RESEARCHES REGARDING THE VARIABILITY OF CYTOLOGICAL AND BIOCHEMICAL PARAMETERS OF BULL SEMEN USING FACTORIAL ANALYSIS

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Abstract. There were analysed cytological parameters (sperm concentration/ml x 10^9, sperm motility %) and biochemical parameters (pH, total protein %, albumin %, GOT, GPT-IU, fructose mg %, alkaline and acid phosphatase activities IU) of the semen taken from 80 bulls of Bruna race. The semen volume was also measured. Semen parameters showed a high variability, less pH and sperm motility. GPT and phosphatases activities showed variability of over 50%. The protein mean (6.362±1.666%) and plasma fructose mean (602.664±233.603 mg %) values were similar to some state of art data, with differences from author to author. GOT (100,453±20,091 IU) and GPT (12,558±7,087 IU) activities were lower than data in literature. Generally, low transaminase activities indicate a good quality sperm and a well-structured cell membrane. The phosphatases activity was higher than some literature data that is 2996, 098 IU or alkaline phosphatase and 903,300 IU for acid phosphatase. Increase of phosphatases activity may inhibit sperm respiration due to the massive release of phosphorus ions. Very significant correlations were recorded between protein-albumin ($r = 0.798$, $p < 0.001$), concentration-motility ($r = 0.647$, $p < 0.001$), fructose-concentration ($r = -0.389$, $p < 0.001$) and fructose-protein ($r = 0.378$, $p < 0.001$). Most correlations were characterized by low correlation coefficients and factorial analysis was used. It resulted 5 principal components, which can describe 72% of the total variability. The first principal component described satisfactorily semen quality, being correlated with mobility (0.799 saturation level), concentration (0.869), acid phosphatase activity (0.557) and fructose content (-0.559).

Keywords: biochemical parameters, cytological parameters, factorial analysis, seminal material.

Rezumat. Cercetări privind variabilitatea parametrilor citologici şi biochimici ai spermei de taur utilizând analiza factorială. S-au analizat parametrii citologici (concentraţia în spermatozoizi/mlx10^9, mobilitatea spermatozoizilor %) si biochimici (pH, proteină totală %, albumină %, GOT, GPT-UI, fructoză mg %, activitatea fosfatazei alcaline si acide UI) ai spermei prelevate de la 80 de tauri de rasă Brună şi s-a măsurat volumul. Parametri spermei au prezentat o variabilitate ridicată, mai puţin pH-ul şi mobilitatea spermatozoizilor. Activităţile GPT şi a fosfatazelor au prezentat variabilităţi de peste 50 %. Valorile medii ale proteinei (6.362± 1.666%) şi fructozei plasmatice (602.664±233.603 mg %) au fost similare cu unele date din literatură, cu diferenţe de la autor la autor. Activităţile GOT (100.453±20.091 UI) şi GPT (12.558±7.087 UI) au fost mai mici decât cele din literatură. În general, activităţi transaminazice scăzute indică o spermă de bună calitate şi o membrană celulară bine structurată. Activitatea fosfatazelor a fost mai mare decât unele date din literatură, respectiv 2996,098 IU pentru fosfataza alcalină şi 903,300 IU pentru fosfataza acidă. Corelaţii foarte semnificative s-au înregistrat între: proteină-albumină ($r = 0.798^{***}$), concentraţie-mobilitate ($r = 0.647^{***}$), fructoză-concentraţie ($r = -0.389^{***}$) şi fructoză-proteină $r = 0.378^{***}$). Majoritatea corelaţiilor s-au caracterizat prin coeficienţi scăzuţi de corelaţie şi s-a recurs la analiza factorială. Au rezultat 5 componente principale, care pot descrie 72 % din variabilitatea totală. Prima componentă principală descrie mulţumitor calitatea spermei, fiind corelată cu mobilitatea (0,799), concentraţia (0,869), activitatea fosfatazei acide (0,557) şi conţinutul în fructoză (-0,559).

Cuvinte cheie: analiză factorială, material seminal, parametri biochimici, parametri citologici.

INTRODUCTION

The quality of the semen is an essential condition of its fertility and it is an attribute determined by the interaction of a large number of factors, difficult to include in a single evaluation. The connection between sperm quality and fertility had been investigated in numerous studies and by an impressive number of techniques: macroscopic evaluation tests (volume, colour, consistency, density, pH), microscopic evaluation tests (motility, concentration), morphological tests (for the identification of primary, secondary and tertiary abnormalities), biochemical tests (to determine the concentration of various components of seminal plasma or enzymatic activity), metabolic activity tests (reduction of methylene blue, resazurin, oxygen utilization etc.), immunoassays etc. (DHURVEY et al., 2012). The predictive importance of these tests concerning semen fertility is difficult to assess, because the correlation coefficients values of these parameters with fertility, varies from author to author. For example, in Graham's review on bovine semen (2001), we can see that there are no solid arguments for correlation of sperm motility with fertility, correlation coefficients ranging from 0.15 to 0.84. At the same time, the correlation coefficients between sperm morphology and fertility varied from 0.06 to 0.86, and between the fertility and cell viability, from 0.33 to 0.66. Therefore, we aimed to approach the relationship between different quality parameters of Bruna bull semen (cytological and biochemical) using factorial analysis.

MATERIAL AND METHODS

Semen samples were collected from 80 Bruna bulls in the reproductive season (semen collection was done using the mannequin). The volume of the ejaculates was measured and the spermogram highlighted the following aspects: sperm concentration relative to 1 ml of seminal material (multiplied by 10^9) and gametes motility (%). The methods used are given below:

- **haemocytometric microscopic determination of the sperm concentration** (spermatozoa/ml x 10^9). 1-2 drops of diluted sperm were appropriately diluted with 3% NaCl in the Potain pipette and placed on the Thoma chamber network. The number

of spermatozoa was read in 5 middle squares taken diagonally (80 squares). The calculation formula (depending on the dimensions of the Thoma chamber squares) was used, then it was reported to 1 ml of whole seminal material;

- **determination of sperm motility** (%). The vigorous movements of sperm were judged microscopically (a drop of semen was fixed between the blade and the lamella) and 10 spermatozoa were successive followed in 3 fields (circular or rotational movements were not taken into account). The result of observation was noted in decimal system, the mean was calculated and the final result was expressed in percentages. The determination should be isothermal (blade, lamella, pipettes, etc. should be maintained at 38 ° C). Afterwards, the seminal material was centrifuged for 20 minutes at 2500 rpm. In seminal plasma there were evaluated: pH, total protein, albumin, fructose, GOT activity, GPT activity, alkaline phosphatase activity, acid phosphatase activity, according to the methods below:

- **pH measurement**. pH measurement was made potentiometrically by using a two decimal pH meter;

- **determination of total protein by the biuret method** (%). Variable amounts of seminal plasma, according to species (0.1 ml) were shaken with 5 ml of biuret reagent. The colour developed in 30 minutes, after which optical density (O.D.) was read, using a spectrophotometer at 570 nm. The control consisted of 1% bovine albumin serum (KRUEZIGER & ALLEN, 2009);

- **determination of albumin with bromocresol green reagent** (%). To 0.1 ml of seminal plasma, bromocresol green reagent in citrate buffer pH = 3.8, was added. It rested for 10 minutes, than the sample O.D. was read on the spectrophotometer at 637 nm. The control consisted of 1% bovine albumin serum (GENTRY & LUMSDEN, 1978);

- **determination of glutamate-oxalacetate transaminase GOT activity** (IU). GOT catalyses L-aspartate + α-ketoglutarate ↔ L-glutamate + oxaloacetate reaction. 0.1 ml of semen plasma was incubated with 0.5 ml of substrate solution (aspartic acid, α-ketoglutaric acid in phosphate buffer, pH = 7.4) for 60 min. at 37 °C. It was added 0.5 ml of 1 mM 2,4-dinitrophenylhydrazine solution in 1N HCl. It was stirred, allowed to stand for 20 minutes and then added 5 ml of 0.4 N NaOH. After 5 minutes, the sample O.D. was read on the spectrophotometer at 546 nm (REITMAN et al., 1957);

- determination of pyruvate-glutamate transaminase GPT (IU) activity. GPT catalyses the L-alanine + α-ketoglutarate ↔ L-glutamate + pyruvate reaction. 0.1 ml of the semen plasma was incubated with 0.5 ml of substrate solution (alanine, α-ketoglutaric acid in phosphate buffer pH = 7.4) for 30 min. at 37 °C. It was added 0.5 ml of 1 mM 2,4-dinitrophenyl hydrazine solution in 1N HCl. It was stirred, allowed to stand for 20 minutes and then added 5 ml of 0.4N NaOH. After 5 minutes, the sample O.D. was read on the spectrophotometer at 546 nm (REITMAN & FRANKEL, 1957);

- determination of seminal fructose (mg%). To 2 ml of deproteinized seminal plasma (with a solution of zinc sulphate and sodium hydroxide) and filtered, 2 ml of 0.1% resorcin in alcohol and 6 ml of 30% HCl were added. The sample was placed on the water bath at 80 °C for 10 minutes and then was kept in the dark for 60 minutes. The sample O.D. was read on the spectrophotometer at 540 nm. The control contained fructose solution instead of seminal plasma (JIN-CHUN LU et al., 2007);

- determination of alkaline phosphatase activity (IU). Bessey-Lowry-Brock method - under the action of phosphatases para-nitro-phenyl phosphate is hydrolyzed and the liberated phenol is yellow in alkaline medium and can be read in spectrophotometer. Suitably diluted seminal plasma (0.1 ml) according to the species was incubated for 30 min with 2 ml buffered substrate (7.6 mM sodium p-nitrophenyl phosphate solution in glycol buffer pH = 10.4) at 37^0 C. Afterwards, 10 ml of 0.1 N NaOH was added and the sample O.D. was read on the spectrophotometer at 405 nm. The control contained 2 mM para-nitrophenol solution (GALINDO, 2010);

- determination of acid phosphatase activity (IU). Diluted semen plasma (0.1 ml), according to the species, was incubated with 2 ml buffered substrate (sodium para-nitrophenyl phosphate, citric acid, sodium citrate, pH = 4.8) for 30 min. at 37°C and then the sample was read in spectrophotometer (405 nm). The same treatment was applied to another sample, to which 0.1 ml of 0.2 M sodium tartrate solution was added, to inhibit the activity of acid phosphatase of prostatic origin. After incubation, 10 ml of 0.1 N NaOH was added and the sample O. D. was read on the spectrophotometer at 405 nm. The difference between the first and the second sample is the activity of prostatic acid phosphatase (ŞERBAN et al., 1976).

RESULTS AND DISCUSSIONS

Table 1. hows the mean values and the main characteristics of variability for cytological and biochemical parameters of seminal plasma, taken from 80 bulls of Brună race.

Table 1. Mean values and variability estimates of cytological and biochemical parameters in Brună race seminal plasma.

Parameter	Minimum	Maximum	Mean	Std. Deviation	Variation coefficient (%)
Volume (VOL, ml)	2.00	14.00	5.637	1.963	34.235
Motility (MOT, %) x 10^2	0.50	0.90	0.770	0.058	7.532
Concentration (C, spermatozoa/ml x 10^9)	0.14	1.75	1.160	0.326	28.103
pH	5.90	7.50	6.505	0.302	4.642
Protein (PROT, %)	2.56	11.78	6.362	1.666	26.186
Albumin (ALB, %)	0.64	3.50	1.800	0.624	34.667
GOT (IU)	51.62	151.18	100.453	20.091	20.000
GPT (IU)	3.08	39.41	12.558	7.087	56.434
Fructose (FRU, mg %)	120.46	1231.41	602.664	233.603	38.761
Alkaline phosphatase (ALP, IU)	234.09	8947.56	2996.098	1870.850	62.443
Acid phosphatase (ACP, IU)	34.81	2463.00	903.030	532.105	58.924

Table 1 shows that, except motility and pH parameters, all of the other parameters exhibited a very high variability. This variability is more than 50% concerning enzyme activities (GPT, alkaline and acid phosphatases activity), which are directly correlated with seminal metabolism.

The mean values obtained for **cytological parameters** are the same with those reported in the literature for normal semen quality (5 to 6 ml volume of ejaculate, motility 30-90%, sperm concentration 0.2-3.0 x 10^9/ml) (CAMPBELL et al., 2009; CHENOWETH & LORTON, 2014).

Regarding the seminal plasma **pH**, the mean values (6.505 ± 0.302) fell within the limits specified by literature 6.2 - 7.5, most of the authors frequently indicating values around 6.8 (TAMBA-BEREHOIU & CONSTANTIN, 2000; KNOBIL & NEILL, 2006;). pH was characterized by the lowest variability of all analyzed parameters, suggesting that its value may be a race characteristic. Low variability also highlights the effectiveness of seminal plasma buffers in maintaining sperm viability.

The **protein content** of the seminal plasma was characterized by a medium to high variability. Numerous studies had associated low levels of seminal plasma protein with reduced sperm quality (VERMA et al., 1985; DHAMI & KODAGALI, 1989; BERGERON et al., 2004, cited by KHAN et al., 2015). However, previous researches did not confirm this relationship (ASSUMPÇÃO et al., 2005). Certainly, the proteins level is a characteristic of race and is largely influenced, even for the same race, by specific environment conditions (season, diet, animal health, age and so on.). The protein mean values obtained for Brună race (6.362 ± 1.666%) are higher than the results reported in literature for other races: 13.5 mg/l for the Neese race (ASSUMPÇÃO et al., 2005), but similar to the values presented in earlier researches 7.04% (GRAHAM, 1978; 2001).

The **albumin fraction** represented on average, approximately 28.3% of the total protein in the seminal plasma. Involvement of albumin in improving the motility of epididymal sperm is invoked in a series of studies (LINDHOLMER, 1974; SŁOWIOSKA et al., 2014).

The mean content of **fructose** in seminal plasma (602,664 ± 233,603 mg%) was similar to that reported by KIRTON et al., 1964 (681 ± 52 mg%) and higher than the mean values in the range of 460-598 mg%, reported by COLL & CUPPS (1969), who cited various bibliographic sources.

As we have shown before, enzymatic activity of spermatozoa varied within very wide limits. **Transaminase activity**, represented by **glutamate-oxalacetate-transaminase** (GOT) and **glutamate-pyruvate-transaminase** (GPT), showed lower values than those reported by other literature studies, e. g: 100. 453 ± 20.558 IU and 12.558 ± 7.087 IU versus 166.72 IU and 34.56 IU reported By CHAUHAN & SRIVASTAVA (1973) concerning bison seminal plasma, or 594.25 IU and 40.93 IU reported by HUSSAIN et al. (2016) for Holstein bulls seminal plasma. Some authors considered the GOT/GTP ratio as a quality index for the seminal material, but this ratio shows a very large variability in various studies: 8:1 in this study, 5:1 and 14.5:1 in the above mentioned studies, 42:1 declared by FLIPSE (1960) or 19:1 declared by ROUSSEL & STALLCUP (1965). Transaminase activity of seminal plasma may be an indicator of semen quality, because it occurs at least partially, as a result of an increase of sperm membrane permeability, followed by GOT and GPT enzyme discharge into the environment. Therefore, GOT and GPT activity in seminal plasma can provide a good characterization of the degree of sperm damage and implicitly of the spermatozoa fertilization capacity.

The **activity of phosphatases** in the seminal plasma, in our researches, showed significant different values from those indicated by a number of authors, respectively: 2996,098 IU for alkaline phosphatase and 903,300 IU for acid phosphatase versus 1687.5 IU and 1702,674 IU as declared by CHENOWETH & LORTON (2014), which quoted sources from literature, or 1990 IU, respectively 700 IU declared by MURDOCH & WHITE (1968). The differences declared in literature could highlight certain race traits. The importance of phosphatase activity for sperm quality is underlined by various authors. Excessive hydrolase activity involves the release of large quantities of phosphorus ions, which can inhibit spermatozoa breathing and provoke the reduction of sperm viability. Also, reduced phosphatases activities had been reported in animals exhausted by excessive reproductive exploitation (TAMBA-BEREHOIU & CONSTANTIN, 2000).

Table 2 presents the main correlations established between biochemical and cytological parameters in seminal plasma samples from Bruna bulls.

Table 2. Correlations between the main cytological and biochemical parameters.

Parameters	VOL	MOT	C	pH	PROT	ALB	GOT	GPT	FRU	ALP	ACP
VOL	1										
MOT	0.166	1									
C	0.003	**0.647*****	1								
pH	-0.182	-0.172	**-0.251***	1							
PROT	0.112	0.146	0.149	-0.092	1						
ALB	0.176	0.107	0.037	0.015	**0.798*****	1					
GOT	-0.084	-0.134	-0.008	-0.094	0.047	0.123	1				
GPT	0.148	**0.226***	0.186	-0.037	0.165	0.172	0.011	1			
FRU	0.137	**-0.232***	**-0.389*****	-0.060	**0.378*****	**0.323****	0.066	0.050	1		
ALP	0.015	0.151	0.104	-0.036	0.155	0.059	-0.159	-0.148	-0.031	1	
ACP	-0.041	**0.295****	**0.284***	-0.199	**-0.231***	-0.155	-0.025	0.096	**-0.264***	0.126	1

* Correlation is significant at the 0.05 level (2-tailed), ** Correlation is distinct significant at the 0.01 level (2-tailed), *** Correlation is very significant at the 0.001 level (2-tailed).

Table 2 shows that there are very significant positive correlations between Total Protein - Albumin (r = 0.798***), Sperm Concentration - Motility (r = 0.647***) and also, a distinct significant positive correlation between Acid phosphatase - Motility (r = 0.295**).

Fructose is the parameter that established most correlations with a high degree of significance with other parameters, namely: a very significant negative correlation with sperm Concentration (r = -0.389***), a very significant positive correlation with plasma Protein (r = 0.378***) and a distinct significant positive correlation with Albumin (r = 0.323**). Otherwise, most correlations are characterized by poor correlation coefficients, even if some were significant.

In order to interpret synthetically the relationships between parameters, we chose to process these results through a **factorial analysis**. The factorial analysis takes into account the correlations between the initial parameters values and synthesizes a smaller number of variables, called **principal components**. Table 3 presents the matrix of the 5 principal components extracted from the initial parameters values. These components covered 72% of the total variation.

Table 3. Rotated component matrix of analysed parameters.

PARAMETER	PRINCIPAL COMPONENTS (PC)				
	1	2	3	4	5
VOL	-0.040	0.147	**0.667**	0.238	0.419
MOT	**0.799**	0.173	0.104	0.084	0.207
C	**0.869**	0.110	0.002	0.041	-0.065
pH	-0.257	0.015	**-0.781**	0.130	0.299
PROT	0.050	**0.938**	0.039	-0.048	-0.030
ALB	-0.001	**0.900**	0.005	0.058	-0.056
GOT	-0.059	0.102	0.048	0.116	**-0.859**
GPT	0.268	0.222	0.071	**0.716**	0.117
FRU	**-0.559**	0.469	**0.331**	0.019	-0.016
ALP	0.219	0.194	0.042	**-0.750**	0.276
ACP	**0.557**	-0.311	0.202	-0.099	-0.088
Extraction Method: Principal Component Analysis. Rotation Method: Quartimax with Kaiser Normalization.					
a. Rotation converged in 10 iterations.					

From Table 3 we can see that the **first principal component** (PC1) is positively correlated with sperm motility (saturation level **0.799)**, semen concentration (**0.869)**, acid phosphatase activity (**0.557)** and negatively with fructose content (**-0.559)**. Principal component PC1 clearly described the quality of the semen, being positively correlated with both the number and the motility of sperm. The decrease of seminal plasma fructose level, as the sperm motility and concentration increase, was due to its properly consumption for the sperm energy needs. The presence of acid phosphatase in the component described above (PC1), was probably linked to its character of androgenity and hormonal level marker.

The **second principal component (**PC2**)** described the amount of seminal plasma protein, including albumin.

The **third principal component** (PC3) included two parameters that are not significantly correlated in the primary correlation matrix, namely: sperm volume (positive correlation) and pH (negative correlation). Some studies revealed the correlation between the two parameters in the seminal material from other species, including humans. Thus, SHA'ABANI & NASIRIAN (2014) described a significant negative correlation between the ejaculate volume and semen pH, in a study about semen from 200 infertile individuals. PETER et al. (2008) and UCHECHUKWU et al. (2015) have cited the same relationship between the two parameters in rooster semen. It is unclear to what extent this principal component (PC3) could be correlated with sperm quality, but a hypothesis may be that PC3 is a marker of the intensity of glycolytic metabolism in sperm, mediated by fructose (saturation level 0.331). Practically, in this situation pH decreases due to the accumulation of lactic acid resulted from fructose metabolism.

The diagram of the first three main components (PC1, PC2, PC3) is shown in Fig. 1.

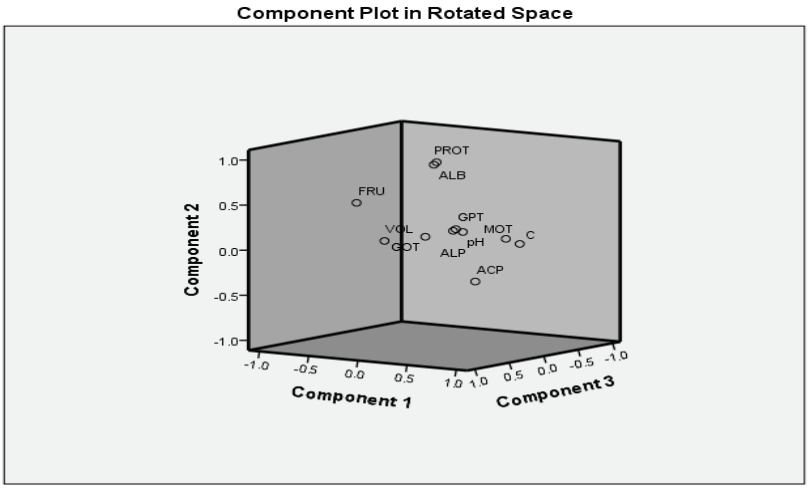

Figure 1. Diagram of the first three main components.

The **fourth principal component** (PC4) involved the activity increase of pyruvate transferase (GPT) and activity decrease of alkaline phosphatase (ALP). The significance of these relationships seemed to be related to spermatic membrane lysis phenomena, given the intracellular origin of GPT, as well as the involvement of alkaline phosphatase in the transport of phosphate groups and glucides through the spermatozoa membrane. In fact, lowering alkaline phosphatase activity may also have the effect of lowering glucidic metabolism, with direct effects on sperm motility and sperm fertility. If the third principal component (PC3) is a marker of the intensity of glucidic metabolism, the fourth component (PC4) may be considered as a marker of the metabolic intensity decrease. The correlation between the decrease in alkaline phosphatase activity and the decrease of sperm fertility was reported by various authors (TANG & HOSKINS, 1975; DHAMI & KODAGALI, 1990).

The **fifth principal component** (PC 5) was related to the decrease of GOT activity. GOT activity was not correlated with any of the cytological or biochemical quality parameters of semen, as seen in the primary correlation matrix presented in Table 1.

CONCLUSIONS

The results concerning Bruna bulls semen quality were similar to those reported in the literature for sperm cytological parameters (concentration 1.16 sp / ml x 10^9 and mobility 0.77 x 10^2%), but also for semen volume (5.6 ml), pH (6.5) and protein (6.36%) parameters. At the same time, the mean content of seminal plasma fructose (602.7 mg %) was higher than that reported by some authors, quoting sources in the literature.

Enzymatic activity of seminal plasma was characterized by a high variability. Thus, transaminase activity recorded lower values than those indicated in the literature (100.4 IU for GOT and 12.56 IU for GPT).

The activity of phosphatases was significantly different from that reported by most authors, higher for alkaline phosphatase (2996.1 IU) and lower for acid phosphatase (903.3 IU).

Except the correlations between total protein - plasma albumin ($r = 0.798$, $p < 0.001$), sperm concentration - motility ($r = 0.647$ $p < 0.001$), fructose-concentration ($r = -0.389$ $p < 0.001$) and fructose-protein ($r = 0.378$ $p < 0.001$), most of the correlations between the analyzed parameters were poor.

Data processing through factorial analysis (the principal component method) allowed the identification of 5 principal components covering together 72% of the variance of the analyzed parameters. The first principal component (CP 1) was positively correlated with sperm motility, concentration, acid phosphatase activity and negatively with fructose content.

The second principal component (CP 2) covered the total protein fraction in the seminal plasma (including albumin). The third principal component (CP 3) appeared to be a marker of the intensity of glycolytic metabolism, being positively correlated with the semen volume, the amount of fructose and negatively with seminal plasma pH. The fourth principal component (PC 4) was correlated with GPT activity increase and alkaline phosphatase activity decrease, probably in relation with plasma membranes damages and reduction of cellular metabolism intensity. The fifth principal component (PC5) was related to the decrease in GOT activity.

We consider that the obtained results are able to exemplify the utility of factorial analysis in identifying the patterns of relationships between the biochemical and cytological parameters of semen. These are solid considerations for directing further researches, to reduce the use of resources and to increase efficiency.

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