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Use of grayscale histogram to assess testicular changes in ram caused by heat stress

[*Uso de histograma em escala de cinza para avaliar alterações testiculares em carneiros causadas por estresse térmico*]

"Scientific Article/Artigo Científico"

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Abstract
Because it detects discrete changes in tissue density, ultrasound allows an early diagnosis of physiologic changes, especially when dealing with sub-clinical changes. The study aimed to evaluate the use of quantitative analysis of the grayscale of ultrasound images of ovine testicles as a method for early diagnosis of testicular changes caused by heat stress. Testicles from ten rams were insulated and then evaluated regarding echogenicity, echotexture, anatomical measurements, and seminal characteristics. Echogenicity was the first variable to show changes during the insulation period, as well as the first one to show evidence of regeneration of those changes. There was a correlation ($p < 0.05$) between echogenicity and sperm vigor, sperm motility, and mass sperm motility, as well as a correlation ($p < 0.05$) between echotexture and motility. Echogenicity, sperm motility, vigor, and mass sperm motility decreased ($p < 0.05$) within four days of testicular insulation, and there was no change ($p > 0.05$) in echotexture. Testicular measurements were only altered ($p < 0.05$) after insulation. Echogenicity is an efficient ultrasound parameter for the early diagnosis of a testicular degenerative process as well as for the early diagnosis of its regeneration.

Keywords: ovine; reproduction; semen; testicle.

Resumo
Por detectar discretas alterações na densidade tecidual, o ultrassom permite o diagnóstico precoce de alterações fisiológicas, principalmente quando se trata de alterações subclínicas. Objetivou-se avaliar o uso da análise quantitativa da escala de cinza de imagens ultrassonográficas de testículos de ovinos como método para diagnóstico precoce de alterações testiculares causadas por estresse térmico. Testículos de dez carneiros foram insulados e avaliados quanto à ecogenicidade, ecotextura, medidas anatômicas e características seminais. A ecogenicidade foi a primeira variável a apresentar alterações durante o período de insulação, bem como a primeira a evidenciar a regeneração dessas alterações. Houve correlação ($p < 0,05$) entre ecogenicidade e vigor espermático, motilidade espermática e motilidade espermática em massa, bem como correlação ($p < 0,05$) entre ecotextura e motilidade. Ecogenicidade, motilidade, vigor e motilidade espermática em massa diminuíram ($p < 0,05$) dentro de quatro dias de insulação testicular, e não houve alteração ($p > 0,05$) na ecotextura. As medidas testiculares só foram alteradas ($p < 0,05$) após a insulação. A ecogenicidade é um parâmetro ultrassonográfico eficiente para o diagnóstico precoce de um processo degenerativo testicular, bem como para o diagnóstico precoce de sua regeneração.

Palavras-chave: ovinos; reprodução; sêmen; testículo.

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Introduction

Testicular temperature in mammals is regulated via thermoregulation and is maintained between 2 and 6°C below body temperature to ensure the production of viable spermatozoa (Durairajanayagam et al., 2015). Elevations in testicular temperature may occur due to the elevation in ambient temperature, local or systemic infections, or even because of failure of the thermoregulation system (Kahwage et al., 2018).

Regarding ambient thermal stress, animals exposed to high temperatures have their fertility disrupted because of the difficulty in or impediment to dissipating heat from the scrotal sac (Moreira et al., 2001). Scrotal insulation, despite increasing the temperature and the metabolism of the testicles, does not increase blood flow, causing hypoxia of these gonads (Setchell, 1998). Fragmentation of DNA and cell apoptosis resulting from this testicular dysfunction (Hamilton et al., 2018), lead to degenerative changes in the germinal epithelium, which interfere in the formation of spermatozoa according to the extent of the lesion (Setchell, 1998).

Because it detects discrete changes in tissue density (Chandolia et al., 1997), ultrasound allows an early diagnosis of physiologic changes (Bastos et al., 2018), especially when dealing with subclinical changes (Belay et al., 2016). It also minimizes the impact from testicular disorders, thus confirming its importance for andrology (Lotti and Maggi, 2015). Ultrasound images are made up of a pixel matrix which represents the density of the tissue shown in a variety of shades of gray (Pierson and Adams, 1995) and may reflect morphological and physiological changes based on the resulting grayscale. As such, the present objective was to evaluate the quantitative analysis of the grayscale of ultrasound images of ovine testicles as an early method to determine changes following thermal stress.

Materials and Methods

The experiment was performed at the Department of Veterinary Medicine of the Federal Rural University of Pernambuco, located in the metropolitan mesoregion of Recife. This region has a mean annual temperature of 25°C, rainfall of 2.500 mm, and relative air humidity of 70%.

Ten Santa Inês rams, with ages ranging from 18 to 20 months were used. The animals were placed in individual stalls with access to a solarium and feeding consisted of Tifton hay (*Cynodon* spp.). The mineralized salt and water were available *ad libitum*. Before the start of the experiment, these animals underwent an adaptation period of 20 days to acclimate to the semen collection methods using an artificial vagina, as well as to evaluate their health, sexual maturity, and semen quality, as recommended by Henry et al. (2013).

The experiment was divided into the following periods: pre-insulation (D0-D4), insulation (D5-D12), and post-insulation (D13-D83). In the pre-insulation period, which lasted five days, seminal evaluation, testicular measurements, and acquisition of ultrasound images to observe echogenicity and echotexture were performed to establish normal values. These evaluations were done twice, four days apart, with the last one performed the day prior to placement of the scrotal pouch for testicular insulation. In the insulation phase, the testicles were enveloped an insulation pouch made with a double layer of cloth interspaced with a 5 mm layer of hydrophobic cotton. In this phase, which lasted eight days (Rocha et al., 2015), semen was collected on the fourth day. The post-insulation phase started with the removal of an insulation pouches. In this phase, semen collection, ultrasound evaluation, and testicular measurements were performed every seven days. During these three periods, morphometric parameters for testicular length and width were obtained using a caliper. Data regarding the scrotal perimeter were obtained with a measuring tape and testicular volume using the formula suggested by Camela et al. (2019).

The semen was collected using a female as a mannequin. It was evaluated macroscopically regarding volume, aspect, and color. Microscopically, the semen was evaluated for mass sperm motility (0-5), progressive motility (0-100%), vigor (0-5), and sperm concentration (x109 cells/mL) (Henry et al., 2013). Sperm concentration was determined with the aid of a Neubauer chamber, diluting semen samples (1:400) in formal saline. Morphological analysis of the spermatozoa was done using the moist preparation, in which 200 cells were counted to determine the percentage of spermatozoa with morphological changes (Henry et al., 2013). All microscopic evaluation of semen was always done by the same technician and with the aid of a phase contrast microscope (Nikon Eclipse E200, Melville, USA).

The testicles were evaluated via ultrasound (Mindray®, DP 2200) equipped with a 10MHz linear transducer and was always done by the same technician. After clipping the fur from the posterior region of the scrotal sac, ultrasound images were obtained in the sagittal plane, locating the testicular mediastinum, as well as on the transverse plane. Afterwards, these images were transferred onto a flash drive. Quantitative computer analysis of the echotexture and echogenicity was done via gray level histogram analysis using GIMP® 2.8 software. In each image of the testicles, ten 25-mm diameter regions were selected within the parenchyma, six in the sagittal plane (above and below the mediastinum) and four in the transverse plane. Each selected region of the image was then used to generate a gray level histogram with GIMP, which indicated the mean pixel intensity values (echogenicity) and standard deviation (echotexture). An overall mean of the data was then calculated for each testicle.

Statistical analyses were performed using GraphPad Prism 5.01®. Normality of the data distribution was tested using the Kolmogorov-Smirnov method. Data obtained as a percentage were transformed into arcsine $\sqrt{x(\%)}$ for analysis of the parametric data using ANOVA (variance analysis). Comparison between the means was done using Tukey's test. The relationship between echogenicity, echotexture, vigor, mass sperm motility, motility, concentration, volume, and sperm disorders were evaluated using Pearson's correlation. Non-parametric data were evaluated using Kruskal Wallis and Dunn tests. Significance level was set as 5%.

Results and Discussion

This study was done under the hypothesis that the ultrasound images would be able to provide greater predictability regarding degenerative changes prior to visualization via conventional semen exams. Although the Santa Inês breed is adapted to the tropical weather, we were able to identify testicular changes due to the heat stress. In agreement with other authors (Garcia, 2006; Barros et al., 2015), their results corroborate our findings showing that the increased temperature affect the testes.

Mean values for testicular echogenicity in the pre-insulation period were higher ($p < 0.05$) than those obtained in the insulation and post-insulation periods until the 56th day (Figure 1).

Testicular echogenicity was one of the most sensitive evaluations to heat stress, being able to identify early on a reduction in echogenicity values during insulation, as well as a return to normal values afterwards. The decreased echogenicity detected on day 4 of testicular insulation was contrary to that reported by Mieusset et al. (1992), which only detected the first effects of heat stress on echogenicity after day 15 of scrotal insulation. It is possible that the differences between the present results and those obtained by Mieusset et al. (1992) were the result of interference from ambient temperature on testicle temperature inside the insulation pouch.

However, it is worth noting that sperm cells have been shown to be affected by heat stress, regardless of the post-insulation period. It is also worth highlighting that among sperm cells, spermatozoa and spermatids are more sensitive to heat stress, while spermatogonia are more resilient (Setchell, 1998; Lotti and Maggi, 2015). It is thus possible that this fact contributed to the decrease in echogenicity observed in this study. Also, changes to echogenicity values may result from mitotic activities of the germinative cells for recovery of the lesions. Cellular changes such as mitosis and apoptosis affect tissue echogenicity (Rojas et al., 2017). Likewise, oscillations may occur in the quantitative analysis of echogenicity in testes of prepubertal lambs during the first wave of spermatogenesis due to proliferation of germ cells (Giffin et al., 2014). In the evaluations on day 69, 76, and 83, mean values for this parameter did not differ ($p > 0.05$) from those obtained in the pre-insulation period (Table 1).

There was a decrease ($p < 0.05$) in mean testicular echotexture values between day 55 and day 62 post-isolation (Table 1). The deleterious effects of heat stress on testicular parenchyma are known. According to Rocha et al. (2015), the exposure of animals to high temperatures affects their reproductive efficiency, which is particularly prevalent in tropical areas where the air temperature is many times higher than the thermoregulation capacity.

Changes observed between days 55 and 69 could cause disorganization in the tissue structure with subsequent changes in echotexture. It is also interesting to note that this supposition is supported by Florentino et al. (2003), who reported the loss of integrity of the seminiferous tubules with points of calcification in the parenchyma after a period

greater than 12 days of heat stress in caprine testicles.

There was a correlation ($p < 0.05$) between echogenicity and vigor, as well as between motility and mass sperm motility. A correlation ($p < 0.05$) was also observed between echotexture and motility (Table 2).

The high correlation observed between echogenicity and spermatic parameters related to spermatic motility may be related to anatomical changes of the seminiferous tubules after heat injury.

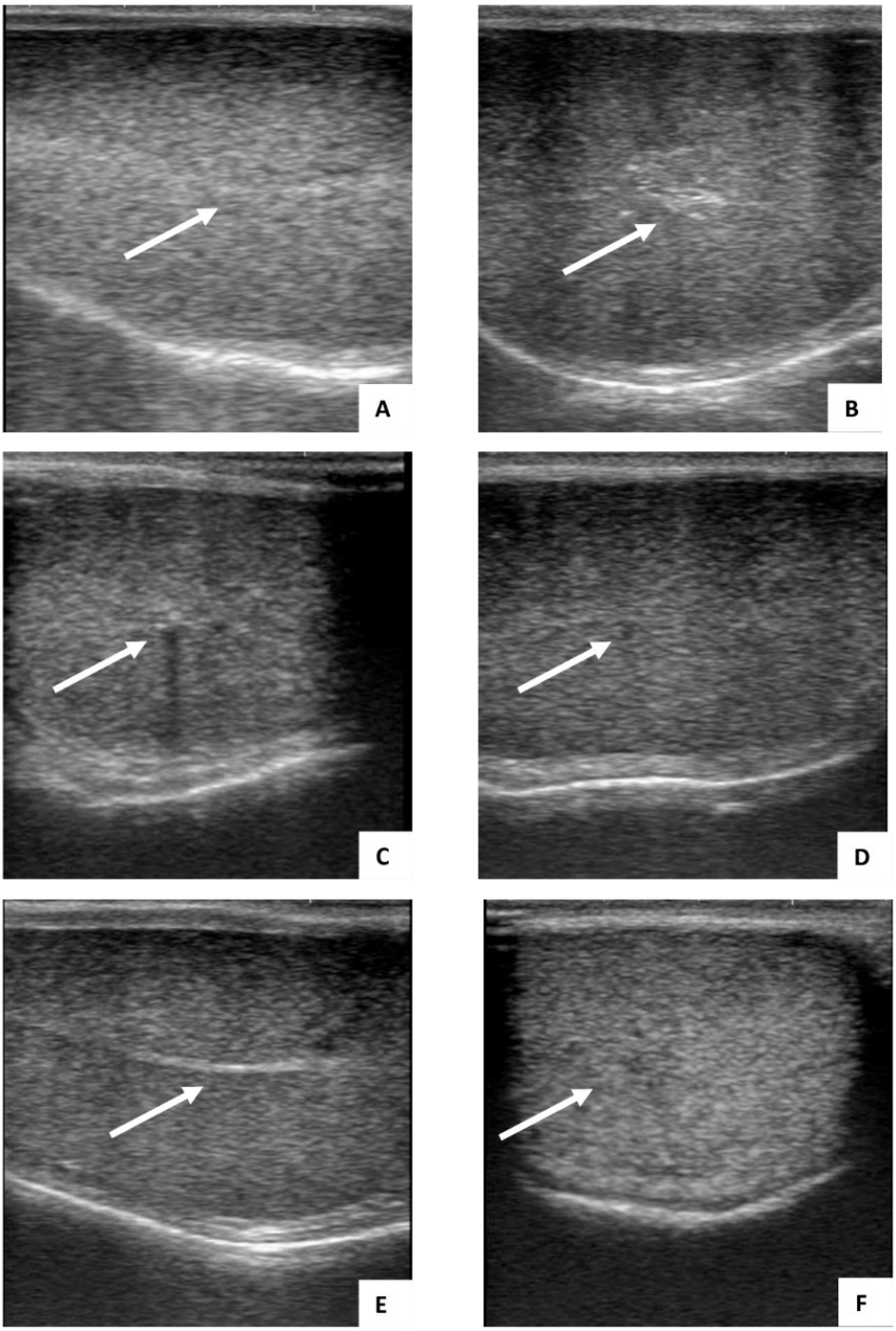

Figure 1. Testicular echogenicity of rams in the pre- and post-insulation period. Arrow: mediastinum; A: onset of heat stress; B: Five days heat stress; C: 10 days heat stress; D: 15 days heat stress; E: 20 days heat stress; F: 25 days heat stress.

Studies evaluating the relationship between echogenicity and changes in the seminiferous tubules related to sexual maturity, showed differences in echogenicity of the testicular parenchyma as the male becomes capable of reproducing (Ayala et al., 2016; Fontoura et al.,

2016). The high correlation between echotexture and motility may also be related to morphological changes in the seminiferous tubules and in the production of fluids by the Sertoli cells originating from the heat stress.

Testicular length and volume were the only parameters that showed a decrease ($p < 0.05$) on the first day of evaluation during the post-insulation period. In this same period, the other testicular parameters for size were decreased ($p < 0.05$) from the second post-insulation evaluation. After day 76 of the post-insulation period, testicular parameters had similar values to those in the pre-insulation period (Table 3).

Table 1. Mean values (± s) for testicular echogenicity and echotexture in rams undergoing scrotal insulation, during pre-insulation (day 0 to 4), insulation (day 5 to 12), and post-insulation (day 13 to 83) periods.

Variables	Day	Echogenicity	Echotexture
Pre-insulation	0	100.0 ± 4.9 [a]	21.6 ± 1.3 [a]
	4	99.7 ± 5.3 [a]	21.4 ± 1.0 [a]
Insulation	8	73.1 ± 14.2 [b]	19.6 ± 1.0 [a]
	13	75.5 ± 12.0 [b]	20.5 ± 1.7 [a]
	20	62.1 ± 7.9 [cb]	19.7 ± 1.5 [a]
	27	67.1 ± 9.1[b]	20.2 ± 1.6 [a]
	34	75.5 ± 7.2 [b]	20.6 ± 1.0 [a]
	41	63.3 ± 7.0 [c]	20.2 ± 1.0 [a]
Post-insulation	48	73.6 ± 10.5 [b]	19.9 ± 1.0 [a]
	55	70.5 ± 12.0 [b]	18.5 ± 1.4 [b]
	62	70.6 ± 13.5 [b]	18.7 ± 1.8 [b]
	69	76.6 ± 7.1 [ab]	20.2 ± 1.6 [b]
	76	88.0 ± 6.1 [a]	22.0 ± 1.0 [a]
	83	91.3 ± 6.9 [a]	22.5 ± 1.6 [a]

Different letters in the same column indicate a difference ($p < 0.05$).

Testicular measurements suffered a negative effect after thermal stress, with a decrease in measurements. This finding agrees with those reported by Moreira et al. (2001) and Florentino et al. (2003) who worked on insulation in ovine and caprine males, respectively. Testicular length and volume were the first parameters to decrease, findings also reported by Moreira et al. (2001). Those authors showed that volume and length measurements are viable indicators of the effect of thermal stress on testicles because they show earlier changes when compared with other testicular measurements. After day 55 of the post-insulation period, testicles recovered their measurements. Scrotal perimeter was the fastest parameter to recover. This may have been a consequence of the scrotal width recovering within the same period and influencing the calculation of the testicular volume.

Semen volume did not vary ($p > 0.05$) between the three experimental periods; however, mean mass sperm motility, vigor, and sperm motility were reduced ($p < 0.05$) in the insulation period. From day 76 forward, the mean values for all evaluated parameters returned to physiologic values (Table 4).

The decrease in mass sperm motility, vigor, and sperm motility with four days of insulation occurred earlier than what was observed by other authors (Santos et al., 1998; Florentino et al., 2003). This is explained by the increase in the number of dead spermatozoa from heat stress. In the same way, these changes occurred earlier than those observed with sperm concentration and testicular measurements (Moreira et al., 2001; Kahwage et al., 2018). Sperm motility, vigor, mass sperm motility, and sperm concentration only returned to physiologic values at the end of the post-insulation period, findings also observed in ovine (Cóser et al., 1979) and in caprine males (Santos and Simplício, 1993). Return of sperm motility to pre-insulation standards can also happen later. This type of finding is probably because an elevated temperature can damage functionality of

the epididymis, which requires more time to be repaired (Moreira et al., 2001).

The decrease in spermatic concentration seen in the first sample of the post-insulation period was also observed by Moreira et al. (2001). This finding can also result from the absorption of spermatozoa with morphological defects in the epididymis (Rao et al., 1980; Sutovsky et al., 2001). The presence of residual bodies in the epidydimal duct, a decrease in spermatozoa, and increase in the number of spermatic defects in the tail of the epididymis was described by Florentino et al. (2003) after six days of scrotal insulation.

The return of spermatic concentration to physiologic levels in the latter third of the scrotal insulation period observed in this study and the one by Florentino et al. (2003) demonstrates that the heat stress causes injury to germinative cells. This return to the prior spermatozoid concentration may be explained by the time needed for the first mitosis of the spermatogonia A until the release of spermatozoa (Cardoso and Queiroz, 1988) associated with the time needed for the transport and maturation of spermatozoa in the epididymis.

Table 2. Correlation efficient between ultrasound findings and semen parameters

Variables	Echog	Echox	Vig	Mot	Con	Vol	MassS	Dis
Echog	1	0.766 *	0.908 *	0.916 *	0.801 *	-0.367	0.906 *	-0.679 *
Echox		1	0.797 *	0.818 *	0.683 *	0.073	0.794 *	-0.509
Vig			1	0.988 *	0.864 *	-0.208	0.982 *	-0.557 *
Mot				1	0.893 *	-0.146	0.997 *	-0.577 *
Con					1	-0.081	0.907 *	-0.670 *
Vol						1	-0.149	0.501
MassS							1	-0.587 *
Dis								1

Echog: echogenicity; Echox: echotexture; Vig: vigor; Mot: sperm motility; Con: concentration; Vol: volume; MassS: mass sperm motility; Dis: diseases *p < 0.05.

Table 3. Mean values (± s) for scrotal measurements (perimeter, length, width, and testicular volume) in rams during the pre-insulation (day 0 to 4), insulation (day 5 to 12), and post-insulation (day 13 to 83) of the testicles

Variables	Day	Scrotal perimeter (cm)	Testicular length (cm)	Testicular width (cm)	Testicular volume (cm^3)
Pre-insulation	0	26.9 ± 2.7 ^{a}	7.5 ± 1.3 ^{a}	4.9 ± 0.7 ^{a}	201.4 ± 77.4 ^{a}
	4	26.6 ± 2.5 ^{a}	7.4 ± 1.2 ^{a}	4.9 ± 0.8 ^{a}	202.4 ± 87.4 ^{a}
Insulation	8	25.4 ± 3.2 ^{a}	7.1 ± 1.1 ^{a}	4.7 ± 0.7 ^{ab}	176.0 ± 73.3 ^{a}
	13	23.3 ± 2.2 ^{ab}	6.5 ± 1.0 ^{b}	4.4 ± 0.6 ^{ab}	140.7 ± 54.6 ^{b}
	20	21.6 ± 1.7 ^{b}	6.1 ± 0.8 ^{c}	4.3 ± 0.5 ^{b}	123.8 ± 42.2 ^{b}
	27	21.4 ± 1.0 ^{b}	6.1 ± 0.9 ^{c}	4.0 ± 0.5 ^{bc}	110.0 ± 39.1 ^{b}
	34	21.3 ± 1.1 ^{b}	6.1 ± 0.9 ^{c}	4.0 ± 0.6 ^{cd}	105.0 ± 39.6 ^{b}
Post-insulation	41	23.0 ± 2.3 ^{b}	6.1 ± 0.7 ^{c}	4.1 ± 0.5 ^{bcd}	113.1 ± 41.4 ^{b}
	48	23.3 ± 2.5 ^{b}	6.1 ± 0.6 ^{c}	4.2 ± 0.7 ^{bcd}	115.5 ± 33.7 ^{b}
	55	24.1 ± 2.6 ^{ab}	6.1 ± 0.4 ^{bc}	4.4 ± 0.8 ^{abcd}	129.8 ± 56.2 ^{b}
	62	24.7 ± 2.8 ^{ab}	6.5 ± 0.6 ^{b}	4.6 ± 0.9 ^{ab}	156.1 ± 62.2 ^{ab}
	69	25.7 ± 2.8 ^{a}	6.5 ± 0.7 ^{b}	4.8 ± 0.8 ^{ab}	163.0 ± 62.0 ^{ab}
	76	26.2 ± 2.8 ^{a}	6.8 ± 0.6 ^{ab}	5.0 ± 0.7 ^{a}	186.5 ± 62.7 ^{a}
	83	26.3 ± 2.5 ^{a}	6.8 ± 0.6 ^{a}	5.0 ± 0.7 ^{a}	190.4 ± 62.8 ^{a}

Different letters in the same column indicate a difference (p < 0.05).

Table 4. Mean values (±s) for parameters related to seminal and spermatic analyses in rams during pre-insulation (day 0 to 4), insulation (day 5 to 12), and post-insulation (day 13 to 83) of the testicles.

Variables	Day	Vol (mL)	MassS (0-5)	Vig (0-5)	Mot (%)	Conc (x10^9/mL)	T Defects (%)
Pre-insulation	0	1.1±0.2 ^{a}	4.6±0.5 ^{a}	4.3±0.8 ^{a}	83.33±5.2 ^{a}	3.2±0.5 ^{a}	11.2±2.0 ^{a}
	4	1.1±0.2 ^{a}	4.3±0.5 ^{a}	4.5±0.6 ^{a}	81.7±7.5 ^{a}	3.1±0.7 ^{a}	12.0±2.0 ^{a}
Insulation	8	1.2±0.3 ^{a}	2.3±0.5 ^{b}	1.8±0.7 ^{b}	35.0±20.7 ^{b}	3.3±0.8 ^{a}	13.3±3.0 ^{a}
Post-insulation	13	1.2±0.2 ^{a}	0.0 ^{c}	0.3±0.5 ^{c}	3.3±5.2 ^{c}	1.7±0.4 ^{b}	22.0±3.8 ^{b}
	20	1.2±0.1 ^{a}	0.0 ^{c}	0 ^{d}	0.0 ^{d}	0.1±0.1 ^{c}	52.7±8.5 ^{c}
	27	1.3±0.1 ^{a}	0.0 ^{c}	0 ^{d}	0.0 ^{d}	0.1±0.1 ^{c}	68.8±6.8 ^{d}
	34	1.1±0.2 ^{a}	0 *	0 *	0 *	0 *	0 *
	41	1.3±0.3 ^{a}	0.0 ^{c}	0.0 ^{d}	0.0 ^{d}	0.1±0.1 ^{c}	52.4±17.7 ^{c}
	48	1.2±0.2 ^{a}	0.0 ^{c}	0.0 ^{d}	0.0 ^{d}	0.1±0.1 ^{c}	51.0±5.6 ^{c}
	55	1.2±0.3 ^{a}	0.0 ^{c}	0.0 ^{d}	0.0 ^{d}	0.1±0.1 ^{c}	44.0±5.1 ^{c}
	62	1.1±0.2 ^{a}	0.0 ^{c}	0.8±0.7 ^{c}	0.7±0.8 ^{e}	0.1±0.1 ^{c}	42.4±9.8 ^{c}
	69	1.1±0.2 ^{a}	1.3±1.4 ^{b}	2.2±1.5 ^{b}	25.0±22.6 ^{b}	0.7±0.4 ^{c}	38.8±9.7 ^{c}
	76	1.2±0.2 ^{a}	4.0±1.0 ^{a}	4.3±0.8 ^{a}	78.3±14.7 ^{a}	2.7±1.2 ^{a}	24.3±4.3 ^{b}
	83	1.3±0.2 ^{a}	4.3±0.7 ^{a}	4.5±0.6 ^{a}	81.7±7.6 ^{a}	3.1±0.7 ^{a}	20.3±5.0 ^{b}

Vol: Volume; MassS: mass sperm motility; Vig: vigor; Mot: sperm motility; Con: concentration; T Defects: total defects. Different letters in the same column indicate a difference ($p < 0.05$). *Azoospermia

Scrotal insulation has a deleterious effect on the quality of the spermatozoid. The severity of the defects is related to the duration of the thermal stress (Moreira et al., 2001). The increase in spermatic issues right after the removal of the scrotal insulation pouch occurred similarly to what was described by Moreira et al. (2001). Those authors, as well as Garcia-Oliveros et al. (2020), stated that there is a relationship between the time of the heat stress and the appearance of spermatic defects. In the same way, Florentino et al. (2003) and Kahwage et al. (2018) observed that an insulation period greater than six days resulted in a decrease in quantity, not only of the spermatids, but also of spermatogonias and Sertoli cells. They also observed that the seminiferous tubules had the most destruction of the germinative epithelium, vacuolization areas, and increase in the number of multinucleated cells, aspects which hindered spermatozoid formation. The reports from those authors support the statement that these changes can justify the quantity of spermatic changes observed in this work during the 70 days of post-insulation period.

The absence of changes to semen volume after scrotal insulation is justified by the fact that the greatest contribution to volume of the ejaculate comes from the accessory glands of the male reproductive system (Moule and Waites, 1963).

Conclusion

It is concluded that echogenicity is an important ultrasound parameter for the early diagnosis of a degenerative testicular process after heat stress, as well as for showing early signs of its regeneration. For this reason, it is acceptable to suggest ultrasound as a supplemental tool in an andrological examination.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this article.

Ethics Committee

This work was performed in accordance with the regulations of the Ethics Committee in the Use of Animals (CEUA) of the Federal Rural University of Pernambuco (UFRPE), under protocol 112/2015

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