



Hormonal and Histopathological Fertility Assessments of Male Dogs in Kosovo

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ABSTRACT

This study is aimed to evaluate the concentration of certain hormones (testosterone, LH and FSH), in male dogs from different regions of Kosovo and to histopathologically evaluate testicular tissue from these examined dogs. Testosterone, LH and FSH concentrations were determined by ELISA and testicular tissue samples were processed and subsequently stained with haematoxylin and eosin for histological examination with light microscopy. For mixed-breed dogs, higher testosterone levels were found in the Malisheva, while lower testosterone levels were recorded in the Vushtrria. LH levels in mixed-breed dogs were higher in the Malisheva and lower in the Kamenica. Furthermore, FSH levels in mixed-breed dogs were higher in the Malisheva and lower in the Kamenica. Histopathology revealed different histological changes in testicular tissues, ranging from normal tissue structure to mild, moderate and severe structural alterations. It can be concluded that hormone analyses and histopathological examinations could be considered for fertility assessments.

Key words: Dogs, Histopathology, Kosovo regions, Sexual hormones.

INTRODUCTION

Testosterone is an androgenic hormone produced primarily by the testes and ovaries, with a small contribution by the adrenal gland. Testosterone is responsible for male secondary sexual development in reproductive organs and has positive effects on bone, skin and the cardiovascular system (Perusquia *et al.*, 2010). Spermatogenesis depends on the action of testosterone, which diffuses directly into the seminiferous tubules or binds an albumin carrier that carries it into these tubules. A previous study reported high testosterone levels surrounding the seminiferous tubules due to the presence of Leydig cells (Castro *et al.*, 2002). In male dogs, testosterone is responsible for changes within the basolateral nuclear group of the amygdaloid body, which is associated with aggression (Jacobs *et al.*, 2006). Normal testosterone levels are between 56 and 2800 pg/ml. Low testosterone levels in male dogs are associated with reduced libido, erectile dysfunction, lower sperm production and consequent infertility. Some researchers have stated that testosterone levels vary with age and season, with the highest levels in spring and autumn (Zhang *et al.*, 2011). Luteinizing hormone (LH), also known as interstitial cell-stimulating hormone in males, is a glycoprotein produced by the anterior pituitary gland that triggers testosterone production by Leydig cells in the testes. Normal LH levels are between 0.1 and 7 ng/ml. Follicle-stimulating hormone (FSH) is another glycoprotein produced by the anterior pituitary gland. In both males and females, FSH stimulates germ cell maturation and helps regulate growth and sexual maturation. Normal FSH levels are between 0.6 and 40 ng/ml. Together, testosterone, LH and FSH are the pivotal endocrine factors controlling testicular function and they are all necessary for quantitatively normal spermatogenesis

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(Martins *et al.*, 2006). In dogs androgens are involved in every aspect of prostate development, growth and function, from early in male embryogenesis to later in prostatic hyperplasia (Wilson, 2011). Reproductive analyses in dogs can be performed by methods such as physical examination, semen and endocrine parameter evaluations, testicular biopsy, epididymal marker measurements and sperm aspiration (De Souza *et al.*, 2004).

MATERIALS AND METHODS

Animals

In September 2017, we conducted a scientific study within a castration involving histopathological and biochemical investigations in Kosovo (supported by the Ministry of Education, Science and Technology of the Republic of Kosovo under grant number 2-2615). One hundred male

dogs aged 3 months to 11 years were obtained from shelters, streets or owners. All client-owned dogs were enrolled with informed consent from their owners. All dogs were physically examined and lacked a recent history of illness or steroid administration. The approval granted for this study allowed the collection of 7 different breeds of dogs (Hunting, Labrador, mixed, Belgian Malinois, Illyrian Sheepdog, Pitbull and Terrier) from 8 different regions in Kosovo (Malisheva, Deçan, Fush Kosova, Kamenica, Mitrovica, Prishtina, Rahovec and Vushtrria). The study was performed according to the welfare principles in Kosovo (Animal Welfare Law no. 02/L10) and ethical and professional principles (FVA-0360019).

Stray dogs from shelters and streets were randomly selected for this study. Dogs with owners were randomly selected for this study based on breed, age and region and the owners provided written informed consent for participation in this project. The dogs were brought to a clinic the day before castration and identified individually by ear tag. Before the castration procedure, a veterinarian confirmed that the animal was clinically healthy and took blood for biochemical and hormone analyses. After castration, the dogs recovered at the clinic in 1.2 x 1.8 m cages with bedding under standard conditions, including adequate ventilation, lighting and temperature. The air temperature on the premises where the animals were kept was not below +5°C during summer and was compatible with the outside temperature.

The animals were fed according to standard procedures in Kosovo (Administrative Instruction (MAFRD) No 04/2017 on technical conditions to be met by temporary care centres for stray animals). The animals were returned to the shelters, streets and owners. Blood samples were collected from the right cephalic vein.

Table 1: Hormone (testosterone, LH and FSH) analysis of dogs in different regions of Kosovo.

Region	No. of dogs	No. Within normal	No. Outside normal
Deçan	16	13	3
Fushë Kosova	9	9	0
Kamenica	10	10	0
Malisheva	19	16	3
Mitrovica	23	22	1
Prishtina	12	10	2
Rahovec	5	4	1
Vushtrri	6	6	0
Total	100	90	10 (10%)

Table 2: Hormone levels above or below the normal range in 100 tested dogs.

Hormone value	Testosterone	LH	FSH	Total tests
	56 - 2800 pg/ml	0.1 - 7 ng/ml	0.6 - 40 ng/ml	
Above normal	6	4	4	14
Below normal	1	1	1	3
Total tests	7	5	5	17

Hormone analyses of testosterone, LH and FSH levels

Blood samples (10 ml) were centrifuged at 3000 rpm for 15 minutes and the serum was stored at -20°C for biochemical hormone analyses. Testosterone, LH and FSH were measured by enzyme-linked immunosorbent assay (ELISA) using kits from Innova Biotech Company (Keyuan Road, Industry Development Zone, Daxing District, Beijing, China): Canine Testosterone ELISA Kit (In-Cn0019), Canine Luteinizing Hormone (LH) ELISA Kit (In-Cn0024) and Canine Follicle-Stimulating Hormone (FSH) ELISA Kit (In-Cn0015) (Mialot *et al.*, 1988). The analysis of samples was done at Institute of Biochemistry, Faculty of Medicine, University of Prishtina "Hasan Prishtina".

Castration

Surgical castration was performed at Private Veterinary Clinics in Kamenica, Prishtina, Mitrovica, Fushë Kosova and Rahovec using Ket-A-Xyl anaesthesia (Agro Vet market S. A, Lima, Peru, company name of the chemicals; 0.5 - 1 ml/ 10 kg bw; i.m.). After the operation, all dogs were monitored closely for 72 hours and then returned to shelters, streets or owners.

Histopathological studies

Tissue samples were obtained from both testes immediately after organ removal, fixed for 48 hours in 10% neutral buffered formalin, dehydrated in ascending concentrations of ethyl alcohol, cleared in xylene and embedded in paraffin. Sections (5-6 µm thick) were prepared and stained with haematoxylin and eosin (H&E) (Bancroft *et al.*, 2012) for histopathological examination with a light microscope (Olympus BX50, Japan).

Tissue Samples were done at the Institutes of Pathology, Faculty of Medicine, University of Prishtina "Hasan Prishtina" in Kosovo and Department of Pathology, Faculty of Veterinary Medicine, Cairo University in Egypt.

Statistical analysis

The data are presented as the mean ± SEM. Comparisons were conducted by one-way analysis of variance (ANOVA) followed by Tukey's comparisons test. The level of significance was set at P<0.05 and data were analysed using GraphPad software (GraphPad, CA, USA).

RESULTS AND DISCUSSION

Testosterone, LH and FSH levels in the studied animals of all breeds are shown in Table 2. Overall, 10 dogs (10% of the study population) showed alterations in hormone levels. Seven of these 10 dogs had hormone levels above the

normal range, whereas 3 had values below the normal range. The hormone assessments of the affected cases from different regions in Kosovo revealed that dogs from Deçan and Malisheva recorded high levels of testosterone, FSH and LH; dogs from Prishtina recorded low levels of both testosterone and FSH and dogs from Mitrovica showed low levels of only LH (Table 1).

For each tested hormone (testosterone, LH and FSH), the number of dogs with levels above or below the normal range was determined. Our study identified more dogs with

higher hormone levels but only one dog with lower levels of each hormone (Table 2).

Hormone assessments of the tested dogs of different breeds demonstrated changes in only mixed-breed dogs (Table 3).

As shown in Table 4, the mean testosterone value was 557.73 pg/ml in dogs with normal testosterone levels and 4014.8 pg/ml in dogs with levels outside the normal range; the difference in mean testosterone level between these two groups was significant ($p < 0.0001$).

As shown in Table 5, the mean LH level was 1.401 ng/ml in the dogs with normal LH values and 9.17 ng/ml in those with LH levels outside the normal range; the difference between groups was significant ($p < 0.0001$).

As shown in Table 6, the mean FSH level was 6.598 ng/ml in dogs with values within the normal range and 60.64 ng/ml in dogs with values outside the normal range; the difference between groups was significant ($p < 0.0001$).

Microscopic examination of testicular tissues from dogs in various regions of Kosovo and of various breeds revealed histological changes that ranged from normal tissue structure to mild, moderate and severe structural alterations.

Testicular tissues of mixed-breed dogs from the Malisheva, Fushë Kosova, Kamenica and Vushtrria regions showed no

Table 3: Hormone parameters (testosterone, LH and FSH) in tested dogs in Kosovo according to breed.

Breed	No. of dogs	No. Within normal	No. Outside normal
Hunting dog	5	5	0
Illyrian sheepdog	3	3	0
Labrador	1	1	0
Terrier	2	2	0
Belgian malinois	1	1	0
Pitbull	1	1	0
Mixed	87	77	10
Total	100	90	10

Table 4: Mean testosterone levels in tested dogs within or outside the normal range.

Testosterone	All tested dogs	Dogs within the normal range	Dogs outside the normal range	Dogs with values above the normal range	Dogs with values below the normal range
Number of points	100	90	10	6	1
Mean	903.44	557.73	4014.8	6519.3	87
SD deviation	1660.6	508.67	3978	3106.7	
SD error	166.06	53.618	1258	1268.3	
Minimum	87	102	87	3327	
Maximum	11404	2481	11404	11404	
Median	350	333.5	3710.5	6075.5	
Lower 95% CI:	573.47	451.02	1169.3	3258.5	
Upper 95% CI:	1233.4	664.45	6860.3	9780.1	
		$p < 0.0001$		$p < 0.0001$	

Table 5: Mean LH level in tested dogs within or outside the normal range.

LH	All tested dogs	Dogs within the normal range	Dogs outside the normal range	Dogs with values above the normal range	Dogs with values below the normal range
Number of points	100	90	10	4	1
Mean	2.178	1.401	9.17	18.65	0.1
SD deviation:	4.164	1.167	10.812	11.243	
SD error:	0.4164	0.123	3.419	5.622	
Minimum:	0.1	0.2	0.1	9.1	
Maximum:	34.3	5.8	34.3	34.3	
Median:	0.9	0.9	7.65	15.6	
Lower 95% CI:	1.351	1.156	1.436	0.762	
Upper 95% CI:	3.005	1.646	16.904	36.538	
		$p < 0.0001$		$p < 0.0001$	

histological changes, with apparently normal seminiferous tubules, spermatogonia and spermatozoa (Fig 1a and b).

Mild histological alterations were detected in testes of mixed-breed dogs from the Mitrovica and Rahovec regions; such alterations included oedema between seminiferous

tubules and congestion in the interstitial blood vessels (Fig 1c and d).

Moderate changes were reported in testes of mixed-breed dogs from the Prishtina region; these changes were represented by the initiation of fibrous connective tissue

Table 6: Mean FSH level in tested dogs within or outside the normal range.

FSH	All tested dogs	Dogs within the normal range	Dogs outside the normal range	Dogs with values above the normal range	Dogs with values below the normal range
Number of points	100	90	5	4	1
Mean	9.745	6.598	60.64	75.7	0.4
SD deviation	15.786	5.266	42.047	29.073	
SD error	1.579	0.555	18.804	14.537	
Minimum	0.4	1.3	0.4	48.8	
Maximum	114.1	25.5	114.1	114.1	
Median	5.2	4.95	58.3	69.95	
Lower 95% CI	6.608	5.493	8.44	29.445	
Upper 95% CI	12.882	7.703	112.84	121.96	
		p<0.0001		p<0.0001	

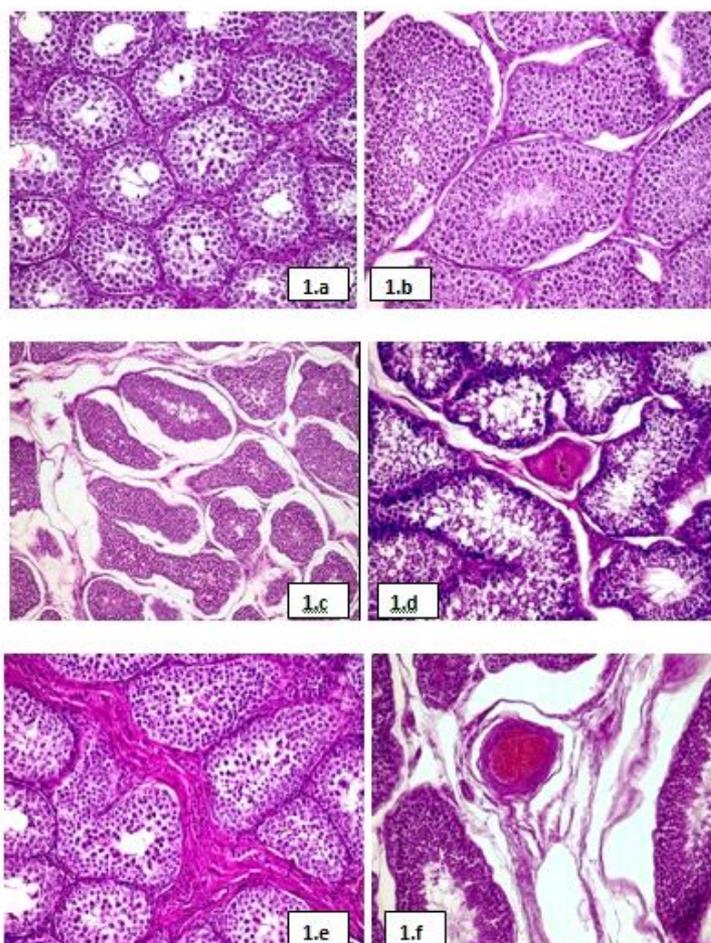


Fig 1: Testes of mixed-breed dogs from different regions of Kosovo: **a)** Normal seminiferous tubules, spermatogonia and spermatozoa, H&E X200, **b)** Normal seminiferous tubules, spermatogonia and spermatozoa, H&E X400, **c)** Oedema between seminiferous tubules, H&E X100, **d)** Congestion in the interstitial blood vessels, H&E X200, **e)** Fibrous connective tissue proliferation between seminiferous tubules, H&E X200, **f)** Oedema between seminiferous tubules and congested blood vessels, H&E X200.

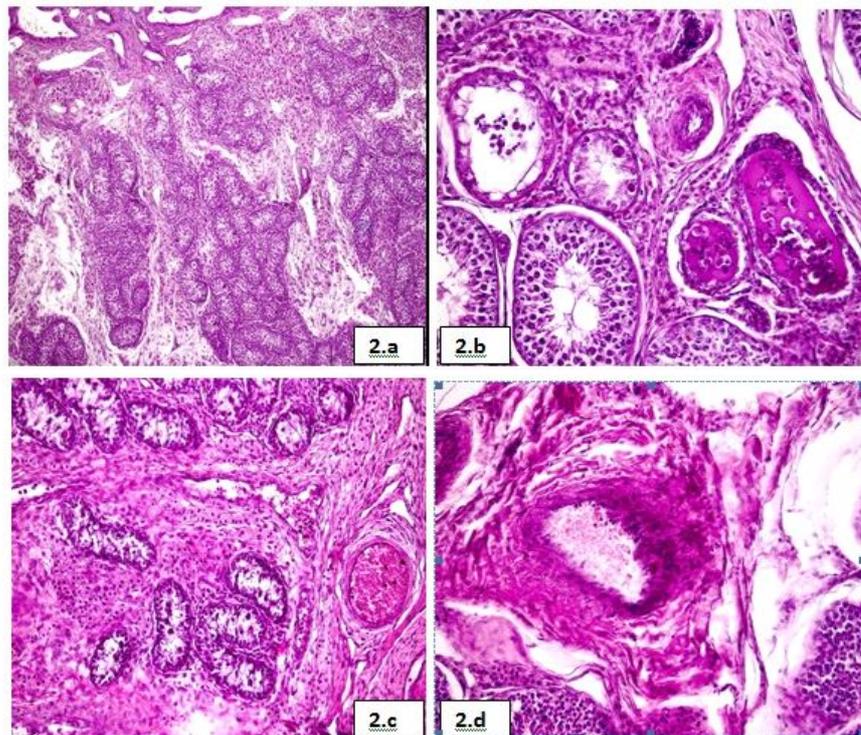


Fig 2: Testes of mixed-breed dogs from the Deçan and Malisheva regions of Kosovo: **a)** Severe fibrosis with atrophied seminiferous tubules, H&E X100, **b)** Hyaline degeneration of some seminiferous tubules, H&E X200, **c)** Degenerated and necrosed seminiferous tubules, H&E X100, **d)** Congested blood vessels with a thickened muscular wall, H&E X400.

proliferation, oedema between seminiferous tubules and congested blood vessels (Fig 1e and f).

Severe testicular histological changes were noted in mixed-breed dogs from the Deçan and Malisheva regions, including severe fibrosis with atrophied seminiferous tubules (Fig 2a); hyalinized (Fig 2b), degenerated and necrosed seminiferous tubules (Fig 2c) and congested blood vessels with a thickened muscular wall (Fig 2d).

One hundred male dogs of various breeds from different regions of Kosovo were assessed for hormone levels and histopathological indicators of fertility (De Gier *et al.*, 2012). After analysing the levels of hormones such as testosterone, LH and FSH, we found that only 10% of the dogs had hormone levels outside the normal range (Frank *et al.*, 2003). Of the 10 breeds that were evaluated in this study, only mixed-breed dogs showed hormonal changes. Dogs from Deçan and Malisheva had high levels of testosterone, FSH and LH and these changes were confirmed histopathologically by the presence of severe testicular pathology, including severe fibrosis with atrophied seminiferous tubules; hyalinized, degenerated and necrosed seminiferous tubules and congested blood vessels with a thickened muscular wall (Fukuda *et al.*, 2000). Dogs from Prishtina had low levels of both testosterone and FSH, which was reflected histopathologically as slight fibrous connective tissue proliferation, oedema between seminiferous tubules and congested blood vessels (Mendis-Handagama *et al.*, 2005). Testicular degeneration and leydig cell atrophy have been

observed (Shahar *et al.*, 2017). Moreover, dogs from Mitrovica had low levels of only LH, which appeared histologically as oedema between seminiferous tubules and congestion in the interstitial blood vessels (Kellom and O'Conner, 1991). The mean testosterone level in dogs with values within the normal range was 557.73, while that for dogs with values outside the normal range was 4014.8. Moreover, the mean LH level was 1.401 in dogs with values within the normal range and 9.17 in those with values outside the normal range (Nett *et al.*, 2002). Lastly, the mean FSH level was 6.598 in dogs with values within the normal range and 60.64 in those with values outside the normal range. In comparisons of the mean value of each of the three hormones in the dogs within and outside normal, the two groups were significantly different in all three comparisons ($p < 0.0001$) (Ortega-Pacheco *et al.*, 2006).

Our study concluded that hormonal analysis and histopathological examination are good parameters for assessing fertility and the obtained results provide valuable guidance for fertility determinations in male dogs in Kosovo.

CONCLUSION

In our study, we found high levels of testosterone, FSH and LH and these changes were confirmed histopathologically by the presence of severe testicular pathology, including severe fibrosis with atrophied seminiferous tubules; hyalinized, degenerated and necrosed seminiferous tubules. This is a pioneer work which could be explored for evaluating biochemical and histopathological alterations in male dogs in Kosovo.

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