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DETERMINATION OF ENZYME ACTIVITY IN RABBIT SEMINAL PLASMA AND ITS RELATIONSHIP WITH QUALITY SEMEN PARAMETERS

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Abstract: The objective of this study was to determine rabbit seminal plasma enzyme activity. Furthermore, correlations between semen parameters and enzyme activity and male age were examined. The study was performed using 17 New Zealand White males from 5 to 9 mo old. Overall, 252 semen samples were collected from bucks from May to September. Semen characteristics were analysed and the seminal plasma was obtained by centrifugation. The activities of alanyl aminopeptidase (APN), aspartate transaminase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH) and alkaline phosphatase (ALKP) in the seminal plasma fluid were measured. Significant differences between males were found in APN, GGT, LDH, ALKP and ALT activities ($P<0.05$). No significant differences between enzyme activity and male age were found. We also observed significant positive correlations between male age and sperm concentration (r=0.26), progressive motility (r=0.17) and amplitude of lateral head displacement (r=0.21), and negative ones between male age and average path velocity (r=–0.56), velocity of the sperm head along its actual curvilinear path (r=–0.61), straight line velocity (r=–0.50), linearity index (r=–0.13), and cytoplasmic droplet (r=–0.33). Furthermore, a significant negative correlation between APN activity and the status of the acrosome (r=–0.20) and significant positive correlations between APN activity and the sperm abnormalities (r=0.21), GGT activity and sperm concentration (r=0.34) and the status of the acrosome (r=0.31), and ALKP activity and sperm concentration were observed (r=0.41). In our study, APN and GGT seem to be the most predictive enzymes for rabbit semen quality.

Key Words: rabbit, seminal plasma, enzyme activity, seminal parameters.

INTRODUCTION

In mammals, seminal plasma is a complex mixture of secretions from the epididymis and from the various accessory sex glands (La Falci *et al.,* 2002). A large variety of enzymes is present in seminal plasma, but in many instances the gland responsible for their production has not been identified (Dogan *et al.,* 2009). Determinations of biochemical constituents of seminal plasma are needed for semen evaluation, as physical characteristics of semen alone are not completely satisfactory for semen appraisal in current practice (Mann and Lutwak-Mann, 1981). For instance, alkaline phosphatase (ALKP), aspartate transaminase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) are essential for metabolic processes which provide energy for survival, motility and fertility of spermatozoa (Sirat *et al.,* 1996). Likewise, γ-glutamyl transpeptidase (GGT) has been shown to be correlated with sperm motility in horse (Pesch *et al.,* 2006; Dogan *et al.,* 2009). Furthermore, mammalian seminal plasma aminopeptidases like alanyl aminopeptidase (APN) are involved in many physiological processes. Alterations in APN activity may contribute to the subfertility of human semen in terms of sperm motility, viability, and spermatogenesis, due to its positive correlation with sperm motility and the percentage of dead sperm and its negative correlation with

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the total number of spermatozoa per millilitre (Irazusta *et al.,* 2004). Additionally, APN is involved in many peptide metabolism pathways by catalysing the degradation of peptides related to these pathways (Cheng *et al.,* 2012). Therefore, estimates of these enzymes in seminal plasma have been recommended as markers for semen quality, as they indicate sperm damage (Sirat *et al.,* 1996). Nevertheless, there are a limited number of studies focused on the presence of these enzymes in rabbit semen. Okab *et al.* (2007) reported that the leakage of ALP and LDH in rabbit seminal plasma during summer and spring, respectively, could reveal a positive correlation between enzyme release and sperm cell integrity and acrosomal damage. In other works in rabbit, enzyme activity in seminal plasma has been studied to evaluate the effect of diet supplementation with different substances on semen quality (Yousef, 2005, Yousef *et al.,* 2003, 2004, 2006, 2010; Attia and Kamel, 2012).

Thus, this work was undertaken to investigate the enzyme activities (ALKP, AST, ALT, LDH, GGT and APN) in New Zealand White rabbits' seminal plasma and evaluate the effect of male and age on these enzyme activities. The correlations between semen parameters and enzyme activity and male age were also examined.

MATERIAL AND METHODS

Unless stated otherwise, all chemicals in this study were purchased from Sigma-Aldrich Química S.A (Madrid, Spain).

Animals

All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013.

To study the effect of male and age on seminal plasma enzyme activity, a total of 17 New Zealand White adult bucks were used. All males were kept individually in flat deck cages under 16 h light/8 h dark conditions at the experimental farm of the Animal Technology and Research Centre (CITA-IVIA, Segorbe, Castellón, Spain) and fed *ad libitum* with the same commercial diet (17.5% crude protein, 2.3% ether extract, 16.8% crude fibre, 2600 kcal digestible energy/kg) and free access to water.

Semen collection and evaluation

All males began their training with an artificial vagina at 5 mo of age and their ejaculates were collected until they were 9 mo old. One ejaculate was collected per male each week for the first 2 wk. After this period, 2 ejaculates per male were collected once a week, with a minimum period of 30 min between ejaculates. Only ejaculates containing urine or with a volume equal to or less than 0.1 mL were rejected. Ejaculates coming from the same male and the same day were pooled together. After collection, sperm evaluation was performed to assess the initial seminal quality. A 20 µL aliquot was diluted 1:50 with 0.25% glutaraldehyde solution to calculate the concentration (CONC), sperm abnormalities (ABNOR) and acrosome status (sperm with intact apical ridge, NAR). Concentration was evaluated using a Thoma chamber. Percentage of NAR was observed by phase contrast at a magnification of 400×. The motility characteristics of sperm were evaluated using a computer-assisted sperm analysis system (ISAS, version 1.0.17, Proiser, Valencia, Spain) operating at 30 video frames/s (30Hz), with particle area settings from 20 to 80 µm and a search radius of 13 mm. Sperm motility was assessed at 37°C using a ×10 negative phase-contrast objective on a Nikon Eclipse 90i (Nikon Corporation Instruments Company; IZASA, Barcelona, Spain) connected to the computer through a monochrome Basler A312f video camera (Basler Vision Technologies, Proiser, Paterna, Valencia, Spain). The sperm concentration of each sample was adjusted to 7.5×10^6 sperm/mL with Tris-citrate-glucose extender-BSA. Five microlitres of the subsamples were placed in a Mackler chamber (Counting Chamber Mackler, Sefi-Medical Instruments) pre-warmed at 37°C on a thermal plate, and the data for a minimum of 200 sperm from 4 different fields were collected. Individual sperm tracks were visually assessed to eliminate possible debris and wrong tracks. The following kinetic parameters were evaluated: percentage of total motile sperm (MOT, number of motile sperms/total×100); average path velocity (VAP, velocity of the sperm head along its average; µm/s); curvilinear velocity (VCL, velocity of the sperm head along its actual curvilinear path; µm/s); straight line velocity (VSL, velocity of the sperm head along a straight line; µm/s); linearity index (LIN, linearity of the curvilinear path, average value of the ratio VSL/VCL, %); straightness coefficient (STR, straightness of the path velocity VSL/VAP, %); percentage

progressive spermatozoa (PROG, percentage of spermatozoa with a VAP>40 μm/s and straightness >80%) and amplitude of lateral head displacement about its average path (ALH; μm).

Seminal plasma preparation

Semen samples were centrifuged at 7400 g for 10 min at 22 ºC. The resulting supernatants were collected and centrifuged again (7400 g for 10 min) to remove residual spermatozoa and cell debris. The resulting pellets were discarded, whereas the supernatants were stored at –80°C until use.

Measurement of enzyme activity

APN activity in seminal plasma was determined according to Viudes-de-Castro *et al.* (2014). Briefly, samples were incubated with the substrate (alanine-β-naphthylamide) for 30 min at 37°C, after which the reaction was stopped with 0.1 M sodium acetate buffer (pH 4.2). The release of β-naphthylamide as a result of enzyme activity was determined by measuring the fluorescence intensity at 460 nm with excitation at 355 nm. Fluorescence values obtained with the experimental samples were transformed into picomoles of β-naphthylamide released by comparison with a standard curve previously obtained. Protein concentration of semen samples was measured using the bicinchoninic acid method, using BSA as standard protein (Smith *et al.,* 1985). APN activity and protein concentration were measured in triplicate. Eleven determinations of APN activity per male were made and the enzyme activity was expressed as pmol of β-naphthylamide released per milligram of protein per minute.

Activities of AST, ALT, GGT, LDH and ALKP in seminal plasma were determined using a VetTest® 8008 Chemistry Analyser (Idexx Laboratories, Cergy Pontoise, France). The VetTest apparatus requires 10 μL of plasma for each parameter. The different biochemical tests are available as dry slides that include all necessary reagents. As the sample filters through the layers, biochemical reactions take place within the film, producing progressive colour changes. The system uses three reflectometers operating at 6 wavelengths to perform both end point and rate measurements. The results of the analyses can be read after a maximum of 6 min. Four measurements per male and enzyme were taken and the enzyme activity was expressed as International units per litre (IU/L).

Statistical analysis

Data were statistically evaluated with Statgraphics® Plus 5.1 library procedures (Statistical Graphics Corp., Rockville, MO, USA). To analyse the effect of male (1 to 17) and age of male (24 to 40 wk) on enzyme activity, a 2-way analysis of variance (ANOVA) was used. Means were separated using Fisher's Least Significant Difference (LSD) test at a fixed 5% error level and the results are presented as least square mean values (LSM) ±the standard error (SE). Correlation analysis (2-tailed Pearson's correlation test) was used to assess the relationship between enzyme activities and morphologic and kinetic seminal parameters.

RESULTS AND DISCUSSION

Seminal plasma enzyme activity

Enzyme activity was detected in all seminal plasma samples. No significant association was found between male age and enzyme activity. However, significant differences in APN (Figure 1), GGT (Figure 2), LDH (Figure 3), ALKP (Figure 4) and ALT (Figure 5) activities were found between males ($P<0.05$). Conversely, AST activity was not significantly different between males (Figure 6). Values of LDH, AST and ALT activities obtained in this work are in the range of previous studies in rabbits (Yousef *et al.,* 2003, 2004, 2005, 2010). However, in comparison with the results of Okab *et al.* (2007), our ALKP, AST and ALT values are lower and our LDH values are higher.

APN activity has previously been reported in seminal plasma of different species of mammals (Agrawal and Vanha-Perttula, 1986; Huang *et al.,* 1997; Osada *et al.,* 2001; Fernández *et al.,* 2002; Irazusta *et al.,* 2004; Viudes-de-Castro *et al.,* 2014). Recently, Viudes de Castro *et al.* (2014) showed that the bioavailability of buserelin acetate when added to the seminal dose appears to be determined by the activity of the existing seminal plasma aminopeptidases. In this work, 2 groups of males can be clearly distinguished regarding APN activity: males with high or low activity

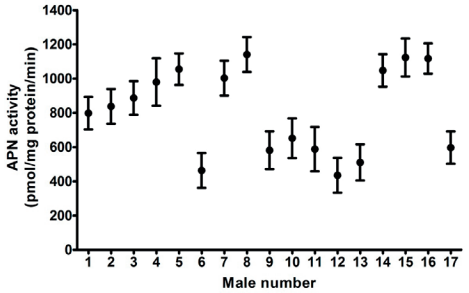

Figure 1: Mean alanyl aminopeptidase (APN) activity in the seminal plasma of the seventeen New Zealand White male rabbits studied.

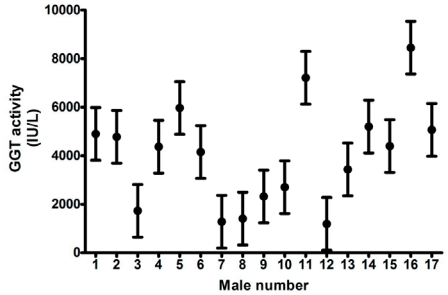

Figure 2: Mean γ-glutamyl transpeptidase (GGT) activity in the seminal plasma of the seventeen New Zealand White male rabbits studied.

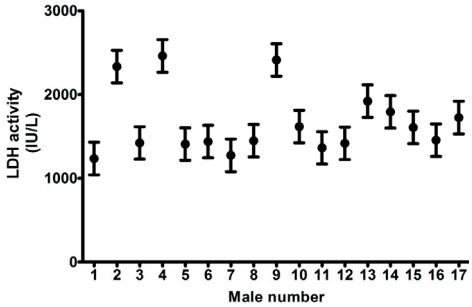

Figure 3: Mean lactate dehydrogenase (LDH) activity in the seminal plasma of the seventeen New Zealand White male rabbits studied.

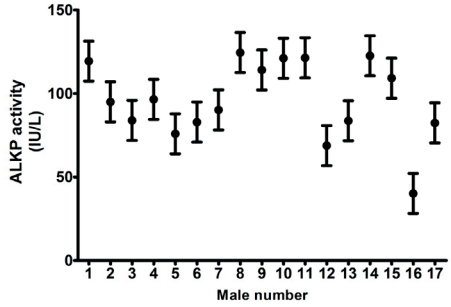

Figure 4: Mean alkaline phosphatase (ALKP) activity in the seminal plasma of the seventeen New Zealand White male rabbits studied.

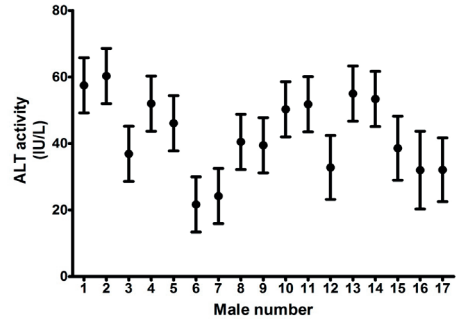

Figure 5: Mean alanine aminotransferase (ALT) activity in the seminal plasma of the seventeen New Zealand White male rabbits studied.

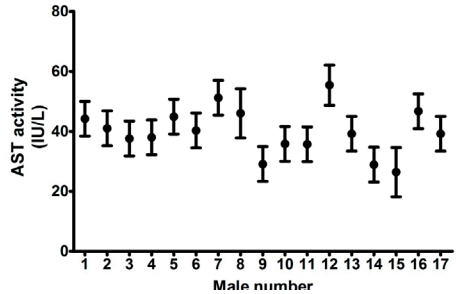

Figure 6: Mean aspartate transaminase (AST) activity in the seminal plasma of the seventeen New Zealand White male rabbits studied.

(Figure 1). What is interesting in this data is that APN activity of one group is almost twice than the other group and that approximately half of population can be configured in each group. Therefore, it could be that when less APN activity is present in the male seminal plasma, a lower amount of hormone should be added to semen extender to trigger female ovulation. So, the use of males with low APN activity could increase the analogue bioavailability and help diminish its concentration in the extender when intravaginal administration of GnRH analogues is used.

Correlation between sperm traits and male age

The results of the correlation study between quality sperm characteristics and male age are shown in Table 1. Male age was correlated with the majority of kinetic characteristics studied, but not with the enzyme activity measured in seminal plasma, which suggests the quality of movement is age dependent in the male rabbit. Significant positive correlations between male age and sperm concentration (r=0.26), progressive motility (r=0.17) and amplitude of lateral head displacement (r=0.21) and negative ones between male age and average path velocity (r=–0.56), velocity of the sperm head along its actual curvilinear path (r=–0.61), straight line velocity (r=–0.50), linearity index (r=–0.13), and cytoplasmic droplet (r=–0.33) were found. In rabbits, parameters such as mean ejaculate volume and mean sperm concentration, as well as the fertility and litter size at birth, are influenced by the age of the male (Alvariño, 2000). In general, quality parameters are observed to improve with age (Miros and Mikhno, 1982; Luzi *et al.,* 1996; Theau-Clément *et al.,* 2015).

Correlation between sperm traits and enzyme activity

The results of the correlation study between quality sperm characteristics and enzyme activity are shown in Table 2. Kinetic parameters were not correlated with any enzyme activity. However, we did find significant correlations between enzyme activities and other sperm traits. For instance, a significant negative correlation between APN activity and the status of the acrosome (r=–0.20) and a significant positive correlation between APN activity and the sperm abnormalities (r=0.21) was found. Samples with greater APN activity were related to higher abnormal sperm rates and lower percentages of normal apical ridge. Consequently, this can influence the fertility of the ejaculate.

Several studies have demonstrated an inverse relationship between abnormal sperm rate and fertility reduction (Xu *et al.,* 1998; Farrell *et al.,* 1993; Lavara *et al.,* 2005). Considering that APN activity does not vary with age, combined with its correlation between the status of the acrosome and the abnormalities and the relationship between these characteristics and fertility, the determination of APN activity may be included as a new quality parameter in the basic analysis of semen during the male rabbit training period.

On the other hand, the cellular damage in the semen could be the result of an improper balance between reactive oxygen species generation and scavenging activities. One of the biological functions of GGT is the regulation of glutathione levels, which counteracts the effects of oxidative stress in sperm cells. This agrees with the significant positive correlation between GGT activity and acrosome status (r=0.31) observed in the present study. In similar works in stallion, GGT seminal plasma activity correlated significantly with motility and progressive motility

Table 1: Pearson's correlation coefficients between sperm traits and male age.

Sperm traits	Mean (SE)	No	Male age (correlation coefficient)
CONC	272 (9)	243	0.26[c]
MOT	63.6 (1.4)	252	0.08
PROG	23.2 (1.0)	251	0.17[b]
VAP	25.6 (0.8)	251	–0.56[c]
VCL	47.4 (1.2)	251	–0.61[c]
VSL	18.1 (0.7)	251	–0.50[c]
LIN	38.1 (0.9)	249	–0.13[a]
ALH	2.33 (0.04)	251	0.21[c]
STR	69.0 (0.7)	250	–0.67
NAR	89.8 (0.9)	243	–0.01
CD	9.32 (0.63)	241	–0.33[c]
ABNOR	12.4 (0.5)	242	–0.11

CONC: Sperm concentration per mL; MOT: % of total motile sperm cells; PROG: % of progressive spermatozoa; VAP: average path velocity (μm/s); VCL: velocity of the sperm head along its actual curvilinear path (μm/s); VSL: straight line velocity (μm/s); LIN: linearity index (%); ALH: amplitude of lateral head displacement (μm); STR: straightness coefficient (%); NAR: % of normal apical ridge; CD: % of cytoplasmic droplet; ABNOR: % of sperm abnormalities; SE: standard error; No: sample size; [a]$P<0.05$; [b]$P<0.001$; [c]$P<0.0001$.

Table 2: Pearson's correlation coefficients between sperm traits and enzyme activity.

Sperm traits	APN activity	GGT activity	LDH activity	ALKP activity	ALT activity	AST activity
CONC	0.02	0.34[b]	–0.10	0.41[b]	0.01	–0.08
MOT	–0.05	0.13	0.18	0.14	0.08	–0.13
PROG	–0.01	0.09	0.06	–0.01	–0.02	–0.09
VAP	–0.01	–0.12	–0.09	0.08	0.01	–0.11
VCL	–0.08	–0.21	–0.08	0.14	0.03	–0.12
VSL	0.03	–0.08	–0.12	0.01	–0.04	–0.09
LIN	0.06	0.12	–0.01	–0.06	–0.05	–0.04
ALH	–0.12	0.05	–0.02	–0.01	–0.22	–0.13
STR	0.01	0.05	–0.04	–0.18	–0.17	–0.04
NAR	–0.20[b]	0.31[a]	0.18	0.12	0.23	–0.09
CD	–0.06	–0.16	–0.09	–0.09	0.23	–0.09
ABNOR	0.21[b]	0.05	–0.20	–0.19	–0.12	–0.02

CONC: Sperm concentration per mL; MOT: % of total motile sperm cells; PROG: % of progressive spermatozoa; VAP: average path velocity (µm/s); VCL: velocity of the sperm head along its actual curvilinear path (µm/s); VSL: straight line velocity (µm/s); LIN: linearity index (%); ALH: amplitude of lateral head displacement (µm); STR: straightness coefficient (%); NAR: % of normal apical ridge; CD: % of cytoplasmic droplet; ABNOR: % of sperm abnormalities. APN: alanyl aminopeptidase, GGT: γ-glutamyl transpeptidase, LDH: Lactate dehydrogenase, ALKP: Alkaline phosphatase, AST: aspartate transaminase, ALT: alanine aminotransferase; [a]$P<0.05$; [b]$P<0.001$.

(Pesch *et al.,* 2006) and negatively with progressive motility (Dogan *et al.,* 2009). However, our results failed to find a significant relationship between GGT levels and sperm motility. Moreover, a significant positive correlation between GGT and ALKP activities and sperm concentration was also found (r=0.34 and r=0.41, respectively). This finding agrees with the results of Pesch *et al.* (2006) in stallion, which showed a correlation of the enzymes GGT and ALKP and sperm concentration, as well as Kiso *et al.* (2013) in elephant and Kozdrowski and Dubiel (2004) in boar, who reported a significant correlation between sperm concentration and seminal plasma ALKP activity. The main source of the ALKP in rabbits is the epididymis (Muller, 1983). Furthermore, seminal plasma ALKP activity is involved in sperm capacitation, acrosome reaction, hyperactivation, and zona pellucida binding (Salzberger *et al.,* 1992; Urner and Sakkas, 2003). The positive correlation between sperm concentration and GGT and ALKP activities suggest these enzymes may serve as potential diagnostic markers for testicular function in rabbits.

Finally, in the present study we could not find any correlation between transaminase activities (AST-ALT) or LDH activity and seminal characteristics.

In conclusion, our results suggest that GGT and APN seem to be the most predictive enzymes for rabbit semen quality, as their activities are related with the status of the acrosome and/or sperm count and abnormal spermatozoa, and they could be used as additional biomarkers along with classic semen analysis to assess semen quality.

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