Unilateral testicular degeneration in dogs: Effects on spermatozoal characteristics, testis and cauda epididymis

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Abstract

Testicular degeneration is an important cause of poor fertility in dogs, but there is little knowledge on its effects on spermatozoa in affected dogs. The study investigated the specific effects of unilateral testicular degeneration (UTD) on spermatozoal characteristics in the testis and epididymis. Ten sexually mature Nigerian indigenous breed of dogs, comprising 5 normal dogs and 5 dogs with UTD were used for the study. The testis and epididymis were removed via orchidectomy for morphological and histopathological evaluation. Sperm in the testis and cauda epididymis were analysed. The samples were grouped into four as N1 (normal right testis), N2 (normal left testis), ND (non-degenerated testis in UTD dogs), and D (degenerated testis in UTD dogs). Data were analysed using one-way ANOVA. There were significant decreases (p<0.001) in testes weight, length, width and volume, and the gonado-somatic index in the D testes compared to the contralateral ND and the N groups. The D group also had significantly lowered epididymal sperm total and progressive motility, viability and concentration (p<0.001), and a decreased testicular sperm concentration (p<0.01). Moreover, there was a decreased percentage (p<0.001) of morphologically normal sperm, with increased prevalence of sperm abnormalities in the D group compared to the other groups. In comparison with the N groups, the ND group had a significantly lowered (p<0.05) epididymal sperm progressive motility, with increased percentage (p<0.01) of sperm with proximal cytoplasmic droplets and looped tails. The findings demonstrated that UTD in dogs adversely affected spermatozoa in the testis and cauda epididymis. There was also evidence of compromised spermatozoa in the epididymis contralateral to the degenerated testis.

Keywords: Canine; Epididymis; Spermatozoa; Testicular atrophy; Testicular degeneration

Introduction

Dogs are mainly bred for companionship, showmanship, security, hunting, and to provide valuable source of income for breeders. In addition, the slaughter and consumption of dog meat is popular in some countries (Podberscek, 2009; Ehimiyein *et al*., 2014). Therefore, infertility is likely to cause significant economic losses in canine breeding.

The testis is the primary organ of reproduction in male animals and functions in spermatogenesis (sperm production) and steroidogenesis (testosterone production). Testicular degeneration (TD) is considered as one of the most common causes of poor semen quality and acquired infertility in male dogs (Fontbonne, 2011; Domingos and Salomão, 2011) with a prevalence of 15-58% (Ortega-Pacheco *et al*., 2006; Câmara *et al*., 2014). Testicular degeneration involves deterioration in the structure of the testis with a consequent loss of testicular function (Turner, 2007). It may affect one testis (unilateral) or both testes (bilateral), involving parts of the testis or the whole testis. The seminiferous epithelium of the testis is highly susceptible to damage, with a wide variety of agents causing reversible or irreversible degeneration (Parkinson, 2001). Acute TD can result secondary to a known insult such as exposure of the testes to toxins, radiation, extreme scrotal temperature, scrotal trauma, autoimmune disease, certain nutritional deficiencies, and infection with pathogenic organisms (Turner, 2007; Obi *et al*., 2013). Idiopathic TD has no identifiable underlying cause and has also been reported in dogs (Rehm, 2000; Fontbonne, 2011).

Spermatozoa can be recovered from the cauda epididymis of dogs by percutaneous epididymal aspiration, following castration, or at post mortem; for use in the investigation of sperm quality and spermatozoal characteristics (Varesi *et al*, 2013; Chima *et al*., 2017; Bhanmeechao *et al*., 2018), and for artificial breeding of bitches (Wydooghe *et al*., 2016). Ejaculated semen comprises sperm from both testes as well as secretions of the accessory sex glands. Thus, while evaluation of the ejaculate is used to assess semen for fertility potential, it precludes the investigation of specific testis/epididymis-derived effects of testicular disorders, and their impact on spermatozoa.

A few studies reported adverse effects of various testicular disorders, including TD on testicular and sperm morphology (Ortega-Pacheco *et al*., 2006; Câmara *et al*., 2014). In addition to sperm morphology, several other sperm character-

istics and parameters (e.g. motility, viability, concentration) are known to have major influence on the integrity, functionality and fertility potential of spermatozoa (Johnston *et al*., 2001; Robert *et al*., 2016; Kolster, 2018). However, there is little knowledge on the effect of TD on these sperm characteristics in the testis or epididymis of affected dogs. Therefore, the aim of the study was to investigate the effects of unilateral testicular degeneration (UTD) on spermatozoal characteristics in canine testis and epididymis.

Materials and methods

Animals

Ten sexually mature (9-12 months old) apparently healthy Nigerian indigenous breed of dogs comprising 5 normal dogs: N (both testes normal and present in the scrotum) and 5 dogs with UTD: one non-degenerated (ND) and one degenerated (D) scrotal testes, were used for the study. Only dogs with no other observed reproductive pathology during clinical examination, but with a presentation and history of progressive unilateral scrotal testicular atrophy were selected. Animal welfare was observed in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), and the protocol was approved by the Research Ethics Committee of the Faculty of Veterinary Medicine (2016/10-173537). The animals had body weights ranging from 7.8-9.7 kg. Following an elective request for castration, orchidectomy (open castration) was performed as previously described (Hassan and Hassan, 2003). The surgery was performed following premedication with atropine sulphate (Pauco Atropine®, Jiangsu Huayang Pharmaceutical, Jiangsu, China) at 0.04 mg/kg b.w., IM and xylazine hydrochloride (AnaSed®, Akorn, Lake Forest, IL, USA) at 0.5 mg/kg b.w., IM., and under general anaesthesia using ketamine hydrochloride (Ketmin®, Laborate, Panipat, India) at 5 mg/kg b.w., IM. Post-surgical pain was alleviated with tramadol hydrochloride (Tradyl®, PT Interbat, East Java, Indonesia) at 2.5 mg/kg b.w., IM twice daily for four days. The recovered testis and epididymis were dissected, and then utilized for gross morphometry, histopathology and sperm analysis. Each testis/epididymis was considered as the experimental unit. Thus, the samples were grouped into four as N1 (normal right testis), N2 (normal left testis), ND (non-degenerated testis in UTD dogs), and D (degenerated testis in UTD dogs).

Morphological and histopathological evaluation

The weights of testis and epididymis were measured using an electronic weighing scale (Ohaus, Pine Brook, NJ, USA). Testis length and width, and epididymal length were measured with a vernier calliper. Testes volume was measured by the water displacement method (Sakamoto *et al*., 2007). The gonado-somatic index (GSI, g/kg) was calculated by dividing the testis weight (g) by the body weight (kg). Likewise, the epididymo-somatic index (ESI, g/kg) was calculated by dividing the epididymis weight (g) by the body weight (kg) (Omari *et al*., 2018).

Small portions of cauda epididymal and testicular tissues were immersed in Bouin's fixative solution for 24 h and then transferred to 70% ethanol until processing. Histopathological sections were prepared and stained with haematoxylin and eosin as previously described (Slaoui and Fiette, 2011). Sections were evaluated for histopathology under light microscopy (Figure 1A-D). Samples were designated as normal (N) in the absence of testicular or epididymal histologic abnormalities. Testicular degeneration was confirmed based on the presence of characteristic histopathological features as previously described (Yuan and McEntee, 1987; McGavin and Zachary, 2007; Câmara *et al*., 2014). Samples with absence of spermatozoa (azoospermia) in the epididymides indicated severe testicular degeneration and were excluded from the study, as the aim was to investigate spermatozoal characteristics in the different groups.

Epididymal and testicular sperm analyses

Sperm evaluation was performed as previously described (Seed *et al*., 1996; World Health Organization, 2010). Sperm motility was determined using sperm diffusion in phosphate buffered saline (PBS; pH 7.4, 37 °C) from sectioned cauda epididymis. Sperm motility (%) was determined at ×400 using a phase-contrast microscope (Motic B3; Motic, Carlsbad, CA, USA) equipped with a stage slide warmer (TCS-100; Amscope, Ivrine, CA, USA) set at 37 °C. Sperm viability (%) was evaluated using eosin-nigrosin vital staining, and sperm were categorized as live (unstained head) or dead (marked pink-stained head) under light microscopy at ×1000 magnification. Sperm morphological abnormalities were evaluated using phase-contrast microscopy and eosin-nigrosin staining. All values in percentage were determined by examining 200 sperm cells across different fields in duplicates. Cauda epididymal and testicular sperm concen-

trations were determined following cauda epididymal/testicular tissue homogenization in PBS, and counting of sperm cells using a haemocytometer (Weber, England). Total sperm count was expressed as the number of sperm per gram cauda epididymis, and per gram testis, respectively (Seed *et al*., 1996).

Statistical analysis

Data were analysed with the one-way analysis of variance (ANOVA) tool using GraphPad Prism version 6.01 (GraphPad Software, Inc.), and the results presented as Mean ± standard deviation (SD). Significant differences between means were confirmed using Tukey's honestly significant difference post hoc test. Results were considered statistically significant when $p<0.05$.

Results

Morphological evaluation of testis and epididymis

The results of gross morphological evaluation of the testis and epididymis in normal dogs and UTD dogs were as shown in **Table 1**. UTD affected the right testis in two dogs and the left testis in three dogs. There were significant decreases ($p<0.001$) in testes weight, length, width and volume, and the GSI in the D testes compared to the contralateral ND testes in UTD dogs, and also compared to the N testes in the normal dogs. However, these parameters did not differ ($p>0.05$) between the ND and the N testes. In addition, there were no significant differences in epididymal weight and length, and the ESI in all the four groups.

Table 1. Testis and epididymis morphological characteristics in dogs with normal testes and unilateral testicular degeneration (UTD).

	Normal		UTD	
Parameter	**N1**	**N2**	**ND**	**D**
Testis weight (g)	7.74 ± 0.34[a]	7.80 ± 0.28[a]	7.92 ± 0.31[a]	5.12 ± 0.64[b]
Testis length (cm)	2.78 ± 0.04[a]	2.76 ± 0.04[a]	2.83 ± 0.09[a]	2.01 ± 0.15[b]
Testis width (cm)	2.26 ± 0.04[a]	2.27 ± 0.03[a]	2.29 ± 0.05[a]	1.57 ± 0.10[b]
Testis volume (cm^3)	7.13 ± 0.18[a]	7.14 ± 0.19[a]	7.21 ± 0.21[a]	4.47 ± 0.53[b]
Epididymis weight (g)	1.25 ± 0.11	1.23 ± 0.08	1.25 ± 0.12	1.19 ± 0.09
Epididymis length (cm)	4.81 ± 0.05	4.83 ± 0.04	4.81 ± 0.06	4.72 ± 0.09
Gonado-somatic index (g/kg)	0.89 ± 0.01[a]	0.90 ± 0.02[a]	0.88 ± 0.05[a]	0.56 ± 0.02[b]
Epididymo -somatic index (g/kg)	0.14 ± 0.01	0.14 ± 0.00	0.14 ± 0.00	0.13 ± 0.01

N1 (normal right testis); N2 (normal left testis); ND (non-degenerated testis in UTD dogs); D (degenerated testis in UTD dogs). Values represent mean ± SD, n = 5. Rows with different superscript letters indicate significant differences between groups ($p<0.05$).

Histopathology of the testis and cauda epididymis

Evidences of TD were observed (**Figure 1B**) and these included the presence of decreased seminiferous tubular diameter, reduced thickness of the seminiferous epithelium, and scanty presence of spermatogenic cells. The cauda epididymis of the degenerated testis (**Figure 1D**) showed evidence of regression of epididymal tubules with reduced tubular diameter, scanty or no presence of spermatozoa in the tubular lumen and increased inter-tubular connective tissue.

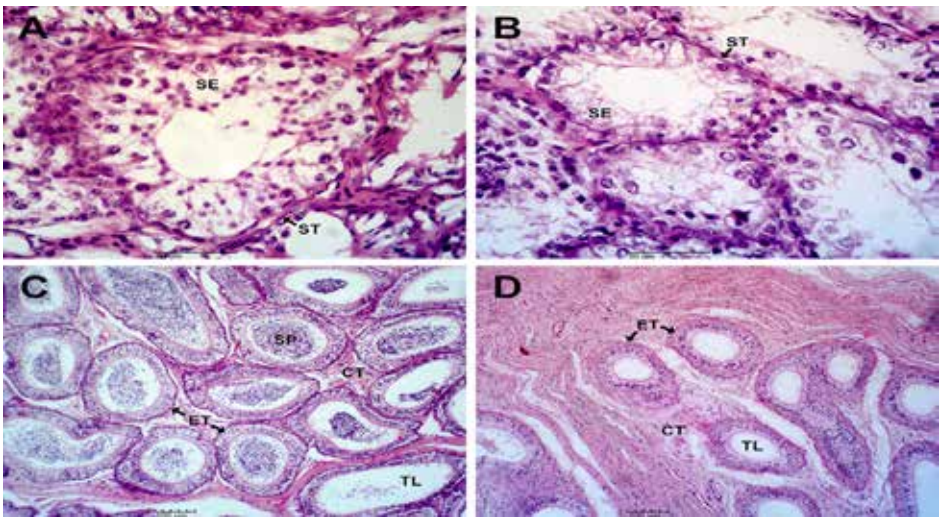

Figure 1. Histomicrographs of the testis and cauda epididymis in dogs with normal testes and unilateral testicular degeneration (UTD).

A: Normal testis showing large diameter of seminiferous tubules (ST), thick multi-layered and active seminiferous epithelium (SE) comprising numerous cells of the spermatogenic lineage. B: testis showing evidence of degeneration including decreased seminiferous tubular diameter, reduced thickness of the SE, and scanty presence of spermatogenic cells. C: Normal cauda epididymis showing large diameter of epididymal tubules (ET), presence of abundant spermatozoa (SP) in the tubular lumen (TL), scanty inter-tubular connective tissue (CT). D: cauda epididymis of degenerated testis showing regression of ET with reduced tubular diameter, scanty or no presence of SP in the TL and increased inter-tubular CT. (H & E stain; scale bar: 50 µm, 100 µm).

Cauda epididymal and testicular sperm analysis

The results of the cauda epididymal and testicular sperm analysis in normal dogs and UTD dogs are shown in **Table 2** and **Figure 2A-F**. There were no significant differences (p>0.05) in all the sperm parameters between the two N (N1 and N2) groups in the normal dogs. In comparison with the N groups, the ND group in dogs with UTD did not differ with respect to epididymal sperm total motility, viability and concentration, and testicular sperm concentration. However, they had a significantly lowered (p<0.05) epididymal sperm progressive motility. The percentage of sperm with normal morphology and the prevalence of detached head, abnormal head, coiled midpiece (Dag defect) and coiled tail did not differ between the ND and N groups. However, there was increased

percentage (p<0.01) of sperm with proximal cytoplasmic droplets (PCD) and looped tails in ND compared to the N groups.

Table 2. Cauda epididymis and testis spermatozoal characteristics in dogs with normal testes and unilateral testicular degeneration (UTD).

Testis/Epididymis	Normal		UTD	
	N1	N2	ND	D
Epididymal sperm parameter (%)				
Total motility	83.6 ± 2.7[a]	84.8 ± 3.0[a]	81.3 ± 5.7[a]	11.5 ± 9.1[b]
Progressive motility	74.5 ± 4.7[a]	72.9 ± 3.8[a]	59.6 ± 7.9[b]	4.4 ± 8.3[c]
Viability	94.6 ± 2.3[a]	94.2 ± 2.8[a]	87.6 ± 4.8[a]	27.2 ± 8.3[b]
Epididymal sperm morphology (%)				
Normal sperm	61.0 ± 4.6[a]	63.4 ± 3.9[a]	55.2 ± 7.2[a]	23.7 ± 11.3[b]
Detached head	4.1 ± 1.5[a]	3.7 ± 1.1[a]	4.8 ± 2.8[a]	13.7 ± 4.2[b]
Abnormal head	1.2 ± 0.8[a]	0.9 ± 0.8[a]	1.4 ± 0.9[a]	5.2 ± 1.8[b]
Bent midpiece	2.9 ± 1.3	3.2 ± 0.9	3.0 ± 1.7	6.5 ± 4.1
Coiled midpiece/Dag defect	0.8 ± 0.8[a]	0.6 ± 0.9[a]	0.8 ± 1.1[a]	4.8 ± 2.5[b]
Proximal cytoplasmic droplets	0.2 ± 0.4[a]	0.4 ± 0.5[a]	2.8 ± 1.1[b]	0.6 ± 0.5[a]
Distal cytoplasmic droplets	21.4 ± 9.9	19.6 ± 10.8	22.6 ± 11.6	27.4 ± 16.5
Looped tail	6.6 ± 1.5[a]	7.0 ± 1.2[a]	10.8 ± 2.5[b]	13.1 ± 3.9[b]
Bent tail	4.5 ± 1.8	4.1 ± 1.6	4.4 ± 2.1	4.6 ± 2.5
Coiled tail	3.4 ± 1.1[a]	4.0 ± 1.0[a]	4.5 ± 1.5[a]	8.6 ± 2.6[b]
Sperm concentration				
Epididymis (x10^6 sperm/g)	926.0 ± 23.9[a]	912.4 ± 19.1[a]	874.0 ± 78.7[a]	3.29 ± 2.1[b]
Testis (x10^6 sperm/g)	31.6 ± 5.7[a]	29.3 ± 4.8[a]	23.2 ± 9.3[a]	5.81 ± 2.9[b]

N1 (normal right testis); N2 (normal left testis); ND (non-degenerated testis in UTD dogs); D (degenerated testis in UTD dogs). Values represent mean ± SD, n = 5. Rows with different superscript letters indicate significant differences between groups (P<0.05).

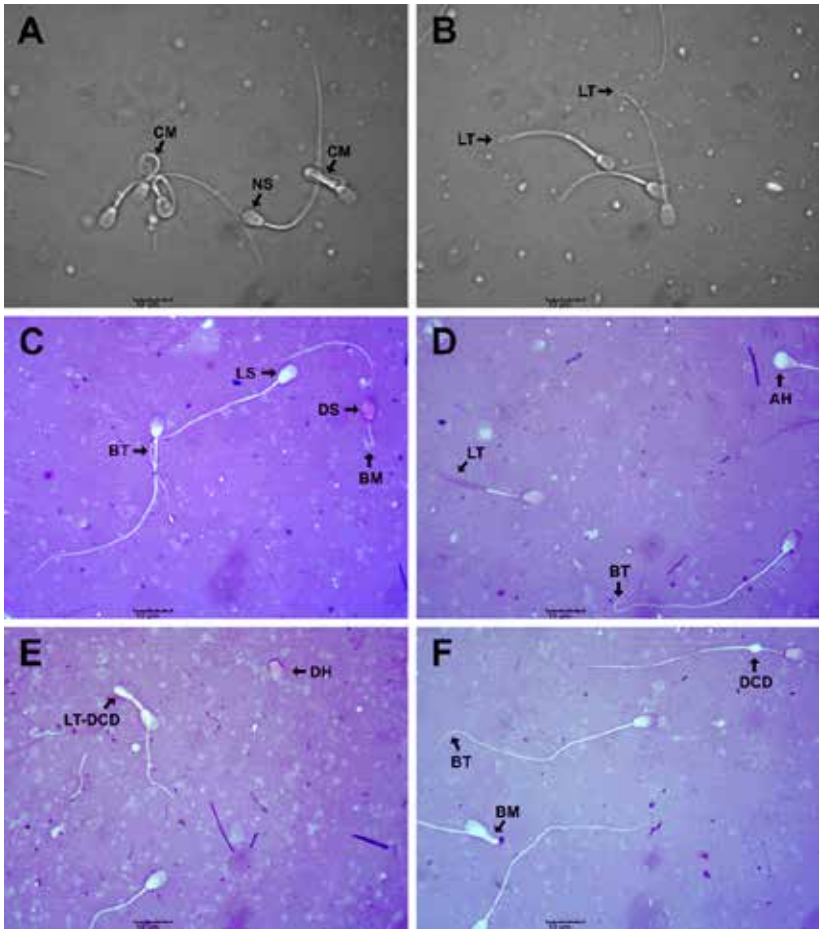

Figure 2. Micrographs of cauda epididymal sperm characteristics in dogs with normal testes and unilateral testicular degeneration (UTD).

A and B: Evaluation of sperm morphology using phase-contrast microscopy. Note the normal sperm (NS) and the presence of coiled midpiece (CM) or Dag defect and looped tail (LT) abnormalities. C-F: Evaluation of sperm viability and morphology using eosin-nigrosin staining. Note the live sperm (LS) and dead sperm (DS), and sperm with bent tail (BT), bent midpiece (BM), LT, pear-shaped abnormal head (AH), detached head (DH), looped tail enclosing a distal cytoplasmic droplet (LT-DCD), and distal cytoplasmic droplet (DCD) abnormalities. Scale bar (10 µm).

On the other hand, there were significant differences in most of the observed sperm parameters in the D group in dogs with UTD, compared to the contralateral ND and the N groups. The D group had a significantly decreased (p<0.001)

epididymal sperm total and progressive motility, viability and concentration, compared to the other groups. Testicular sperm concentration was also significantly decreased in the D group compared to the contralateral ND ($p<0.01$) and the N groups ($p<0.001$). There was decreased percentage ($p<0.001$) of normal sperm, and increased percentage of sperm with detached head ($p<0.001$), abnormal head ($p<0.05$), and coiled midpiece and coiled tail ($p<0.01$) in the D group compared to the other groups. The percentage of sperm with looped tail was also increased in the D group ($p<0.01$), but did not differ from the contralateral ND group. Out of all the observed sperm abnormalities, the percentage of sperm with bent midpiece, bent tail and distal cytoplasmic droplets (DCD) did not differ across all the groups.

Discussion

The study involved only dogs with a history of progressive unilateral testicular atrophy, and with histological evidence of TD and the presence of spermatozoa in the cauda epididymis. This differentiated the dogs from animals with testicular hypoplasia. Therefore, the selected animals were considered to be in a state of progressed TD but not in the advanced or terminal stage characterized by total loss of seminiferous epithelia and the absence of spermatozoa (azoospermia). We applied the term 'non-degenerated' rather than 'normal' in the contralateral ND group for two reasons. First, there were no specific information on the aetiologies of the TD, and to what extent they may have affected the contralateral testes. Second, we observed some sperm morphological abnormalities in the contralateral ND group compared to the normal dogs.

The results showed evidence of adverse effects of UTD in canine testes and spermatozoa. All the testicular gross morphological parameters (weight, length, width, volume and GSI) were markedly reduced following TD. This is consistent with other reports of a decline in testis size and weight in severe cases of TD or testicular atrophy in male animals (Parkinson, 2001; Ortega-Pacheco *et al.*, 2006; Teankum *et al.*, 2013). In contrast, epididymal weight and length and the ESI were not significantly altered by TD. This observation is also in agreement with previous reports of unaltered epididymal size or weight, concurrent with testicular atrophy in cases of TD or testicular atrophy in the stallion and boar (Blanchard and Varner, 1993; Teankum *et al.*, 2013). Ortega-Pacheco *et al.* (2006) reported a decreased epididymal weight only in dogs with advanced TD but not partial TD, compared to the contralateral normal testes.

The percentage motility and viability of cauda epididymal spermatozoa observed in this study for the N groups were comparable to the values reported previously in normal dogs (Chima *et al*., 2017; Bhanmeechao *et al*., 2018). Testicular degeneration decreased the epididymal sperm total and progressive motility in the D group. A low percentage of progressively motile sperm was also reported in the semen of stallions with TD (Blanchard *et al*., 2000). Decreased sperm motility may be related to abnormal sperm formation, and the observed increase in the proportion of sperm with morphologic abnormalities. Testicular degeneration also caused an increase in the percentage of dead epididymal sperm (decreased sperm viability) in the D group. This may be related to a disruption of the normal processes of spermatogenesis and sperm epididymal maturation, as a consequence of testicular insult and degeneration. Sperm motility is a critical indicator of normal structural and functional competence of spermatozoa, and there is positive correlation between the proportion of progressively motile sperm and sperm with normal morphology (Robert *et al*., 2016). Lowered fertility has been associated with <70% total and progressive sperm motility and with increased proportion of dead sperm in the semen of dogs (Johnston *et al*., 2001; Oguejiofor, 2018).

The mean proportion of morphologically normal cauda epididymal sperm in the N groups in the study (62%) was lower than the previous reports of 80% in epididymal sperm (Ortega-Pacheco *et al.*, 2006) and 81% in vas deferens sperm (Câmara *et al*., 2014). This difference was attributed to the exclusion of epididymal sperm with DCD from the proportion of morphologically normal sperm in the study. However, Ortega-Pacheco *et al.* (2006) considered the presence of DCD as normal for sperm harvested from the cauda epididymis; a site for sperm maturation and storage. Although the proportion of sperm with DCD did not vary across the different groups, we observed a lower mean prevalence (21%) in the N groups, in contrast to the 49% reported by that study (Ortega-Pacheco *et al.*, 2006). A high prevalence (76%) of total morphologically abnormal sperm was observed in the D group. Similarly, more than 80% cauda epididymal sperm abnormality was reported in cases of partial TD with oligospermia (Ortega-Pacheco *et al*., 2006). TD reduced the proportion of normal sperm in the study from 62% to 24%. A decrease in normal sperm from 81% to 55% was also observed in vas deferens sperm of dogs with severe TD (Câmara *et al*., 2014). In addition, the findings here are consistent with the previous reports of a high prevalence of sperm with detached head abnormality (Ortega-Pacheco *et al.*, 2006; Câmara *et al*., 2014), and other abnormalities

including acrosomal defects, abnormal midpiece, and bent tails in severe TD (Ortega-Pacheco *et al.*, 2006). Remarkably, these sperm defects are considered to have significant negative effect on canine fertility (Kolster, 2018).

Furthermore, TD decreased the testicular and cauda epididymal sperm concentrations. This may be a consequence of atrophy of the testicular parenchyma, degeneration of seminiferous epithelia, and decreased spermatogenesis. Since the cauda epididymis is the site of sperm storage, the lowered epididymal sperm concentration reflected a decrease in sperm production in the testis. Similarly, there was lowered daily sperm production per millilitre of testis and decreased sperm concentration in stallions with TD (Blanchard *et al.*, 2001). Oligospermia and azoospermia may result in dogs with TD depending on the severity of the condition (Ortega-Pacheco *et al.*, 2006; Fontbonne, 2011; Câmara *et al.*, 2014). Lowered sperm numbers can lead to subfertility or infertility in affected dogs (Robert *et al.*, 2016).

Interestingly, the findings suggest that TD in one testis may impair spermatozoa in the contralateral epididymis. In comparison with the N groups, the ND group had decreased sperm progressive motility and increased prevalence of PCD and looped tail sperm abnormalities. However, the mechanism for this impairment (whether direct or indirect) was unclear due to the unknown underlying cause of UTD in the affected dogs. Most of the sperm parameters were not significantly different between the N testes in normal dogs and the contralateral ND testis in UTD dogs. However, it is likely that the overall fertility outcome in dogs with UTD will depend on the progression and severity of TD. Extragonadal (epididymal) sperm reserve have been reported to positively correlate with gonadal sperm reserve and testicular weight (Ajani *et al.*, 2015), and these in addition to testicular volume, were all significantly decreased by TD. Lowered testicular volume has also been associated with oligospermia and subfertility in males (Tijani *et al.*, 2014). Taken together, TD caused decreased testicular and epididymal sperm concentration, decreased epididymal sperm motility, viability and concentration, and increased epididymal sperm abnormalities, and these defects can adversely affect fertility in dogs (Johnston *et al.*, 2001; Robert *et al.*, 2016; Kolster, 2018; Oguejiofor, 2018).

Conclusion

The study demonstrated that UTD in dogs decreased testicular sperm concentration. It also adversely affected epididymal spermatozoal motility, viability, morphology, and sperm concentration in the testis and epididymis. Moreover, there was evidence of compromised spermatozoa (decreased sperm motility and increased sperm abnormalities) in the epididymis contralateral to the degenerated testis. These findings provide more information on alterations in sperm quality in relation to UTD. Although sperm cells may be recovered from the cauda epididymis of affected dogs for application in assisted reproductive technologies, further studies could investigate the functional capacity and fertilizing potential of such recovered spermatozoa.

Conflict of interest

The authors declare that there is no conflict of interest.

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