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IMPACT OF SEMINAL PLASMA INSULIN ON SOME BIOLOGICAL CHARACTERISTICS OF STALLION SEMEN: A PRELIMINARY STUDY

(With 2 Tables)

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تأثير إنسولين بلازما السائل المنوى على بعض الخصائص البيولوجية للمنى في طلائق الخيول: دراسة تمهيدية

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أجريت هذه الدراسة على ٨١ قذفة منوية تم تجميعها من ٢٣ حصان (طلوقة) عربي أصيل تتراوح أعمارهم ما بين ٣ إلى ١٩ سنة ، و كان الهدف منها تعيين وجود الإنسولين في السائل المنوى للفصيلة الخيلية . و لقد تم تقييم كل قذفة منوية معمليا من حيث الحجم و تركيز أيونات الهيدروجين و الحركة الأمامية للحيامن و تركيز الحيوانات المنوية و دليل حيوية السائل المنوى عند درجة حرارة ٣٠م، وكذلك دليل حيوية الحيوانات المنوية المحفوظة عند درجة حرارة ٥م في مخفف الترس. أيضا تم قياس تركيزات الإنسولين والجلوكوز وكذلك البروتين الكلى في بلازما السائل المنوي لكل قذفة منوية. ولقد أظهرت النتائج وجود الإنسولين في بلازما السائل المنوي بتركيزات مختلفة تتراوح ما بين ٩,٠١ و ٦٩٢,٨٦ ميكرو وحدة دولية/مللى. ولقد لوحظ من تلك الدراسة أن اختلاف تركيزات الإنسولين و الجلوكوز في بلازما السائل المنوي لم تتأثر معنويا باختلاف عمر الحصان . أظهرت النتائج أيضا أن القذفات المنوية التي تحتوى على أعلى تركيزات من الإنسJJلولين (٥٢,٦١ – ٦٩٢,٨٦ ميكرو وحدة دولية/مللى) تتميز بوجود أعلى نسبة من الحركة الأمامية للحيامن (٧١,٢٥ ± ٣,٤٩٪) ، واعلى قيمة لدليل الحيوية عند درجة حرارة ٣٠م (٠,٩٢ ± ٠,٠٥) ، وأعلى تركيز للجلوكوز (٠,٦٧ ± ٠,٠٩ ملجم/مللى) ، وكذلك أعلى تركيز من البروتين الكلى (٢٤,٥٣ ±٣,٦٨ ملجم / مللى) في بلازما السائل المنوي .

SUMMARY

In this study, 81 ejaculates were collected from 23 Arabian stallions (3-19 years old) to determine whether insulin was present in equine semen. Each semen sample was evaluated for volume, pH, sperm motility, sperm concentration, viability at 30°C and preservability in egg yolk-

Tris extenders at 5°C. Insulin was quantified in seminal plasma by immunoradiometric assay and the concentrations of glucose and total protein were also determined. The results revealed presence of appreciable levels of insulin in stallion seminal plasma, which varied from 9.01 to 692.86 µIU/ml. The age of stallion did not significantly affect the concentrations of insulin and glucose in seminal plasma. Semen samples containing the highest levels (52.61-692.86 µIU/ml) of insulin had the maximum values of sperm motility (71.25± 3.49%), viability index at 30°C (0.92± 0.05), glucose (0.67±0.09 mg/ml) and total protein (24.53±3.68mg/ml) concentrations in seminal plasma.

Key words: *Stallion; Semen; Insulin; Glucose: Protein.*

INTRODUCTION

Spermatozoa rely primarily on extracellular substrates to meet their energy requirements (Engle *et al.*, 1975). Stallion semen possesses a low level of fructose and an appreciable content of sorbitol and glucose (Mann and Lutwak-Mann, 1981). Unlike spermatozoa from other species, stallion sperm can not metabolize sorbitol and possess a limited capacity to utilize fructose (Mann, 1964). Other exogenous substrates such as glucose, lactic acid and amino acids, could be utilized by stallion sperm (Mann, 1964; Engle *et al.*, 1975).

Insulin is a normal constituent of human seminal plasma which is detected to have the ability to regulate the metabolism of freshly ejaculated spermatozoa (Hicks *et al.*, 1973; Baccetti *et al.*, 2002). Insulin increases hexose utilization, oxygen uptake, pyruvate decarboxylation and carbon dioxide production by sperm cells (Hicks *et al.*, 1973).

Accordingly, the objectives of this study were 1) to determine whether insulin was present in seminal plasma of stallions; 2) to investigate the effect of stallion age on concentrations of insulin, glucose and total protein in seminal plasma; and 3) to examine the impact of seminal plasma insulin concentrations on the quality of stallion semen.

MATERIALS and METHODS

Unless otherwise stated, all chemicals used in this study were of the highest available grade and were purchased from Sigma-Aldrich Co., Deisenhofen, Germany.

Throughout a one – year period, eighty – one semen ejaculates were collected by means of CSU model artificial vagina from twenty-

three Arabian stallions (3-19 years old) belonging to El-Zahra Arab Horse Stud, Cairo, Egypt. At the time of collection (early in the morning before offering the daily ration), an estrous mare was used as a mount animal. Prewarmed collection bottles fitted with a nylon filter were used for separation of gel from semen. Each animal was housed in a hygienic separate pen and fed 2 to 4 kg of a balanced grain ration as well as 5 to 7 kg of barseem hay daily. Clinical examination of the external genitalia and accessory organs of these animals showed no abnormality and revealed their soundness.

Immediately after collection, semen samples were transferred to the laboratory and kept in a water bath at 30 °C for evaluation of ejaculate volume (gel and gel – free semen), pH of gel-free semen (pH paper, range 6-14 with 0.30 grades) and sperm progressive motility in one drop of gel-free semen under a phase-contrast microscope (400x) equipped with a thermal stage at 37 °C. Also, sperm motility was reassessed at hourly intervals after incubation of aliquots (2ml) of gel-free semen at 30 °C for 4 hours. The viability index of incubated semen was calculated from the following formula (Milovanov *et al.*, 1964):

$S = \Sigma\left[A \times \frac{T-R}{2}\right]$, Where S is the viability index, Σ is a sign for the sum total, A is the percentage of sperm motility, T is the time of next determination of motility and R is the time of previous determination of motility. The concentration of spermatozoa in gel-free semen was estimated by direct cell count using the Thoma ruling of the improved Neubauer haemocytometer. The total number of progressive motile sperm per ml of gel-free semen was calculated.

Following the initial evaluation, a 5ml aliquot of gel-free semen was centrifuged at 1000 × g for 10 minutes and the supernatant seminal plasma was aspirated without disturbing the sperm pellet. The seminal plasma was then centrifuged at 3000 × g for 15 minutes and aliquots of the supernatant were stored at – 20 °C until determination of insulin, glucose and total protein concentrations. Insulin was quantified in seminal plasma using immunoradiometric assay kit (INSI – CTK IRMA, DiaSorin, Italy) and the results were expressed in terms of μIU/ml. The specificity (cross reactivity with horse insulin) and sensitivity of the assay were 41.80 % and 0.30 μ IU/ml at 95% confidence limit, respectively. The precision of assay in terms of within – and between – assay coefficient of variations (C.V.) were 2.60% and 4.70%, respectively. The accuracy of assay in terms of recovery percentages checked by the dilution (up to 1:16, 7.60 μ IU/ml) and recovery (100.00

μ IU/ml added concentration) tests were 98.70% and 95.30%, respectively. Glucose and total protein were determined in seminal plasma using colorimetric methods kits (Biodiagnostic, Egypt) and the results were expressed in terms of mg/ml.

Within five minutes after collection, each semen sample was prepared for short-term storage at 5 °C in the egg yolk – Tris diluent (Samper, 1992) which was composed of Tris (hydroxymethyl) amino methane (2.40g), glucose (0.45g), citric acid monohydrate (1.25g), fresh chicken egg yolk (22.00 ml), penicillin G sodium (100,000 IU), streptomycin sulfate (100 mg), and glass-distilled water to 100 ml. In brief, a 3 ml aliquot of gel-free semen was diluted (1:1) at 30 °C with the egg yolk – Tris extender and centrifuged at 500 ×g for 5 minutes. The supernatant was aspirated and sperm pellet was resuspended in 3 ml of a prewarmed (30 °C) Tris – buffered solution which was composed of the same ingredients and concentrations of the above mentioned diluent without egg yolk. The sperm cell suspension was then diluted (1:1) at 30 °C with the egg yolk – Tris extender and incubated in the refrigerator at 5 °C for 72 hours. The number of progressively motile sperm per ml of diluted semen was 50 to 250 × 10^6. Sperm progressive motility was assessed after dilution as well as after 6, 24, 48 and 72 hours of incubation period and the viability index of incubated semen was calculated as previously described for freshly ejaculated semen.

Statistical analysis of the results was carried out according to Snedecor and Cochran (1980). The variations among semen parameters due to age of stallion and the concentration of insulin in seminal plasma were examined by the Student's test (*t*-test) at 1% and 5% levels of probability. Also, linear correlation coefficients were carried out to clarify the relationships between insulin concentrations in seminal plasma and some physico-chemical characteristics of stallion semen.

RESULTS

The overall mean concentrations of insulin, glucose and total protein in seminal plasma of stallions were 38.04 ± 8.72 μ IU/ml (C.V. = 206.31%) , 0.56 ± 0.04 mg/ml (C.V. = 67.76%) and 15.03 ± 1.55 mg/ml (C.V. = 75.78%), respectively. The maximum values of insulin, glucose and total protein were 692.86 μ IU/ml, 2.49 mg /ml and 45.88 mg/ml, respectively. The corresponding minimum values were 9.01 μ IU/ml, 0.14 mg/ml and 0.62 mg/ml, respectively.

As indicated in Table 1, seminal plasma of stallions aged 11 to 19 years was characterized by presence of non-significant higher levels of insulin, glucose and insulin per mg glucose as well as a significant ($P< 0.01$) greater concentration of total protein than that in seminal plasma of stallions aged 3 to 10 years. On the contrary, the concentration of insulin per mg protein in seminal plasma was significantly ($P< 0.05$) decreased by increasing the age of stallion. The mean concentrations of insulin / mg protein for 3-10 and 11-19 years old stallions were 6.09 ± 1.35 and 3.14 ± 0.51, respectively.

Table 1: Effect of age on concentrations of insulin, glucose and total protein in stallion seminal plasma.

Stallion age (years)	Insulin (µIU/ml)	Glucose (mg / ml)	µIU insulin per mg glucose	Total protein (mg / ml)	µIU Insulin per mg protein
3-10 (n = 28)*	25.18 ± 3.70	0.50 ± 0.04	52.31 ± 6.68	8.55 ± 1.61^{A}	6.09 ± 1.35 ^{a}
11-19 (n = 53)**	45.67 ± 12.93	0.59 ± 0.05	65.04 ± 10.04	17.52 ± 1.92^{B}	3.14 ± 0.51^{b}

Means ± SEM with dissimilar superscripts in the same column are significantly different at $P<0.01$ for letters A, B and at $P<0.05$ for letters a, b.
n = Number of ejaculates obtained from 11* and 12** stallions.

Frequency distribution of insulin concentrations in seminal plasma revealed that 44.44%, 40.74% and 14.82 % of semen ejaculates fell in the ranges of 9.01 – 19.96, 20.02 – 49.27 and 52.61 – 692.86 µIU /ml, respectively. A significant ($P<0.01$) difference was detected between means of these concentration ranges (Table 2).

The overall means of gel volume, gel-free semen volume, pH, sperm motility, sperm concentration and number of motile sperm per ml of semen were 4.12 ± 0.57 ml, 25.10 ± 4.00 ml, 7.46 ± 0.04, 58.49 ± 3.66%, 372.82 ± 43.58 × 10^6/ ml and 232.11 ± 33.89 × 10^6, respectively.

As can be seen from Table 2, increasing the concentrations of insulin and protein in seminal plasma was associated with a significant ($P<0.01$) augmentation in the concentrations of insulin / mg glucose as well as a pronounced improvement in sperm motility percentages and viability indices at 30 °C. The maximum values of sperm motility (71.25 ± 3.49%), viability index at 30 °C (0.92 ± 0.05), motile sperm / ml (257.87 ± 39.06 × 106), glucose concentration / ml (0.67 ± 0.09 mg), insulin concentration / mg glucose (172.68 ± 53.93 µIU) and protein concentration /ml (24.53 ± 3.68 mg) were recorded for semen samples

which contained the highest levels (52.61 – 692.86 µIU/ml) of insulin in seminal plasma. On the other hand, none of the other semen parameters including volume, pH, viability index at 5 °C, sperm concentration and insulin concentration / mg protein were significantly affected by the variation of insulin concentrations among semen samples.

Table 2: Influence of seminal plasma insulin on the quality of stallion semen.

Semen Parameters	Insulin concentrations (µIU / ml)		
	9.01 – 19.96 12.88 ± 0.46^{a} (n = 36)	20.02 – 49.27 32.28 ± 1.32^{c} (n = 33)	52.61 – 692.86 127.34 ± 52.61^{d} (n = 12)
Volume of gel (ml)	4.75 ± 1.21	4.43 ± 0.95	3.53 ± 0.55
Volume of gel-free (ml)	24.04 ± 3.02	30.70 ± 4.81	23.25 ± 4.17
pH	7.53 ± 0.07	7.39 ± 0.04	7.47 ± 0.02
Motility (%)	45.47 ± 4.54^{a}	58.75 ± 2.94^{b}	71.25 ± 3.49^{c}
Viability index (30°C)	0.56 ± 0.07^{a}	0.82 ± 0.08^{bc}	0.92 ± 0.05^{c}
Viability index (5 °C)	44.17 ± 4.69	44.24 ± 4.01	45.09 ± 4.26
Concentration (×10^{6} /ml)	390.13 ± 40.67	366.26 ± 39.72	362.08 ± 50.34
Motile sperm (× 10^{6} /ml)	196.04 ± 31.70	242.43 ± 30.90	257.87 ± 39.06
Glucose (mg / ml)	0.51± 0.04	0.58 ± 0.07	0.67 ± 0.09
µIU insulin / mg glucose	32.30 ± 2.84^{a}	61.84 ± 3.81^{c}	172.68 ± 53.93^{d}
Protein(mg / ml)	10.73 ± 2.47^{a}	15.13 ± 1.87^{ab}	24.53 ± 3.68^{c}
µIU insulin / mg protein	3.74 ± 0.94	3.35 ± 0.64	5.41 ± 1.43

Means ± SEM in the same row with dissimilar letters are significantly different at P<0.01 for a – c, c – d, a – d and at P< 0.05 for a – b, b – c, ab – c, a – bc. (n = number of ejaculates).

Computation of correlation coefficients disclosed significant (P<0.01) positive relationships between insulin concentrations in seminal plasma and each of sperm motility percentages (r = + 0.53) and glucose concentrations in seminal plasma (r = + 0.52). Also, there was a significant (P<0.01) positive correlation coefficient (r = + 0.74) between the concentrations of insulin / mg glucose in seminal plasma and sperm motility percentages. However, low but significant (P<0.05) positive correlation coefficients were obtained between sperm motility percentages and each of protein (r = + 0.27) and glucose (r = + 0.36) concentrations in seminal plasma. With the exception of protein concentrations in seminal plasma (r = + 0.57; P< 0.01), none of the remainder semen parameters correlated significantly with the concentrations of insulin in seminal plasma.

DISCUSSION

The data presented herein declared, for first time, the presence of significant levels of insulin in equine semen. The mean insulin concentration (38.04 ± 8.72 μIU/ ml) in stallion seminal plasma was higher than that (19.20 ± 2.70 μIU / ml) found in human seminal plasma (Hicks *et al.*, 1973). Decidedly, the significant positive relationships that were observed in the current study between sperm motility and each of insulin and glucose concentrations in seminal plasma might, in part, reflect the importance of this hormone for initiation and regulation of sperm metabolic activity. For instance, increasing the availability of insulin per mg glucose from 32.30 ± 2.84 to 61.84 ± 3.81 and 172.68 ± 53.93 μIU was accompanied by a remarkable amelioration in sperm motility percentages from 45.47 ± 4.54 to 58.75 ± 2.94 and 71.25 ± 3.49%, respectively. Concomitantly, the number of motile spermatozoa per ml of gel-free semen was also increased from 196.04 ± 31.70 to 242.43 ± 30.90 and 257.87 ± 39.06 × 10^6, respectively. In agreement with our findings, Younis *et al.* (1998) detected a significant improvement in sperm motility after supplementation of chimpanzee semen with insulin. However, in human semen, Paz *et al.* (1977) and Makler *et al.* (1980) ruled out the role of insulin in regulation of sperm kinematic activity.

The insignificant dependence of seminal plasma insulin and glucose on age of stallion might point out the necessary need of ejaculated sperm cells at all ages for satisfactory levels of insulin and glucose capable of initiating their progressive motility.

Considering insulin as an important factor in the regulation of carbohydrate metabolism (Baccetti *et al.*, 2002), the favourable influence of insulin on sperm motility might be attributed to its ability to bind with specific receptors on sperm plasma membrane, increase fusion of glucose transport proteins with plasma membrane and, in turn, facilitate influx of glucose, amino acids, potassium ions and phosphate ions into sperm cells (Hicks *et al.*, 1973; Guyton and Hall, 1996). Likewise, it was claimed that insulin was capable of stimulating oxygen uptake, pyruvate utilization and carbon dioxide production by sperm cells (Hicks *et al.*, 1973) as well as increasing the initial phosphorylation of intracellular glucose by activating a local protein kinase which in turn caused phosphorylation and activation of glucokinase, a key enzyme that involved in stimulation of the pentose shunt and Krebs cycle (Guyton and Hall, 1996). Also, it was speculated that insulin had a specific effect

on sperm cell metabolism through activation of intracellular pyruvate dehydrogenase and citrate-synthase (Hicks *et al.*, 1973).

Concerning sperm viability, it was evident from our data that maintenance of sperm motility over the incubation periods was dependent on presence or absence of seminal plasma and its constituents. Increasing the concentrations of insulin and total protein in seminal plasma was associated with a stepwise augmentation in the viability indices of whole semen at 30°C. However, after centrifugation and removal most of seminal plasma (~99%), the viability indices of stored spermatozoa at 5 °C were not significantly influenced by the variation in insulin concentrations among semen samples.

Since there were significant positive relationships between protein concentrations in seminal plasma and each of insulin concentrations and sperm motility percentages, it seems that seminal plasma may contain a glucose tolerance factor, such a protein bound trivalent chromium, which is responsible for strengthening of insulin action on living cells (Tietz, 1986). In fact, the latter assumption may highlight the significance for existence of an expedient level of seminal plasma during preservation of stallion semen (Braun *et al.*, 1994). It was observed that the motility of chilled-stored epididymal and ejaculated stallion spermatozoa was significantly better maintained in samples containing 25% versus 0% seminal plasma (Braun *et al.*, 1994).

In the present study the overall mean concentration of total protein (15.03 ± 1.55 mg/ml) in seminal plasma was nearly comparable to that (14.74 ± 0.03 mg / ml) reported by Sakran (1996). Moreover, in accordance with our results, the latter author detected a significant augmentation in the concentration of total protein in seminal plasma by advancing the age of stallion.

It is worthy to note although insulin has been identified as a protein hormone (Guyton and Hall, 1996), the clear increase in seminal plasma protein concentrations of stallions aged 11 to 19 years was not associated with an increase in insulin concentrations since at this age, there was a significant reduction in the concentration of insulin per mg protein of seminal plasma. Consequently, the variability in insulin concentrations among semen samples might be ascribed rather for individuality or season than for age of stallion.

In conclusion, seminal plasma of Arabian stallions possessed an appreciable level of insulin. The close associations between insulin, glucose, total protein, sperm motility and viability may suggest the pivotal role of this hormone in regulation of sperm metabolism. Further

research will be prerequisite to 1) investigate the effect of individuality and seasons on the concentrations of insulin in stallion seminal plasma; 2) characterize the source of insulin within stallion reproductive tract; 3) verify presence of insulin receptors on sperm plasma membrane as well as presence of chromium (a glucose tolerance factor) in stallion seminal plasma; 4) explore the influence of endogenous and exogenous insulin on sperm metabolism; and 5) study the effect of in vitro provision of semen extenders with insulin on preservability of stallion spermatozoa.

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