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pa1gocag@lucano.uco.es
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Zangeronimo, M.G.; Silva, D.M.; Murgas, L.D.S.; Sousa, R.V.; Rocha, L.G.P.; Pereira, B.A.; Faria, B.G.; Veras, G.C.

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IDENTIFICAÇÃO DO FATOR DE CRESCIMENTO SEMELHANTE À INSULINA I NO PLASMA SEMINAL DE VARRÕES E SUA INFLUÊNCIA NA QUALIDADE ESPERMÁTICA

Zangeronimo, M.G.[1]*; Silva, D.M.[2]; Murgas, L.D.S.[1A]; Sousa, R.V.[1B]; Rocha, L.G.P.[1C]; Pereira, B.A.[1D]; Faria, B.G.[1E] and Veras, G.C.[3]

[1]Departamento de Medicina Veterinária. Universidade Federal de Lavras. Lavras, Minas Gerais. Brasil. *zangeronimo@prpg.ufla.br; [A]lsmurgas@dmv.ufla.br; [B]rvsousa@dmv.ufla.br; [C]lgvetp@yahoo.com.br; [D]barbaraap.vet@gmail.com; [E]brunofaria13@hotmail.com

[2]Departamento de Zootecnia. Universidade Federal de Lavras. Lavras, Minas Gerais. Brasil. daninemoreira@hotmail.com

[3]Faculdade de Engenharia de Pesca. Universidade Federal do Pará. Campus Bragança, Pará. Brasil. galiveras@hotmail.com

ADDITIONAL KEYWORDS

Boar spermatozoa. Functional membrane integrity. Male sexual function. Spermatic motility.

PALAVRAS CHAVE ADICIONAIS

Espermatozóide de varrão. Integridade funcional da membrana. Função sexual do macho. Motilidade espermática.

SUMMARY

The present research was carried out to investigate the relationships between spermatozoal parameters and concentrations of IGF-I in the seminal plasma of boar. Semen samples (duplicates) were collected from mature boars (n=27). In each ejaculate, the macroscopic and microscopic characteristics were determined. After centrifugation to separate sperm cells from seminal plasma, concentrations of IGF-I in seminal plasma samples were determined by a human-specific immunoassay kit. The mean concentration of the boar seminal plasma (n=54 samples) IGF-I was 1.5±0.2 ng/mL. There was no correlation (p>0.05) between IGF-I with most of the evaluated parameters. There was only a low negative correlation (-0.29; p=0.0331) between the concentration of IGF-I in seminal plasma and the motility degradation rate. This study suggests that IGF-I in the seminal plasma of boars is not related to initial fresh semen parameters. However, the hormone did increase the duration of sperm motility.

RESUMO

A presente pesquisa foi realizada para investigar as relações entre os parâmetros dos espermatozóides e as concentrações de IGF-I no plasma seminal de varrões. Amostras de sêmen (duplicatas) foram coletadas de machos maduros (n=27). Em cada ejaculação, as características macroscópicas e microscópicas foram determinadas. Após a centrifugação para separar espermatozóides do plasma seminal, as concentrações de IGF-I em amostras de plasma seminal foram determinadas por kit de imunoensaio humano específico. A concentração média de IGF-I do plasma seminal de varrões foi de 1,5±0,2 ng/mL (n=54 amostras). Não houve correlação (p>0,05) entre IGF-I com a maioria dos parâmetros avaliados. Houve apenas uma correlação baixa negativa (-0,29, p=0,0331) entre a concentração de IGF-I no plasma seminal e a taxa de degradação da motilidade. Este estudo sugere que o IGF-I no plasma seminal de varrões não está relacionado com os parâmetros seminais iniciais do sêmen *in natura*. No entanto, o hormônio aumentou a duração da motilidade espermática.

INTRODUCTION

Components of male reproductive function are controlled by endocrine and

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local regulatory factors. Investigation of fertility in some species has been focused on local regulators of reproductive events, including growth factors and differentiation factors (Wang and Hardy, 2004; Miah *et al*., 2008; Selvaraju *et al.*, 2009).

Insulin-like growth factor I (IGF-I) is a potent mitogenic, metabolic and differentiating polypeptide that is becoming widely recognized as an important regulator of reproductive functions. In the male reproductive tract, IGF-I has been identified in the testis (Hess and Roser, 2001), where it is secreted by Sertoli and Leydig cells (Roser, 2001) under the control of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), respectively (Lejeune *et al.*, 1996). Receptors for IGF-I have also been identified in these cells (Wang and Hardy, 2004; Vanelli *et al.*, 1998). Additionally, these receptors have been identified in other cells such as secondary spermatocytes, spermatids (Vanelli *et al.*, 1998) and spermatozoa (Henricks *et al.*, 1998).

IGF-I has been suggested to be an important factor for germ cell development, maturation and motility of the spermatozoa (Henricks *et al.*, 1998; Glander *et al.*, 1996; Vickers *et al.*, 1999). Furthermore, IGF-I is believed to be involved in spermatogenesis (Tsutura and O'Brien, 1995) and steroidogenesis (Saez, 1994).

The role of IGF proteins as post-testicular regulators of reproductive function has also been examined and an effect of the IGF system on sperm physiology and function in some species has been demonstrated. The concentration of IGF-I in human seminal plasma was correlated (r= 0.75, p=0.00001) to sperm concentration and morphology in one study (Glander *et al.*, 1996). In animals, *in vitro* addition of IGF-I at physiological concentrations stimulates spermatozoal motility in bovine, stallion and buffalo semen (Selvaraju *et al.*, 2009).

In boars, there was no observed correlation between IGF levels and semen parameters (Hirai *et al.*, 2001). However, more recently it was verified that progressive motility and the induction rate of capacitation and acrosome reaction were increased by IGF-I (Miah *et al.*, 2008). Thus, the relationship between the IGF system and characteristics of semen still remains unclear, especially in production animals, and the role of this hormone on reproductive function must be investigated. Therefore, the present investigation was carried out to investigate the relationships between spermatozoal parameters and concentrations of IGF-I in the seminal plasma of these animals.

MATERIAL AND METHODS

Animals and semen collection

The boars used in this study (18 Agroceres-PIC, 8 DB-Danbred and 1 Genetic Pork) were 7-34 months old and were housed in a commercial farm. Whole ejaculates (without the gelatinous fraction) were collected once a week during routine farm operations in January 2010 (2 ejaculates per boar, totalling 54 ejaculates). All ejaculates fulfilled the minimum requirements for use in artificial insemination (>70 % motile spermatozoa, <20 % cytoplasmatic droplets, total sperm number $>10x10^9$). Immediately after semen collection and semen analysis, sample of fresh semen was centrifuged at 3360 g for 10 minutes and the supernatant was removed and stored at –20 °C until IGF-I analysis.

Sperm analysis

The ejaculate appearance was visually determined and classified as 1 for aqueous, 2 for serum-milky and 3 for milky aspect. The total ejaculate was weighed on a digital scale. Motility was determined by the amount of moving spermatic cells after putting one drop of semen between a slide and coverslip under optical microscopy and 100x magnification. Sperm vigor was observed under the same conditions by the progressive forward motility of the spermatozoids from

0 (low) to 5 (high). The live and dead test (%) was conducted by counting the number of live cells (without colour) and dead (pink) under optical microscopy (400x) using one drop of semen colour with one drop of eosine-nigrosine (Mies Filho, 1982). The sperm concentration (spermatozoa/mL) was determined by spectrophotometry (Sperma-Cue, Minitub, Germany). The motility degradation rate was determined by incubating 2.0 mL of semen at 37 °C for 2 hours. The rate was observed at the beginning and at the end, and the motility difference between the two moments was divided by the initial motility (Salgueiro *et al.*, 2003). The osmotic resistance was evaluated by adding 100 mL of semen to 1.0 mL of a 100 mOsm/L solution (two parts of distilled water in one part of BTS diluent - Beltsville Thawing Solution). The samples were incubated at 37 °C for 40 minutes. Then the amount of spermatozoa (percentage) with twisted (stable membrane) and straight tails (disrupted membrane) were evaluated under optical microscopy (400x). The spermatic morphology (%) was evaluated using a mix of semen and formol-citrate (2.94 %) using a phase contrast microscope (100x). The percentages of acrosome and tail abnormalities, the presence of proximal cytoplasmatic droplets and the total amount of morphological abnormalities were calculated.

In this study, all samples were evaluated by the same operators (three) and sample handling followed the same protocol throughout the study.

Quantification of IGF-I in seminal plasma

Immediately after collection, the semen samples were centrifuged (3 ºC, 3360 g for 10 minutes) to remove the supernatant containing the seminal plasma. The seminal plasma was further centrifuged twice to remove more spermatozoa. The separated seminal plasma was stored at –10 °C until assayed for free IGF-I concentration. The IGF-I concentration in seminal plasma was determined by a specific immunoassay kit (Human IGF-I ELISA RayBiotek-USA) according to the manufacturer's protocol. This kit was used to assess boar seminal plasma IGF-I because the homology of the amino acid sequence of human and swine IGF-I has been reported to be 100 % (Tavakkol *et al.*, 1998). Concentrations of free IGF-I were expressed as nanograms per millilitre (ng/mL) of seminal plasma. The assay had a sensitivity of 0.2 ng/mL, as determined by the manufacturer.

Statistical analysis

Statistical analysis was performed with SAS System for Windows. The relationships between free IGF-I concentrations in seminal plasma (n=54 ejaculates) and the semen parameters were examined using the Spearman's correlation coefficient because the IGF-I data were not normally distributed (as determined by the Shapiro-Wilk test). Values of $p \leq 0.05$ were considered significant.

RESULTS

As shown in **table I**, the mean boar seminal plasma IGF-I concentration (mean ± SD) was 1.50 ± 0.20 ng/mL, ranging from 1.39 to 2.44.

Levels of IGF-I in seminal plasma were not related ($p > 0.05$) to the majority of semen parameters. Only a low negative correlation ($p < 0.05$) was observed between the IGF-I concentration in seminal plasma and the motility degradation rate.

DISCUSSION

In this research, all values of seminal parameters were technically acceptable, confirming that the boars had normal sexual characteristics and were adequate and sound for reproduction.

The concentrations of free IGF-I in seminal plasma of the boars were low, ranging from 1.39 to 2.44 ng/mL, with a mean of 1.5±0.20 ng/mL. Other previous studies (Hirai

Table I. *Means, standard deviations (SD) and correlation values between the IGF-I concentration in the seminal plasma and the seminal parameters of 27 boars (n=54 ejaculates).* (Médias, desvios padrão (DP) e valores de correlação entre a concentração de IGF-I no plasma seminal e parâmetros seminais de 27 varrões (n=54 ejaculados)).

Variable	Mean	SD	r	p=
Free IGF-I concentration (ng/mL)	1.50	0.20	1.0	0.0000
Ejaculate appearance	2.6	0.4	0.20	0.1488
Ejaculate weight (g)	292.6	162.5	-0.10	0.4665
Sperm motility (%)	81.9	7.9	-0.02	0.8700
Sperm strength movement	3.2	0.4	0.08	0.5519
Agglutination degree (number/area)	2.3	2.0	0.13	0.3668
Live:dead ratio (%)	88.8	5.9	-0.17	0.2112
Sperm concentration (10^6 cells/mL)	405.1	115.3	0.09	0.5249
Motility degradation rate (%)	19.5	19.5	-0.29	0.0331
Osmotic resistance test (%)	88.4	6.8	-0.08	0.5851
Abnormal acrosome (%)	1.4	1.2	-0.15	0.2757
Abnormal tail (%)	8.4	4.2	0.01	0.9308
Proximal cytoplasmatic droplets (%)	0.6	0.9	0.00	0.9539
Total of abnormalities (%)	10.8	4.6	-0.05	0.6938

et al., 2001) obtained total values ranging from 8.4 to 22.2 ng/mL in Pietrain boars, aged 22-44 months, whereas Lackey *et al.* (2002) showed values ranging from 5.0 to 25 ng/mL in Duroc and Yorkshire boars. The variability in results may be related to the presence in the semen of IGFBP (insulin-like growth factor binding protein), that represent a family of proteins that help to modulate IGF action in complex ways that involve inhibiting it by preventing its binding to the IGF-I receptor (Hwa *et al.*, 1999).

The IGF's are known to bind the IGF-I receptor, the insulin receptor, the IGF-II receptor, the insulin-related receptor and possibly other receptors. The IGF-I receptor is the main physiologic receptor for IGF-I, having significantly higher affinity for this hormone. Thus, measuring free IGF-I concentration is important to determine some physiological functions in spermatozoa (Cohen *et al.*, 1991). In other studies, total IGF-I was measured. For instance, in buffaloes, Selvaraju *et al.* (2009) observed values from 41.41 to 198.95 ng/mL. The values ranged from 116 to 144 ng/mL (Henricks *et al.*, 1998) in bovine seminal plasma, and in stallion from 10.2 to 10.4 ng/mL (Macpherson *et al.*, 2002). These values show that the levels in boars and stallions are similar to those in humans (Lackey *et al.*, 2002), but much lower than those found in cattle and buffaloes. According to these authors, differences in ejaculate volume, levels of growth hormone or steroids and nutritional factors may contribute to the variation in semen IGF-I concentrations.

IGF-I in seminal plasma is primarily of testicular or epididymal origin (Glander *et al.*, 1996). IGF-I production is under the control of the gonadotropins FSH and LH (Lejeune *et al.*, 1996) and depends on testicular development. Hence, several factors affecting gonadotropin secretion and testicular growth may influence IGF-I levels in the seminal plasma, such as genetics and nutrition. Nutrition is the most powerful factor that acts on the neuroendocrine system and controls testicular activity in ruminants (Blache *et al.*, 2000; Brito *et al.*, 2007). In the present study, no difference

was observed between the genetic backgrounds (data not shown). In stallions, differences in IGF-I concentrations were not detected between samples from individual animals at different levels of sexual activity, but substantial variation in IGF-I concentrations occurred between stallions (Macpherson *et al.*, 2002).

In the present study, no correlation ($p>0.05$) was observed between the free IGF-I concentration in the seminal plasma of boars and most of the evaluated seminal parameters, except for the motility degradation rate. The low negative correlation for this parameter (–0.29, $p=0.0331$) suggests that IGF-I may have some relation with the duration of motility but not with the intensity of movement of sperm cells right after ejaculation. This result suggests that this hormone maintains the movement of sperm for longer periods of time, which is an important role in male fertility. Macpherson *et al.* (2002) also observed inconsistent results between bovine seminal plasma IGF-I and sperm parameters (sperm numbers, morphology and motility). However, these authors observed that sperm morphology and motility were better in some samples that displayed high IGF-I concentrations.

Possible sources of seminal plasma IGF proteins include the testis, epididymis and accessory glands. The source of seminal plasma IGF-I production in men was either the testicular/epididymal tissue or the accessory glands (Glander *et al.*, 1996). These authors observed that the seminal plasma IGF-I concentrations dropped to approximately one-third after vasectomy, thus implicating an IGF-I production in the testis, epididymus or both. A similar occurrence may be true for pigs; however, specific research has not been performed to determine the origin of seminal plasma IGF-I in this species. The accessory glands of boars contribute with a significant fluid fraction to the final ejaculate, therefore secretions from these organs could likely include IGF-I.

In growth hormone (GH)-deficient dwarf rats, Vickers *et al.* (1999) observed that IGF-I treatment increased the IGF-I concentration in the seminal vesicle fluid but not in the epididymal fluid, suggesting a role for this hormone in the post-testicular metabolism of the spermatozoa after ejaculation. These authors also observed an improvement in sperm motility and morphology mainly due to changes in the IGF-I levels in seminal plasma. It is important to emphasize that in this case, the authors worked with GH-deficient animals, reinforcing the idea of improvement in sperm quality with increa-sing concentrations of IGF in seminal plasma.

The supplementation of IGF-I above physiological concentrations in buffalo semen was shown to increase total and progressive motility when compared with controls during the incubation period (Selvaraju *et al.*, 2009). Similar results were found in previous research using bovine (Henricks *et al.*, 1998), human (Miao *et al.*, 1998), equine (Chanpion *et al.*, 2002) and swine (Miah *et al.*, 2008) samples. IGF-I might act as a chemokinetic factor involved in the regulation of cell movement, because IGF-I receptors have been reported to be found on spermatozoa (Henricks *et al.*, 1998). Spermatozoal motility and velocity indirectly reflect their mitochondrial function. IGFs in neuronal cells prevent mitochondrial dysfunction when exposed to glutathione-depleting agents, maintain calcium homeostasis and increase cell survival (Sortino and Canonico, 1996). If IGFs act as antioxidants, this would then result in increased sperm viability. Thus, it is conceivable that the effect of IGF-I in increasing spermatozoal motility during incubation in the present study might be due to the direct effect through the IGF-I receptor on the spermatozoa.

It was suggested that IGF-I might maintain motility through energy uptake or due to its antioxidant effect in these species, with these effects being linked to spermatozoal metabolism, capacitation and acroso-

mal reaction (Selvaraju *et al.*, 2009), which would be related to the attachment and penetration of the oocyte. These authors observed that IGF-I increased the fructose uptake and decreased the lipid peroxidation level of buffalo spermatozoa. This latter effect suggests a positive effect of IGF-I on functional membrane integrity due to spermatozoal membrane stability.

Both the spermatozoal motility and functional membrane integrity had a direct correlation with the reduction in lipid peroxidation (Suleiman *et al.*, 1996). A direct effect of IGF-I on superoxide dismutase activity and total antioxidant status in rodent (bank vole) testis has been reported (Gancarczyk *et al.*, 2006). Hence, it is possible that higher IGF-I concentrations would improve the spermatozoal membrane integrity during the incubation period. In addition, there are indications that IGFs induce increased ion transport (Miah *et al.*, 2008), which may result in increased intracellular calcium levels. Hyperactivation is known to require extracellular calcium (Suarez *et al.*, 1993) and may therefore be partially regulated by growth factors. This also partially explains the higher duration of motility in samples with a higher IGF-I concentration in the present study.

Based on IGF-I levels, fertile bulls can also be selected for artificial insemination (Yilmaz *et al.*, 1999). Nevertheless, no information is available on the role of IGF-I in boars with low semen fertility. Moreover, the *in vivo* quantity of IGF-I binding protein in the boar spermatozoal membrane is not known. In bovine semen, an average of 1000 receptors per cell was estimated (Henricks *et al.*, 1998). The knowledge of the number of receptors in different species may help to elucidate the true role of IGF-I in spermatozoal metabolism and the usefulness of this hormone in the improvement of artificial insemination programs. Pregnancy rates were higher for horses with high seminal plasma IGF-I levels (Macpherson *et al.*, 2002), which is suggestive of the effects of IGF-I on sperm function.

CONCLUSION

The concentrations of IGF-I in seminal plasma of boars were not related to initial fresh semen parameters, but this hormone may increase the duration of sperm motility.

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