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Serum testosterone, sperm quality, cytological, physicochemical and biochemical characteristics of the prostatic fraction of dogs with prostatomegaly

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Contents

Prostatomegaly is a common finding in older non-neutered dogs. This study compared the serum testosterone, sperm quality and characteristics of the prostatic fraction between healthy dogs and dogs with prostatomegaly. Blood samples of ten dogs (five dogs from each group) were taken for serum testosterone measurement. Sperm motility, vigour, concentration, viability, membrane functionality and morphology were analysed in sperm-rich fraction. Osmolality, pH, cell types, and albumin, haemoglobin, acid phosphatase, alkaline phosphatase, glucose, triglycerides, cholesterol, calcium, phosphorus, magnesium and chloride were analysed in prostatic fraction. Dogs with prostatomegaly have the lowest sperm motility, vigour, concentration and functional membrane. Dogs with prostatomegaly have the highest glucose, triglycerides and cholesterol. Glucose was the only constituent positively correlated with serum testosterone and prostate volume. It can be concluded that dogs with prostatomegaly have poorer sperm quality, and glucose, triglycerides and cholesterol in prostatic fraction can be used as prostatomegaly biomarkers.

1 | INTRODUCTION

Prostate disorders are common in non-neutered, older dogs and often cause prostatomegaly (Johnston, Kamolpatana, Root-Kustritz, & Johnston, 2000). Among the prostate diseases, benign prostatic hyperplasia (BPH) is the most frequent (Polisca, Troisi, Fontaine, Menchetti, & Fontbonne, 2016). Although prostate biopsy is considered the most suitable method for the specific diagnosis of the canine prostate diseases (Motheo et al., 2014), there are limitations and complications as the older dogs sedation, haemorrhage (Kustritz, 2006), peritonitis or spread of neoplastic cells (Smith, 2008). Thus, more research is needed to make possible the use of techniques less invasive, rapid, inexpensive and efficient for an early diagnosis of prostatic diseases.

In dog, prostate is the only accessory sex gland, its fluid constitutes approximately 95% of the ejaculate (Iguer-Ouada & Verstegen, 2001), and it is possible that prostate diseases can cause significant biochemical changes in the seminal plasma. However, few studies quantified the biochemical constituents in seminal plasma of healthy dogs (England & Allen, 1990; Mann & L-Mann, 1981) or dogs with prostatic diseases (Motheo et al., 2014). The biochemical assessment of the canine prostatic fluid may help to identify possible biomarkers of prostatic diseases and may provide important benefits for human medicine (Aquino-Cortez, Silva, Araújo, Menezes, & Moura, 2016), as the dog is used as an experimental model for the study of prostatic diseases in men (Leoci, Aiudi, Silvestre, Lissner, & Lacalandra, 2014).

This study aimed to compare the serum testosterone, sperm quality, cytological, physicochemical and biochemical characteristics of the prostatic fraction of ejaculate of healthy dogs (HD) and dogs with prostatomegaly (DP).

2 | MATERIAL AND METHODS

2.1 | Animals and experimental groups

Ten male dogs were used (five Rottweiler, two German Shepherd, two Labrador Retriever and one Mongrel), with age of 5.0 ± 2.2 years and weight of 34.1 ± 6.7 kg. The Ethics Committee for Animal Use of the State University of Ceará approved this research (protocol number: 6591178/2014). The prostate volume of dogs was estimated by the formula: volume (cm^3) = ([largest craniocaudal diameter X transverse diameter X dorsoventral diameter]/2.6) + 1.8 (Kamolpatana, Johnston, & Johnston, 2000) by B-mode ultrasonography (SonoAce Pico–Medison®—multifrequency linear probe of 5-9 MHz). HD group (n = 5) had no haemospermia and the following prostatic characteristics: volume less than 28.2 cm^3 (Atalan, Holt, & Barr, 1999), moderately hypoechoic, regular to uniform parenchyma and homogeneous echotexture (Russo, Vignoli, & England, 2012), bilobed form in the transverse plane and oval form in the longitudinal plan (Davidson & Baker, 2009). Dogs that had prostate volume greater than 28.2 cm^3, changes in prostate form, echogenicity and/or texture, and haemospermia (Zambelli, Cunto, & Gentilini, 2012) were assigned to the DP group (n = 5).

2.2 | Serum testosterone

Three venous blood samples (3 ml) of each dog were collected in the morning (30 min intervals), by venipuncture of the jugular vein. Blood samples was transferred to tubes without anticoagulant, centrifuged at 1840 *g* for 20 min (Centribio/Daikki 80-2B®), and the serum was frozen at −20°C. For analysis of serum testosterone, it was used the liquid phase radioimmunoassay (RIA) and a commercial kit (ImmuChem™ Double Antibody Testosterone RIA-MP Biomedicals) in a Wizard device detector (PerkinElmer of Brazil Ltda). The three blood samples from each animal were evaluated in duplicate in a single assay. The intra-assay coefficient was 10.21%. All concentrations determined were between the minimum and maximum points of the curve.

2.3 | Sperm quality

Three ejaculates of each dog were collected by digital manipulation (7 days intervals), and was separated in pre-sperm fraction, sperm-rich fraction (SRF) and prostatic fraction (PF). Analyses of sperm quality in SRF were performed immediately after collection by optical microscopy (microscope Physis®). Sperm total motility (%) and vigour (0–5 scale) were evaluated subjectively in 10 μl of SRF (100×). Bromophenol Blue stained (5 μl) was mixed with 5 μl of SRF to analyse the percentage (%) of viable sperm (400×). A 10 μl of SRF was diluted in 150 μl Rose-Bengal stained to evaluate the sperm morphology (1000×) and classified into sperm with primary or secondary defects (Christiansen, 1984). A 10 μl of SRF was diluted in 90 μl distilled water to evaluate the sperm membrane functionality (400×). A 5 μl of SRF was diluted in 1 ml of 1% saline-formal solution to determine the sperm concentration in Neubauer chamber (400×).

2.4 | Cytological evaluation of the PF

Immediately after semen analysed, 1 μl of protease inhibitor cocktail (Sigma-Aldrich P8340) was added to 1 ml of PF (Martins et al., 2013) and was centrifuged at 1840 *g* for 20 min (Centribio/Daikki 80-2B®). The clean supernatant was transferred to clean tubes and stored at −20°C until physicochemical and biochemical evaluation.

The precipitate was used to observe the cell types in the PF (Aquino-Cortez, Cortez, Cardoso, & da Silva, 2003). The smears were prepared with the aid of a cotton swab, and for staining of cells, the slides were submerged in 0.1% triarylmethane solution (10 s), in 0.1% xanthene solution (10 s) and in 0.1% thiazine solution (20 s—Diff Quick stain, Laborclin®). The slides were washed with distilled water, dried and evaluated in light microscopy (Microscope Nikon Eclipse Nis, Software Nis 4.0®; 1000×). Rounded cells with centralized and well-defined nucleus, with large amount of cytoplasm and rounded shape, with the presence of granules were classified as normal prostate cells. Cells with irregular shape were classified as squamous keratinized cells, nucleated or anucleated, when their nuclei have not been observed (Aquino-Cortez et al., 2003).

2.5 | Physicochemical and biochemical evaluation of the PF

An aliquot of 2 ml PF was thawed at room temperature (25°C) for 2 min for pH measurement by pH meter mPA 2010 (MS Tecnopon®) and for evaluation of osmolality by Osmometer PZL-1000 (PZL Technology®). The concentration of biochemical constituent of PF was determined by photocolorimetry (Spectrophotometer digital Coleman® 35D), as previously described (Aquino-Cortez et al., 2014).

For albumin (ALB) measurement (ref. 19, Labtest®), 0.01 ml PF was added to 1 ml of colour reagent (bromocresol green and Brij 35), homogenized and after 2 min, and determined its absorbance (630 nm). For haemoglobin (HB) measurement (ref. 43, Labtest®), 5 ml of colour reagent (potassium ferricyanide, potassium cyanide diluted in distilled water) was added to 0.02 ml PF, was homogenized and incubated (25°C/5 min), and the absorbance was determined (540 nm).

For determination of prostatic acid phosphatase (AcP) (ref. 39, Labtest®), 0.5 ml of substrate (thymolphthalein monophosphate, surfactant buffer and sodium azide) was incubated (37°C/2 min), 0.1 ml PF was added to the substrate, incubated (37°C/30 min). Then, 2 ml of reagent (sodium carbonate, sodium hydroxide and stabilizer) was added, and the solution was stirred for reading (590 nm). For determination of alkaline phosphatase (AP) (ref. 40, Labtest®), 0.05 ml of substrate (thymolphthalein monophosphate) was incubated (37°C/2 min), 0.05 ml PF was added and incubated (37°C/10 min). Then, 2 ml of colour reagent (sodium carbonate and sodium hydroxide) was added and homogenized for determination of absorbance (590 nm).

For glucose (GLU) determination (ref. 133, Labtest®), 0.01 ml PF was added to 1 ml of solution (phosphate buffer, glucose oxidase, peroxidase, 4-aminoantipyrine, phenol, sodium azide and surfactants), intensely homogenized and incubated (37°C/10 min), for determination of absorbance (505 nm). For triglycerides (TG) determination (ref. 87,

Labtest®), 0.01 ml PF was added to 1 ml of reagent (magnesium acetate, 4-chlorophenol, 4-aminoantipyrine, ATP, lipoprotein lipase, glycerolkinase and sodium azide), homogenized and incubated (37°C/10 min), for determination of absorbance (505 nm). For cholesterol (CHO) measurement (ref. 76, Labtest®), 0.01 ml PF was diluted in 1 ml of reagent (phenol, sodium cholate, sodium azide, 4-aminoantipyrine, cholesterol esterase, cholesterol oxidase and peroxidase), homogenized and incubated (37°C/10 min), for determination of absorbance (500 nm).

For calcium (Ca) determination (ref. 90, Labtest®), 0.02 ml PF was added to 1 ml of reagent (o-cresolphthalein complexone, 8-hydroxyquinoline and hydrochloric acid) and homogenized, and the absorbance was read (570 nm). For phosphorus (P) determination (ref. 42, Labtest®), 0.10 ml of PF was diluted in 2.5 ml of distilled water, and it was added one drop of catalyst (polyvinylpyrrolidone and hydroxylamine hydrochloride). The solution was homogenized, one drop of reagent (ammonium molybdate and sulphuric acid) was added, agitated and incubated (20–25°C/3 min). Then, two drops of buffer solution (sodium carbonate and sodium hydroxide) were added, agitated and incubated again (20–25°C/5 min) to be determined its absorbance (650 nm). For magnesium (Mg) determination (ref. 50, Labtest®), 0.02 ml PF was added to 2 ml of reagent (tris-hydroxymethyl aminomethane, potassium carbonate, sodium azide and sulphonated magon), homogenized and maintained at room temperature (20–25°C/2 min), and absorbance was measured (505 nm). For chloride (Cl) determination (ref. 115, Labtest®), 0.01 ml PF was diluted in 1 ml of reagent (mercury thiocyanate, mercuric chloride, ferric nitrate, nitric acid and stabilizer), homogenized and remained at room temperature (20–25°C/2 min), and absorbance was determined (450 nm).

2.6 | Statistical analysis

Data were expressed as mean and standard deviation and analysed using the statistical software Graphpad PRISM® version 5.01 (GraphPad Software Inc., San Diego, CA, USA). All data were tested for normality using Shapiro–Wilk test. The results of diameters of the prostate, prostate volume, serum testosterone, pH, osmolality, HB, Ca and Mg were considered within the normality curve, and the comparison between the groups was performed through the unpaired *t* test (parametric test). The results of sperm motility, viable sperm, sperm with functional membrane and sperm morphology were transformed into arcsine and then subjected to the unpaired *t* test. The results of cells types, vigour, sperm concentration, ALB, AcP, AP, GLU, TG, P and Cl were considered outside the normality curve, and the comparison between groups was performed using the Whitney–Mann test (nonparametric test). CHO was considered outside the normality curve too, but the groups were compared using the Wilcoxon test (nonparametric tests). The correlations between all characteristics of the PF, prostate volume and serum testosterone were evaluated. Therefore, when the two parameters evaluated were within the normal curve, the Pearson test was used, but when at least one of these parameters was located outside the normal curve, the Spearman test was chosen.

3 | RESULTS

The prostate volume of DP was significantly higher than that in HD; however, serum testosterone was similar between the two groups (Table 1). No significant correlations were observed between the serum testosterone and prostate volume ($r = .18$, $p > .05$). DP had the

TABLE 1 Parameters (mean ± SD) of prostate, serum testosterone, sperm quality and characteristics of the prostatic fraction healthy dogs (HD) and dogs with prostatomegaly (DP)

Parameters	HD	DP
Prostatic craniocaudal diameter (cm)	3.5 ± 1.0[a]	5.0 ± 0.6[b]
Prostatic transverse diameter (cm)	3.9 ± 0.8[a]	5.6 ± 0.6[b]
Prostatic dorsoventral diameter (cm)	3.0 ± 0.6[a]	4.4 ± 0.6[b]
Prostatic volume (cm^3)	18.3 ± 7.5[a]	50.9 ± 16.3[b]
Serum testosterone (ng/ml)	0.5 ± 0.7[a]	0.9 ± 0.5[a]
Total motility (%)	98.33 ± 3.01[a]	83.33 ± 17.99[b]
Vigour (0–5 scale)	4.93 ± 0.26[a]	4.07 ± 1.03[b]
Sperm concentration ($\times 10^6$/ml)	718.00 ± 467.00[a]	254.13 ± 151.61[b]
Viable sperm (%)	91.40 ± 7.11[a]	87.87 ± 9.80[a]
Functional sperm membrane (%)	93.13 ± 4.52[a]	84.80 ± 11.11[b]
Primary sperm defects (%)	0.60 ± 0.99[a]	1.47 ± 1.36[b]
Secondary sperm defects (%)	17.00 ± 13.11[a]	25.33 ± 16.75[a]
Morphologically normal sperm (%)	82.40 ± 12.94[a]	73.20 ± 16.80[a]
Normal prostatic cells	0.67 ± 1.11[a]	1.20 ± 2.91[a]
Nucleated squamous keratinized cells	2.40 ± 2.67[a]	2.53 ± 3.34[a]
Anucleate squamous keratinized cells	6.60 ± 5.26[a]	18.73 ± 22.78[a]
Total prostatic cells	9.67 ± 7.31[a]	22.47 ± 24.71[a]
pH	6.28 ± 0.30[a]	6.31 ± 0.15[a]
Osmolarity (Osm/kg)	315.29 ± 15.69[a]	307.80 ± 19.46[a]
ALB (g/dl)	0.05 ± 0.04[a]	0.07 ± 0.08[a]
HB (g/dl)	0.49 ± 0.32[a]	0.65 ± 0.43[a]
AcP (U/L)	33.08 ± 0.85[a]	33.29 ± 0.15[a]
AP (U/L)	114.59 ± 82.38[a]	83.77 ± 71.51[a]
GLU (mg/dl)	10.73 ± 12.12[a]	33.36 ± 2.88[b]
TG (mg/dl)	34.22 ± 60.74[a]	115.02 ± 25.32[b]
CHO (mg/dl)	0.00 ± 0.00[a]	47.14 ± 32.33[b]
Ca (mg/dl)	3.39 ± 0.60[a]	3.85 ± 0.56[a]
P (mg/dl)	9.14 ± 18.06[a]	4.78 ± 8.19[a]
MG (mg/dl)	2.95 ± 0.54[a]	2.72 ± 0.81[a]
Cl (meq/L)	174.71 ± 19.03[a]	180.06 ± 16.11[a]

Different letters in the same line represent differences between groups ($p < .05$).

lowest sperm motility, vigour, concentration and percentage of sperm with functional membrane. The percentage of sperm with primary morphological changes was greater in the DP (Table 1).

Cytological evaluation of the PF showed the presence of normal prostatic cells, nucleated and anucleate squamous keratinized cells and red blood cells and sperm in both groups (Figure 1), these cell types did not differ between groups (Table 1) and were not correlated with prostate volume or serum testosterone ($p > .05$).

GLU was the only one correlated significantly with serum testosterone ($r = .70$; $p < .05$) and prostate volume ($r = .68$, $p < .05$). GLU, TG and CHO were highest in PF of DP ($p < .05$). The other physicochemical and biochemical characteristics of the PF did not differ between the groups (Table 1).

4 | DISCUSSION

Abdominal ultrasound has been routinely used to diagnose prostate diseases (Flores et al., 2016; Motheo et al., 2014; Polisca et al., 2016), and the haemospermia is a common clinical signs in animals with prostate diseases, especially in cases of BPH (Smith, 2008; Zambelli et al., 2012). These two analyses allowed the adequate allocation of dogs in the experimental groups.

Some sperm parameters were different between the two groups, and the haemospermia may be the cause of significant reduction in sperm quality (England & Allen, 1992) in DP. Besides, prostate disorders evolve over a long period in its subclinical form, the prostate diseases may go unnoticed in most dogs and affect fertility (Polisca et al., 2016). Dogs with BPH present higher percentage of minor sperm defects, amplitude of lateral sperm head displacement, medium sperm mitochondrial activity and lower sperm DNA integrity (Flores et al., 2016). In this sense, the evaluation of PF should also be indicated in cases of infertility in an intact male dog and with clinical signs of prostate diseases (Kustritz, 2006).

The cytological evaluation is a valuable technique for the diagnosis of prostatic diseases (Kraft, Brown, & Leroy, 2004), however, the characteristics of these cells types are scarce. Prostatic fluid is hypocellular, and cytological interpretation is complicated by the dilution of all cell types, requiring centrifugation of the sample and the examination of the precipitate (Kustritz, 2006). Occasional red blood cells, white blood cells and squamous epithelial cells are present in the PF of the dog ejaculate (Freshman, 2002). These cytological characteristics are similar in normal men (Smith, 2008). In the present study, there was a greater number of anucleate keratinized cells in both groups, confirm the findings of Aquino-Cortez et al. (2003), who observed the presence of cells with irregular shape, asymmetric edges, absent or very reduced size nuclei, and the presence of cells with rounded shape, symmetrical and normal-sized nuclei in the PF of healthy dogs.

Although canine prostatic growth can be correlated to testosterone (de Souza & Martins, 2005), the influence of testosterone on the aetiology of prostatomegaly remains unclear. Despite the highest level of testosterone was registered in prostate fluid of dogs with enlarged prostate (Wolf et al., 2012), our study demonstrated that the serum testosterone did not differ between the two groups studied, and no correlations with prostate volume.

In dogs, although the semen is fractionated during the collection, PF enters the bitch uterus during natural mating (England & Allen, 1992) and plays a key role in regulating fertility in dogs (England et al., 2012, 2013). These results demonstrate that the increase in prostate volume did not affect the pH or the osmolality of PF, and these two parameters were within the normal range for the species (Aquino-Cortez et al., 2003; Mann & L-Mann, 1981; Wales & White, 1965).

Despite the haemospermia observed in the PF of DP, ALB and HB were similar between the two groups. The ALB present in seminal plasma acts together with antioxidant enzymes, preventing the formation of reactive oxygen species (ROS) (González-Cadavid et al., 2014). In humans, ALB is positively correlated with prostate markers, such as prostate specific antigen and zinc ($r = .1$ with $p = .001$ and $r = .2$ with $p < .001$, respectively) (Elzanaty, Erenpreiss, & Becker, 2007). HB present in seminal plasma derives from the lysis of red blood cells and has a deleterious effect on canine sperm, causing significant reduction in sperm motility (Rijsselaere, Van Soom, Maes, Verberckmoes, & de Kruif, 2004).

The AcP and AP did not differ between groups. AP is used as a biomarker for the diagnosis of azoospermia (Schäfer-Somi, Kluger, Knapp, Klein, & Aurich, 2006), bilateral occlusion of the vas deferens

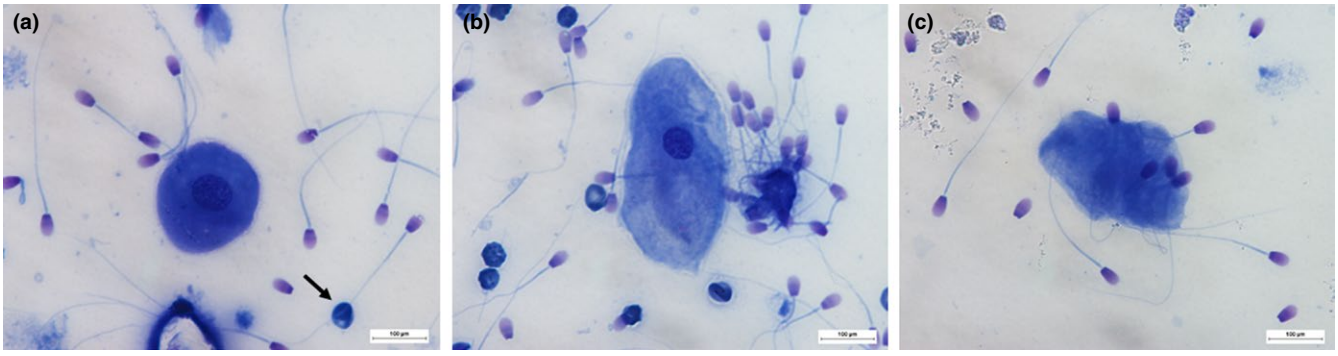

FIGURE 1 The normal prostatic cell (a), nucleated squamous keratinized cells (b) anucleated squamous keratinized cell (c), spermatozoa and red blood cells (arrow) observed in the cytological evaluation of prostatic fraction from dogs with prostatomegaly (Diff Quick stain, Microscope Nikon Eclipse Nis, Nis Software 4.0®. 1,000×)

or epididymis in dogs (Gobello, Castex, & Corrada, 2002), but according to our results, this enzyme would not be indicated for the diagnosis of prostatic diseases. In relation to AcP, this work corroborates that described by Gadelha et al. (2013), who found no correlation between the level of AcP and the prostate size of the animals.

The GLU in PF of DP was three times higher than the in the HD. GLU was the only biochemical constituent of PF which showed a positive correlation with prostate volume and serum testosterone; and it may have some indirect relationship with the lowest sperm quality observed in DP. Studies in patients with diabetes show that glucose metabolism triggers a deleterious effect on motility and integrity of sperm DNA, and this effect would be associated with the production of ROS in seminal plasma (Ding et al., 2015).

In humans, the CHO level in the tissue of increased prostate was higher than in normal prostate (Swyer, 1942), demonstrating its importance in the diagnosis and evaluation of the pathophysiology of accessory sexual glands (Eliasson, 1966). Our work was the first study that demonstrated the highest level of TG and CHO in PF of dogs with prostatomegaly and suggested their quantification in canine seminal plasma as biomarkers of prostate diseases in dogs. CHO in the seminal plasma has been used as a biomarker of sperm quality (Beer-Ljubić et al., 2009). In dogs, a lower concentration of CHO was observed in the seminal plasma of dogs with bad quality after thawing (Schäfer-Somi & Palme, 2016). However, a higher level of CHO in the PF of animals had lower sperm quality in our work. Thus, further in-depth studies are needed to prove this negative action in DP.

The ions in the seminal plasma have shown a role in sperm physiology and pH adjustment of the medium (Hamamah & Gatti, 1998). Ca is involved in the acrosome reaction (Ward & Kopf, 1993) and inhibits the sperm motility (Hong, Chiang, & Turner, 1984). P is related in cell energy metabolism and in activation of enzymes, and Mg is a cofactor in more than 300 enzymatic reactions involved in energy metabolism, spermatogenesis and influences sperm motility (Wong et al., 2001). Although Cl is the ion with higher concentration in seminal plasma (Khan, Yasinzai, & Kakar, 2015), its function on sperm is little studied, and apparently it does not affect sperm quality (de Souza, Martins, & Lopes, 2006). Despite the variations in ionic composition of the seminal plasma (Mann & L-Mann, 1981), the levels of ions in the PF in both groups tested in our work were similar to those reported previously (Aquino-Cortez et al., 2003; Wales & White, 1965) and were not influenced by prostatic enlargement.

DP had lower sperm quality than HD, and they should be carefully evaluated before being included in breeding programs. However, serum testosterone and pH, osmolality and cell types of prostatic fraction were similar between the two groups. The GLU, TG and CHO in the canine PF can be used as biomarkers of prostatic enlargement in dogs. It is believed that biochemical constituents of PF of dog can interfere with fertility of dogs with prostate diseases, but further studies are required to prove such an influence.

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CONFLICT OF INTEREST

The authors have declared that no competing interests exist. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the manuscript.

AUTHOR CONTRIBUTIONS

A Aquino-Cortez and LDM Silva gave substantial contributions to conception and design of the study, for acquisition, analysis and interpretation of data; they drafted the article. BQ Pinheiro, HVR Silva and DBC Lima were responsible for acquisition, analysis and interpretation of sperm quality data. TFP Silva and MB Souza were responsible for acquisition, analysis and interpretation of B-mode ultrasonographic prostatic data. DA Viana, FAF Xavier Júnior and JSAM Evangelista were responsible for analysis and interpretation of data of cytological evaluation of the PF. FZ Brandão was responsible for analysis and interpretation of data of serum testosterone. All authors reviewed it critically for intellectual content and final approval of the version to be published.

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