive/resistant MCs described in some diseases [36] show that the percentage of formalin-sensitive/resistant MCs depends on the local environment of the organ. Therefore we expect that changes in the sex hormone levels during the estrous cycle may affect the ratio of formalin-resistant and formalin-sensitive MCs. This could be the reason why the proportion of formalin-sensitive MCs varies in our study compared to Kube et al. [17].

It was proposed that MCs play a role in the process of ovulation in cats [10]. During the ovulation, the *tunica albuginea* is ruptured so we decided to focus on this area in cats. All MCs in this location were formalin-sensitive. We expect that they belong to one biological subtype which plays a major role in the process of feline ovulation. Further investigation should be provided.

It has been reported that all mucosal MCs in rodents are formalin-sensitive [5]. However, both types as to the fixability by aldehydes were described in the mucosa [e.g. 1, 2, 16, 18] in the majority of species including the dog [1]. Our results confirm this fact – both types of MCs were found in the endometrium.

Based on the results, fixation in IFAA was reliably applicable for detection of MCs in the canine uterus and feline endometrium. However, the total number of MCs was significantly lower in IFAA compared to Mota's BLA in the feline myometrium. This corresponds to a significantly higher number of MCs in IFAA fixed samples compared to formalin reported in the canine skin [2] and in the ovine uterine tube [18].

Carnoy's fluid is widely used for the fixation of MCs in genital organs [e.g. 16, 17, 19] due to the good results of this method. Mota's BLA was used for the fixation of reproductive organs in several studies [14, 15, 16]. Nevertheless, none of these studies described the efficiency of Mota versus other fixation techniques so the appropriateness of this method has been verified only by this study. Our results showed that the numbers of MCs were at their highest after fixation in Carnoy (or Mota) in both organs of both studied species.

To conclude, the results prove the significant effect of the fixation fluid on the total number of detected MCs. This effect should be always taken into account. Since there were no significant differences in comparison with other studies, we would not be afraid to apply the basic results to other organs or domestic animal species.

Conclusions

It follows from our results that the use of Carnoy and Mota was reliable in all examined organs.

Thus, we recommend using Mota's BLA or Carnoy's fluid for performing studies focused on the detection of MCs in the female reproductive organs of domestic carnivores. Studies of the role of MCs in the reproductive processes should be performed in optimally fixed samples. The results of the current study offer this solution. The conclusion of this study is useful for studies describing the total number of MCs in domestic as well as laboratory animals.

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Table 1. Effect of the fixation fluid on the number of MCs/mm² in the ovary

Animal	Part of organ	Fluid	Mean ± SD	Median (min. - max.)
bitch	<i>cortex ovarii</i>	Carnoy	10.6 ± 7.9	8 (3 - 27)
bitch	<i>cortex ovarii</i>	Formalin	1.4 ± 1.8	1 (0 - 5)
bitch	<i>medulla ovarii</i>	Carnoy	21.3 ± 10.7	19 (8 - 38)
bitch	<i>medulla ovarii</i>	Formalin	4.1 ± 2.7	4 (1 - 10)
cat	<i>cortex ovarii</i>	Carnoy	19.0 ± 11.61	18.5 (7 - 34)
cat	<i>cortex ovarii</i>	Formalin	2.7 ± 1.63	2.5 (1 - 5)

cat	<i>cortex ovarii</i>	Carnoy	33.5 ± 34.84	26.5 (4 - 91)
cat	<i>cortex ovarii</i>	Mota	30.7 ± 27.12	28 (4 - 68)
cat	<i>medulla ovarii</i>	Carnoy	20.8 ± 8.54	20.5 (9 - 33)
cat	<i>medulla ovarii</i>	Formalin	12.2 ± 3.43	12.5 (7 - 16)
cat	<i>medulla ovarii</i>	Carnoy	24.8 ± 15.94	21 (10 - 49)
cat	<i>medulla ovarii</i>	Mota	28.7 ± 19.15	25.5 (11 - 53)
cat	<i>tunica albuginea</i>	Carnoy	11.5 ± 5.54	9.5 (6 - 21)
cat	<i>tunica albuginea</i>	Formalin	0.0 ± 0.00	0 (0 - 0)
cat	<i>tunica albuginea</i>	Carnoy	7.8 ± 3.71	7 (4 - 14)
cat	<i>tunica albuginea</i>	Mota	11.7 ± 5.85	11 (6 - 21)

SD = standard deviation. Samples of ovaries from each cat were fixed in Carnoy and Mota or in Carnoy and formalin. For this reason, formalin and Mota can not be compared (pair samples of ovaries fixed in formalin and Mota were not available).

Table 2. Effect of the fixation fluid on the number of MCs/mm² in the uterus

Animal	Part of organ	Fluid	Mean ± SD	Median (min. - max.)
bitch	<i>endometrium</i>	Carnoy	41.9 ± 37.35	32 (5 - 131)
bitch	<i>endometrium</i>	Formalin	7.7 ± 6.73	7 (0 - 22)
bitch	<i>endometrium</i>	IFAA	38.7 ± 36.89	39 (2 - 125)
bitch	<i>endometrium</i>	Mota	39.1 ± 34.58	31 (6 - 119)
bitch	<i>myometrium</i>	Carnoy	52 ± 26.59	53 (18 - 94)
bitch	<i>myometrium</i>	Formalin	20.2 ± 17.28	16 (1 - 48)
bitch	<i>myometrium</i>	IFAA	39.2 ± 23.04	33 (13 - 78)
bitch	<i>myometrium</i>	Mota	55 ± 32.57	39 (23 - 115)
cat	<i>endometrium</i>	Carnoy	11.9 ± 10.53	9 (2 - 34)
cat	<i>endometrium</i>	Formalin	0.9 ± 1.05	1 (0 - 3)
cat	<i>endometrium</i>	IFAA	9.7 ± 9.99	8 (0 - 30)
cat	<i>endometrium</i>	Mota	13.9 ± 11.37	9 (2 - 35)
cat	<i>myometrium</i>	Carnoy	30.9 ± 19.31	31 (11 - 70)
cat	<i>myometrium</i>	Formalin	2.9 ± 2.15	3 (0 - 6)
cat	<i>myometrium</i>	IFAA	19.9 ± 13.05	20 (3 - 41)
cat	<i>myometrium</i>	Mota	40.4 ± 25.86	31 (11 - 81)

SD = standard deviation

Table 3. Percentage of formalin-sensitive MCs in the ovary and uterus (compared to Carnoy's fluid)

Animal	Part of organ	Mean \pm SD	Median (min. - max.)
		[%]	[%]
bitch	<i>cortex ovarii</i>	89.4 \pm 12.1	87.5 (66.7 - 100)
bitch	<i>medulla ovarii</i>	76.8 \pm 18.7	84.2 (41.2 - 92.6)
cat	<i>cortex ovarii</i>	76.8 \pm 27.8	92.7 (28.6 - 95.8)
cat	<i>medulla ovarii</i>	37.8 \pm 12.9	37.7 (22.2 - 57.6)
cat	<i>tunica albuginea</i>	100.0 \pm 0.0	100 (100 - 100)
bitch	<i>endometrium</i>	74.4 \pm 22.5	75.9 (31.2 - 93.9)
bitch	<i>myometrium</i>	62.4 \pm 28.8	69.8 (11.1 - 95.6)
cat	<i>endometrium</i>	90.4 \pm 11.4	97 (75 - 100)
cat	<i>myometrium</i>	85 \pm 11.6	80.6 (72.7 - 100)

SD = standard deviation

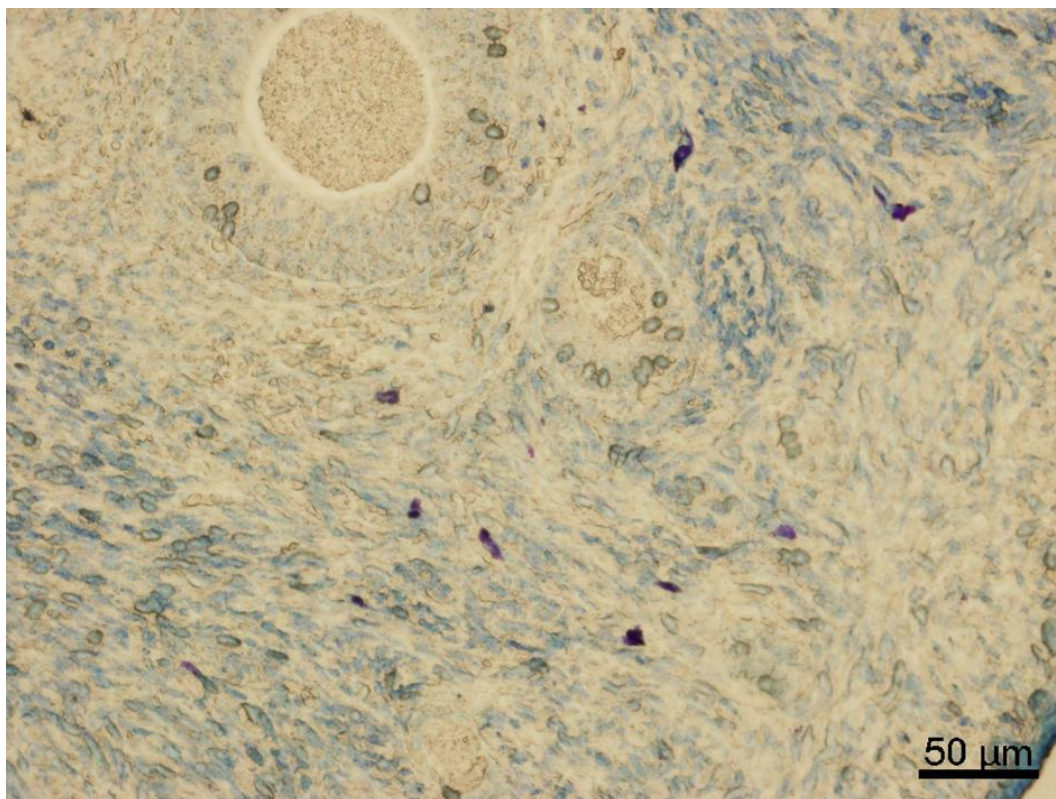


Figure 1. Canine ovary, cortex, Carnoy. The dyeability of MC granules is distinctive. Significantly \uparrow number of detected MCs compared to Formalin (Fig. 2).

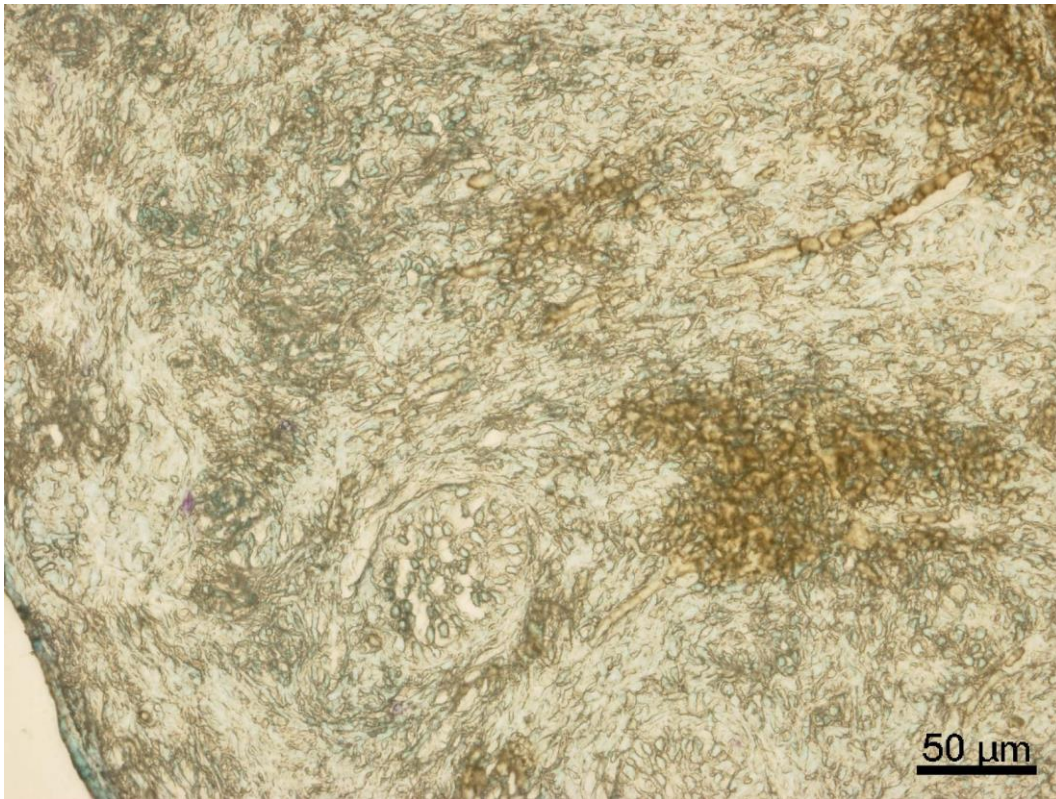


Figure 2. Canine ovary, cortex, Formalin. In detected MCs, the dyeability of granules is weaker compared to Carnoy (Fig. 1). Significantly ↓ number of MCs compared to Carnoy (Fig. 1).

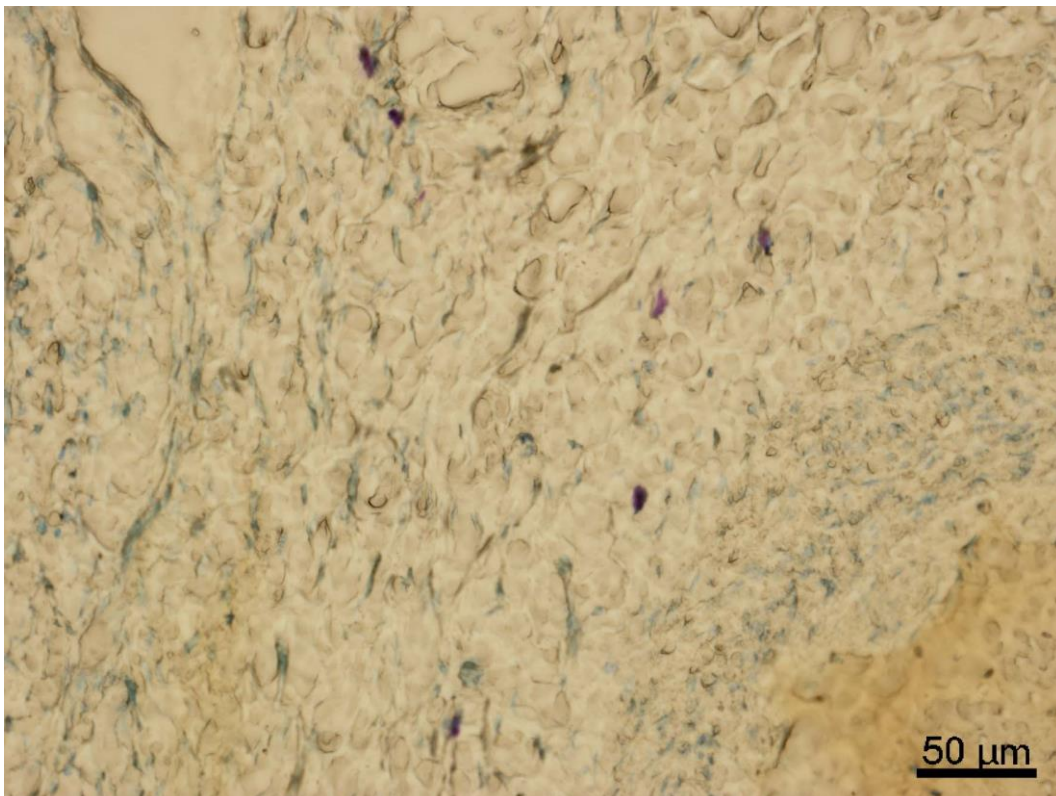


Figure 3. Canine ovary, medulla, Carnoy. Significantly ↑ number of MCs compared to Formalin (Fig. 4).

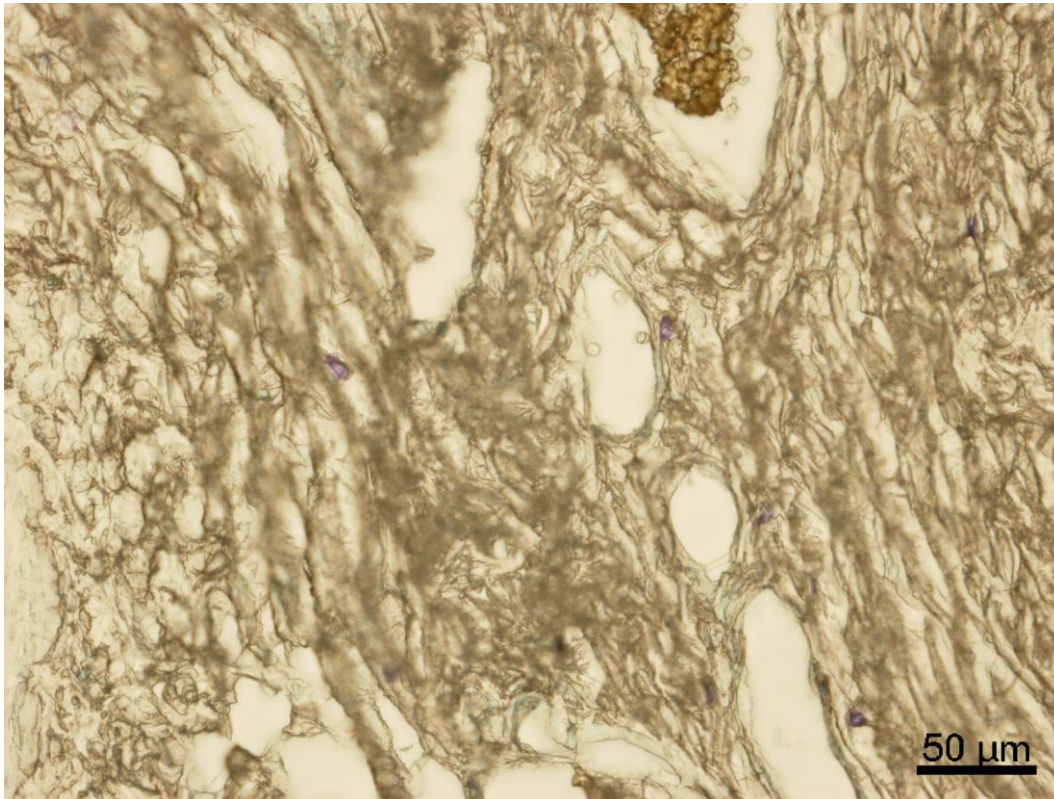


Figure 4. Canine ovary, medulla, Formalin. In detected MCs, the dyeability of MC granules is weaker compared to Carnoy (Fig. 3). Significantly ↓ number of MCs compared to Carnoy (Fig. 3).

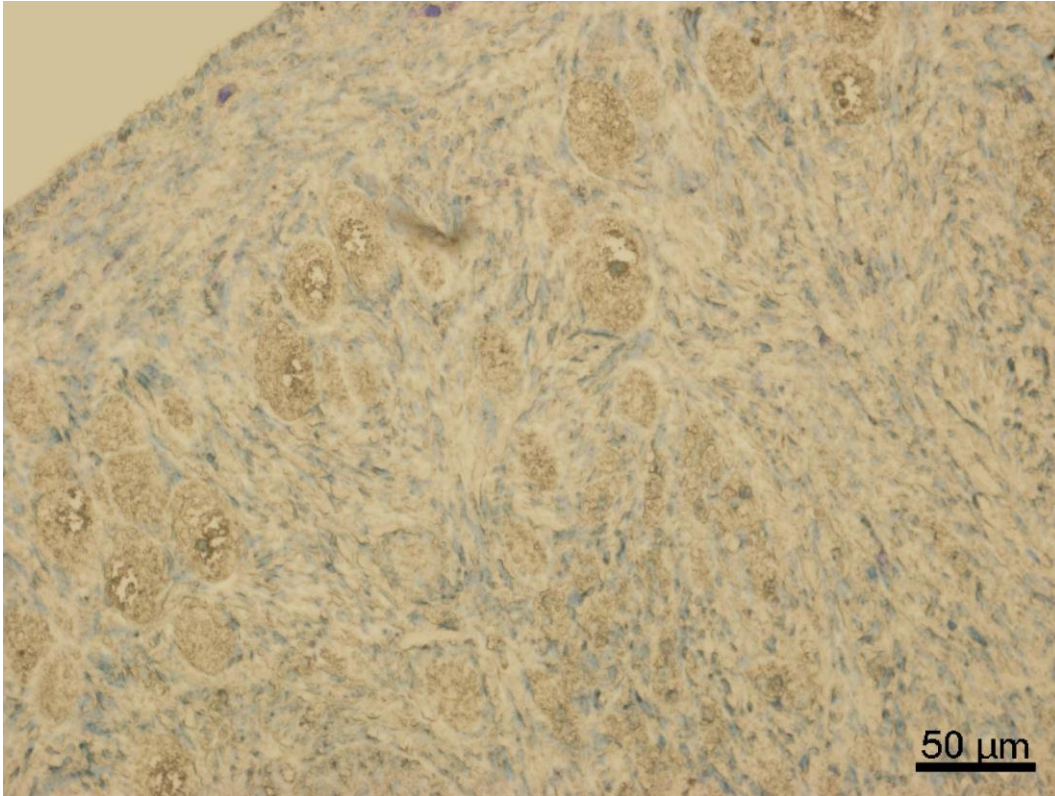


Figure 5. Feline ovary, *tunica albuginea*, Carnoy. No significant difference in the number of MCs, dyeability of granules or in intensity of the background tissue compared to Mota (Fig. 6).

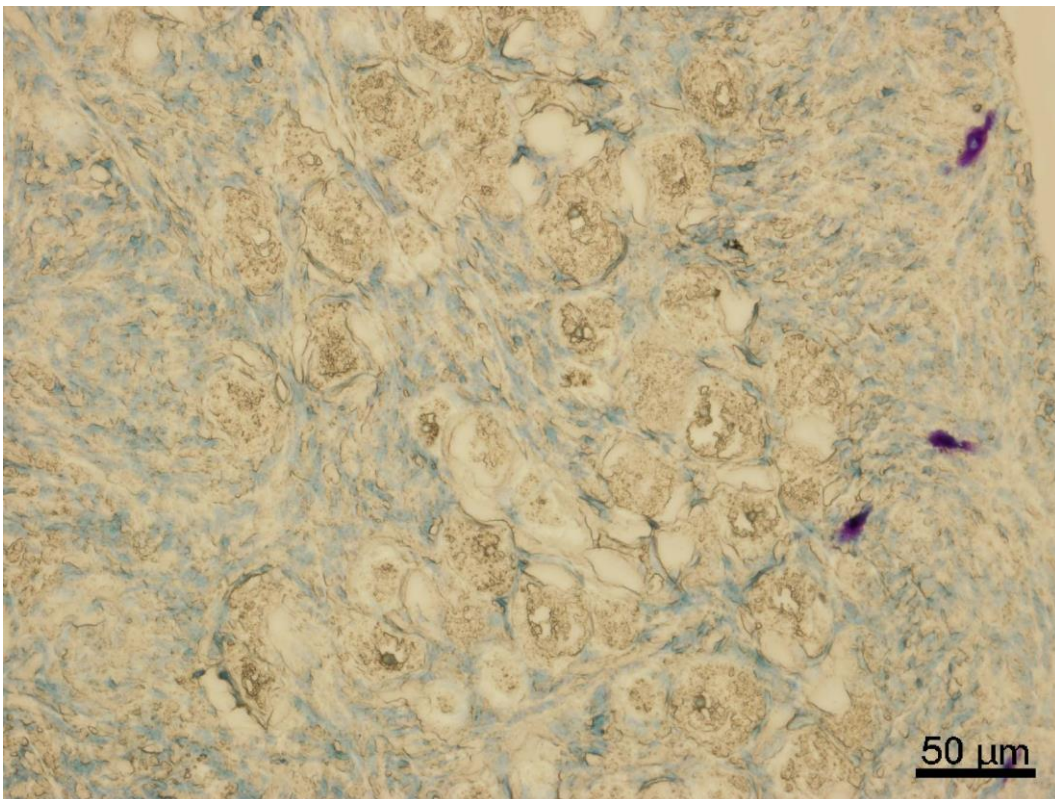


Figure 6. Feline ovary, *tunica albuginea*, Mota. No significant difference compared to Carnoy (Fig. 5).

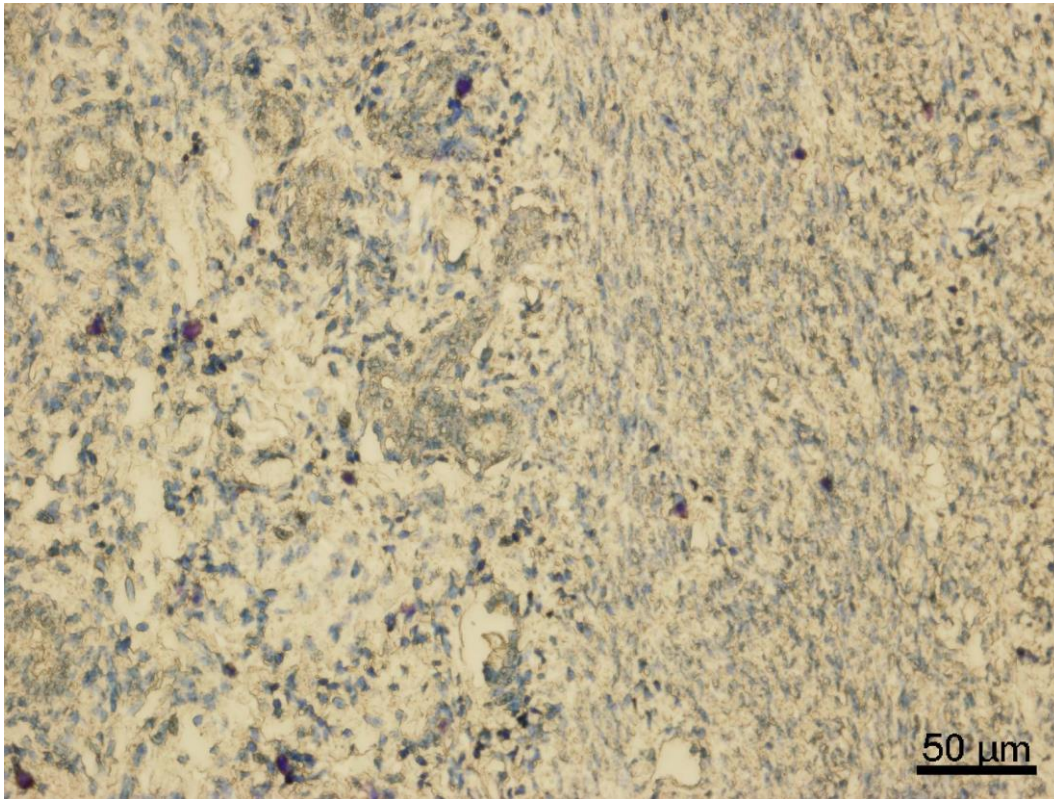


Figure 7. Canine uterus, Carnoy. Significantly ↑ number of MCs compared to Formalin (Fig. 8). The background tissue is highly stained in Carnoy but not in other fluids (see Fig. 8, 9, 10 for comparison).

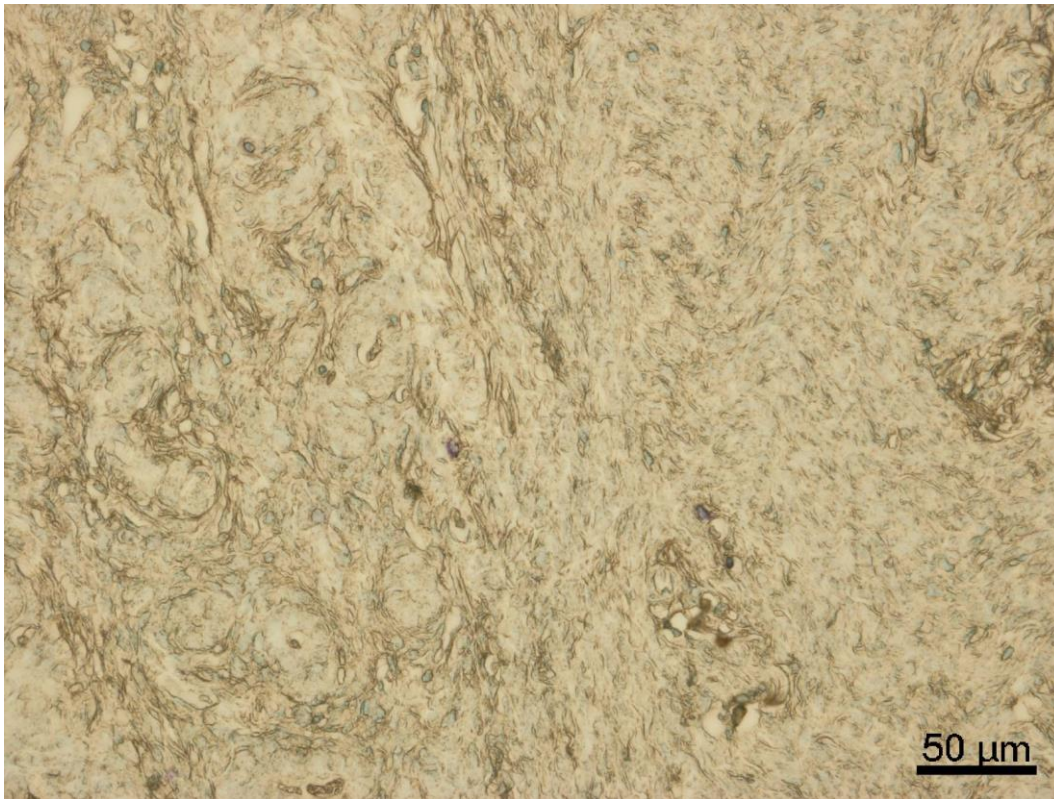


Figure 8. Canine uterus, Formalin. In detected MCs, the dyeability of MC granules is weaker compared to Carnoy (Fig. 7) or Mota (Fig. 10), but similar compared to IFAA (Fig. 9). Significantly ↓ number of MCs compared to Carnoy (Fig. 7), IFAA (Fig. 9) and Mota (Fig.10) was detected.

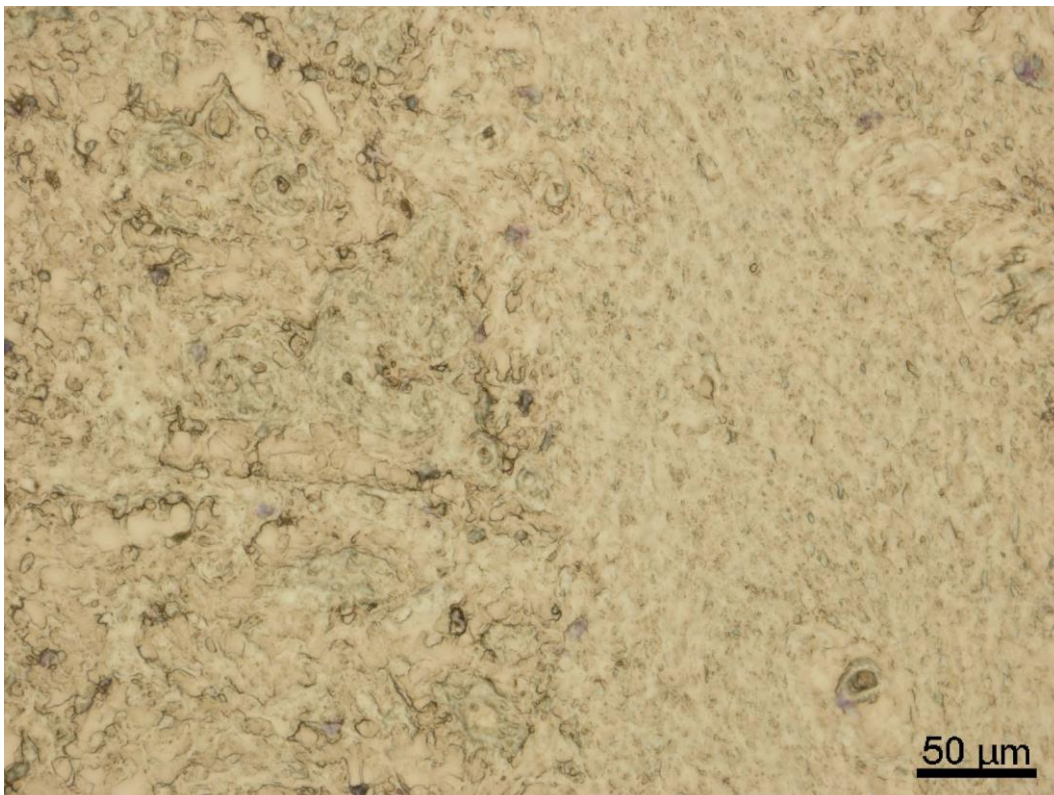


Figure 9. Canine uterus, IFAA. The weak dyeability of granules is similar compared to Formalin (Fig. 8). In the endometrium (left half of the figure), the number of MCs was significantly ↑ compared to Formalin (Fig. 8).

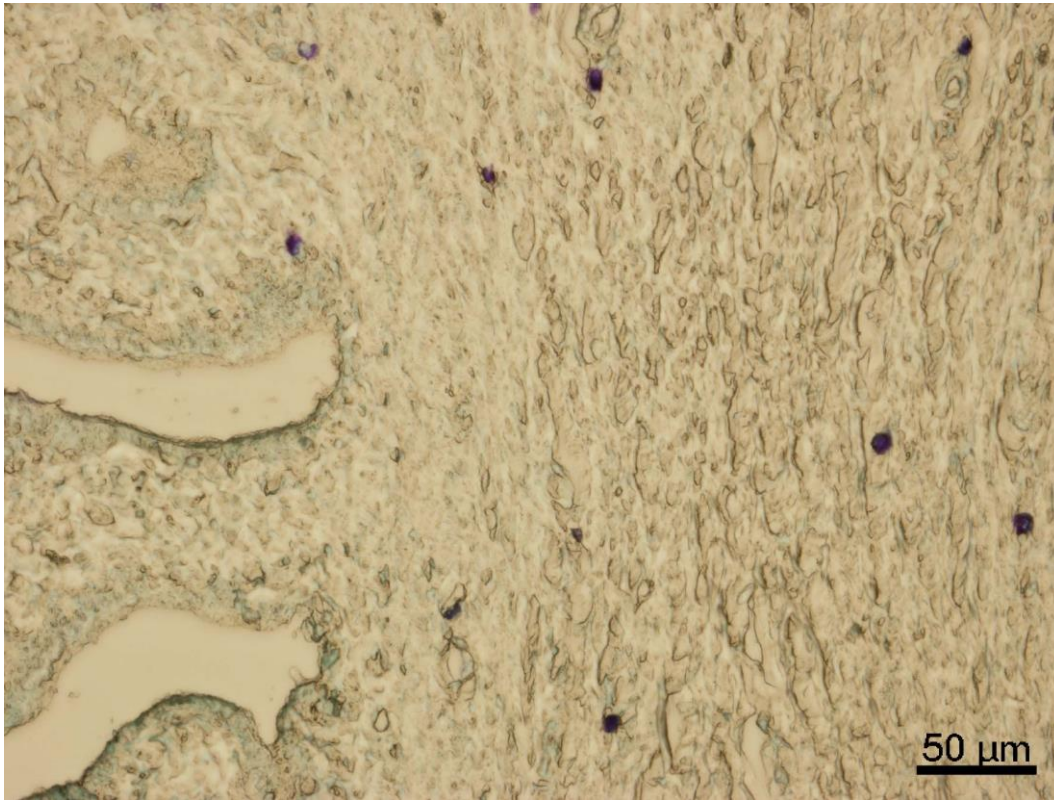


Figure 10. Canine uterus, Mota. The dyeability of granules is as distinctive as in Carnoy and surrounding tissue is less stained compared to Carnoy (Fig. 7). Significantly ↑ number of detected MCs compared to Formalin (Fig. 8).