Egypt. J. Vet. Sci. **Vol. 47**, No.1, pp. 13 – 26 (2016)

Semen Characteristics and Genotyping of Pituitary-Specific Transcription Factor Gene in Buffalo Using PCR-RFLP

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THIS STUDY aimed to evaluate the sperm characteristics in buffalo characteristics in buffalo bulls and to screen the genetic genetic polymorphisms in PIT-1 gene as bases for selection of bulls with good breeding value. The study was performed on 60 buffalo bulls aged 2-8 years. The animals were divided into three groups according to the age. The first group were between 2 to <3 years (n= 35). The second group were between 3 to <5 years (n=14). The third group were between 5 to <8 years (n=11).Three semen collections were obtained from each animal at 15-day intervals and evaluated for volume, individual motility, live sperm and chromatin integrity %. The semen samples were stored at 20 °C until DNA extraction then polymerase chain reaction (PCR) and DNA amplification were carried out. Restriction fragment length polymorphism (RFLP) was used for genotyping of pituitary-specific transcription factor gene using HinfI-RFLP. The results showed that, ejaculate volume, individual motility and live sperm % were significantly lower in old than adult and young buffalo bulls. While chromatin damage percentage had no significant difference among groups. All buffalo bulls were genotyped as BB with the predominance of B allele where PCR 451 bp fragment was digested into two fragments 244 and 207 bp.

It may be concluded that age had adverse effect on semen quality. Monomorphic pattern of the amplicon 451 bp in PIT-1HinfI locus in exon 6 was fixed in Egyptian buffalo with the predominance of B allele and BB genotype in a high frequency (100%).

Keywords: Buffalo, Semen evaluation, PIT-1(POU1F1) gene, Polymorphism, RFLP.

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Semen evaluation is extremely important for a successful artificial insemination (AI) in which a large number of straws is produced from an ejaculate (Barros *et al*., 2010) . Al becomes a tool of disseminating superior genes for economic traits like milk production by propagating the animals with high genetic potential (Baruselli and Carvalho, 2005). From a biological point of view, only viable spermatozoa carrying intact genetic material are potentially fertile and therefore, most of the accurate methods of semen evaluation focus on sperm viability and DNA integrity (Januskauskas and Zilinskas, 2002). Motility is probably the most often used technique for routine semen evaluation. However, the accuracy of this test is low and it depends on the experience of the operator (Graham *et al*., 1980). Probably for this reason, reports on the relationship between sperm motility and fertility are not fixed (Kjaestad *et al*., 1992) but other authors reported a significant correlation between sperm motility and bull fertility (Budworth *et al*., 1988). Chromatin integrity % has been found to have a significant correlation with bull fertility (Kjaestad *et al*., 1992). However, such parameters don't always indicate the level of fertility in the field (Den daas, 1992).

Pituitary-specific transcription factor (PIT-1) is the factor for stimulating expression of growth hormone, prolactin (Cohen *et al*., 1996), the thyroid stimulation hormone (TSH) β-subunit (Steinfelder *et al*., 1991), the gonadotropin releasing hormone receptor genes (Lin *et al*., 1992) and the PIT-1 gene itself (Rhodes *et al*., 1993). PIT-1(POU1F1) gene is located near to the centromeric region of bovine chromosome 1 (Moody *et al*., 1995**).** The bovine PIT-1 gene consists of 6 exons and 5 introns encoding a polypeptide chain of 291 amino acids (Pfäffle *et al*., 1992). The growth and function of mammary gland is mainly controlled by the growth hormone and prolactin, which are secreted in the anterior pituitary gland and their synthesis is regulated under the influence of pituitary specific transcription factor1 (Carsai *et al*., 2012).

Several studies suggested that the PIT-1 polymorphisms have a key role in milk yield and, to a lesser extent, in determining the fat percentage in dairy cattle (Dybus *et al*., 2004). PIT-1/Hinf1 polymorphism was primarily recognized to be associated with milk yield, protein yield and traits of body conformation in Holstein-Friesian bulls in Italy (Renaville *et al*., 1997a). Other authors reported that there are associations of the PIT-1HinfI variants on fat yield (De Mattos *et al*., 2004**).** Moreover, it was stated that PIT-1/HinfI polymorphism had a significant effect on growth performances in Nanyang cattle (Kai *et al*., 2006) and Podolica young bulls (Selvaggi *et al*., 2011**).** Depending on important roles of this gene in milk and growth traits, the objective of the present study was to evaluate the sperm characteristics in buffalo bulls and to screen the genetic polymorphisms in PIT-1 gene as bases for selection of bulls with good breeding value.

Material and Methods

Animals and samples

The study was performed on 60 buffalo bulls aged 2-8 years. The animals belonged to a breeding station at Mahalet Mussa near to Sakha, Kafr el-Sheikh Governorate. The animals were divided into three groups according to the age. The first group was between 2 to < 3 years (n= 35). The second group was between 3 to <5 years (n=14). The third group ages were between 5 to <8 years (n=11). Semen samples were collected using artificial vagina early before feeding at 8.00 a.m. Three collections were obtained from each animal at 15-day intervals and evaluated immediately. The semen samples were stored at -20 °C for DNA extraction. Each animal was subjected to fresh semen evaluation for three times.

Semen evaluation

Volume of the ejaculate was estimated to nearest 0.1 ml. Individual motility was expressed as the percentage of forward motile spermatozoa. Live sperm percentage was estimated using eosin-nigrosin stained smears (Felipe-Pérez *et al*., 2008**)** . Chromatin integrity was recorded using acridine orange (AO) staining technique (Martins *et al*., 2007 and Mahmoud *et al*., 2015). Briefly, smears of semen were prepared on glass slides for each animal, air-dried and fixed for overnight in Carnoy's solution composed of 3:1 methanol and glacial acetic acid. The slides were air-dried and approximately 1 mL of working solution was mounted on each slide for 5 min. The working solution was freshly prepared at the day of examination as follows: 4 ml of 1% AO in distilled water was added to a mixture of 16 ml of 0.1 M citric acid and 1.0 ml of 0.3 M $Na_2HPO_4.7H_2O$. The stained slides were washed in water to remove the background staining, dried and evaluated with a fluorescence microscope. Sperm with normal DNA content presented by a green colored patch but those with an abnormal ssDNA content presented fluorescence that vary from yellow to red colored patch. A total of 200 spermatozoa were evaluated from each bull.

DNA extraction from sperm cells

DNA was extracted from fresh semen according to Weyrich **(**2012) with slight modifications. Fifty µl of semen was washed in 500 µl of 70 % ethanol then the samples were centrifuged for 5 min at 10,000 ×g and the supernatant was removed. The former steps were repeated until the supernatant became clear and an easily visible and a white pellet was obtained. About 500 µl lysis buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, 100 mM NaCl, 1 % SDS aqueous solution) was added to the sperm pellet. Also, 5 µl Triton-X100 (0.5 %), 25µl Dithiothreitol (DTT) 1M and 50 µl proteinase K (20mg/ml) were added. The samples were mixed well and incubated at 50 °C overnight in a thermo shaker. The tubes were centrifuged for 10 min at 15,500 × g and the supernatant was transferred into a new 1.5 ml tube. Sodium acetate (NaAc) 3M was added to the supernatant (bout 1/10 Vol. of the supernatant) and mixed gently. NaAc binds to

the DNA and eases the precipitation. An ice cold absolute ethanol was added to the tubes (2 Vol. of the supernatant). DNA was precipitated at -20°C overnight and pelleted by centrifugation for 20 min. at 15,500 x g. The supernatant was carefully removed by pipetting and the white pellet of DNA remained. DNA was washed by dispensing the pellet in 500 µl ethanol (75 %). The samples were centrifuged for 10 min at 15,500x g at room temperature (25-30 °C). The samples were dried until ethanol was evaporated. Avoid drying the pellet completely because it may affect DNA. DNA pellets were dissolved in 50 µl ddH_2O and concentration was measured using NanoDrop1000 Thermo Scientific spectrophotometer then diluted to working concentration of 50 ng/µl, which is suitable for PCR.

Polymerase Chain Reaction (PCR) and DNA amplification

The DNA fragment of PIT-1gene was amplified through polymerase chain reaction technique developed by Mullis *et al*. (1992). The PCR mixture consisted of 12.5 µl of PCR master mix (2X) composed of 0.1 U/ µl Taq polymerase, 500 µM of dNTP each, 20 µM of Tris-Hcl (pH 8.3), 100 mM of Kcl, 3 mM of MgCl, stabilizer and enhancer. In addition to 1.0 µl of forward primer F (20pM/ µl), 1.0 µl of reverse primer (20pM/ µl), 2.0 µl of DNA (50 ng /µl) and add water up to 25 µl. The sequence of the primers used was F: 5′AAACCATCATCTCCCTTCTT3′ and R: 5′AATGTACAATGTGCCTTCTGAG-3′ (Selvaggi *et al*., 2011**)** for amplification of 451 bp fragment in intron 5 and exon 6 of PIT-1 gene. PIT-1PCR program for amplification was initial denaturation 95 °C for 3 min. then 37 cycle of denaturation 95 °C for 1 min., then an annealing at 54 °C for 1 min., then an extension at 72°C for 1 min. and a final extension at 72°C for 5 min. The PCR reaction products were showed on 2% agarose gel via electrophoresis and stained with red safe to be visualized on UV transilluminator.

Restriction fragment length polymorphism (RFLP)

The PCR products for PIT-1 gene were digested with HinfI restriction enzyme. The restriction mixture for each sample was prepared by adding 2.0 µl of 10 × restriction buffers to 1.0 µl of the appropriate restriction enzyme and the volume was completed to 20 µl by dd H_2O then mixed with 10 µl PCR product. Fast digest restriction enzyme was used. This restriction mixture was incubated at 37°C for 25 min. The digested PCR products were showed on 3% agarose gel by electrophoresis and stained with red safe to be visualized on UV transilluminator.

Statistical analysis

The obtained data of semen were expressed as mean ± SE. The effect of age on the studied semen parameters was tested using analysis of variance (ANOVA) using SPSS version 16 software. Comparison of means was performed by Duncan's Multiple Range Test and the differences were considered to be significant at $P < 0.05$.

Results

Semen evaluation

Data illustrated in Table 1 showed that, ejaculate volume (ml) was significantly higher ($p<0.01$) in bulls aged 3 to < 5 years (3.12 ± 0.26) than 2 to < 3 years (2.55 ± 0.09) and 5 to ≤ 8 years (2.31 ± 0.05). Individual motility percentage was significantly ($P<0.01$) higher in bulls aged 3 to < 5 years (80.15 ± 0.85) and 2 to < 3 years (77.65 ± 0.97) than 5 to ≤ 8 years (73.70 ± 1.12). Live sperm percentage was significantly ($P<0.01$) higher in bulls aged 2 to <3 years (81.75±0.84) and 3 to < 5 years (81.75 ± 0.68) than 5 to ≤ 8 years (78.35 ± 0.92), Table 1 and Fig.1. Chromatin damage percentage had no significant difference among groups (Table 1 and Fig. 2).

TABLE 1. Effect of age on fresh semen characteristics of buffalo bulls (Mean ±SE).

Age (year)	Ejaculate volume (ml)	Individual motility %	Live sperm %	Chromatin damage %
2 to <3(35 animal)	2.55±0.09 [b]	77.65±0.97 [a]	81.75±0.84 [a]	0.96±0.20 [a]
3 to <5 (14 animal)	3.12±0.26 [a]	80.15±0.85 [a]	81.75±0.68 [a]	0.92±0.18 [a]
5 to ≤8 (11 animal)	2.31±0.05 [b]	73.70±1.12 [b]	78.35±0.92 [b]	0.57±0.08 [a]

Values within the same column with different alphabetical letters were significantly different ($p<0.01$).

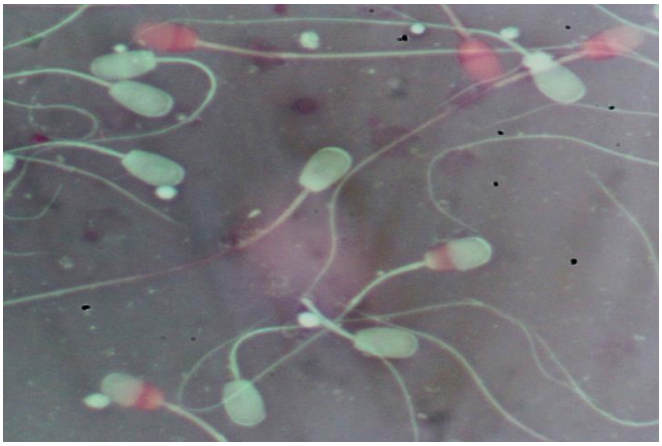

Fig.1. Light microscopy for buffalo spermatozoa stained by eosin nigrosin stain, live sperm appeared white unstained and dead sperm appeared pink or red color of eosin stain (x1000).

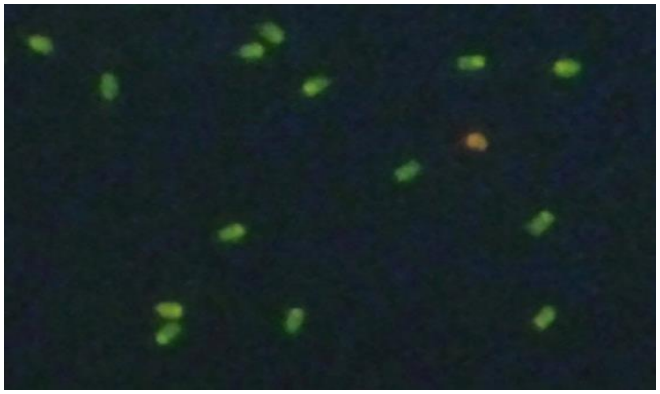

Fig.2. Fluorescence microscopy for buffalo spermatozoa stained by acridine orange stain, damaged chromatin appeared yellow or red color of acridine orange stain and normal chromatin appeared green color (×400).

Genetic characterization of pituitary transcription factor (PIT-1) gene

The present work examined the genetic polymorphism of PIT-1 gene in 60 buffalo bulls using PCR–RFLP technique. The PCR product of PIT-1 gene was 451 bp in all tested buffalo bulls (Fig. 3).

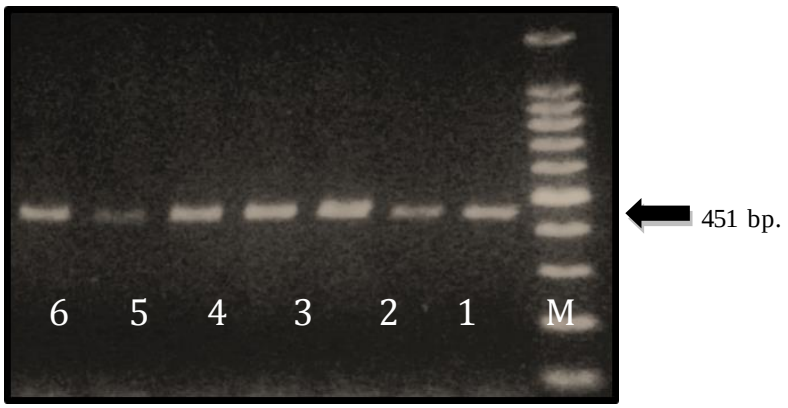

Fig. 3. Red safe-stained gel of PCR products representing amplification of Pit-1 gene in buffalo bulls visualized on 2% agarose gel. M: 100-bp ladder marker. Lanes 1-7 resemble 451-bp PCR products.

By using RFLP technique, digestion of the amplified PCR 451 bp fragment with HinfI restriction enzyme, all buffalo bulls were genotyped as BB with the predominance of B allele where PCR were digested into two fragments 244 and 207 bp (Fig.4). These results proved the presence of HinfI restriction site in this part of PIT-1 gene in all studied buffalo bulls, hence monomorphic pattern of that fragment of this gene.

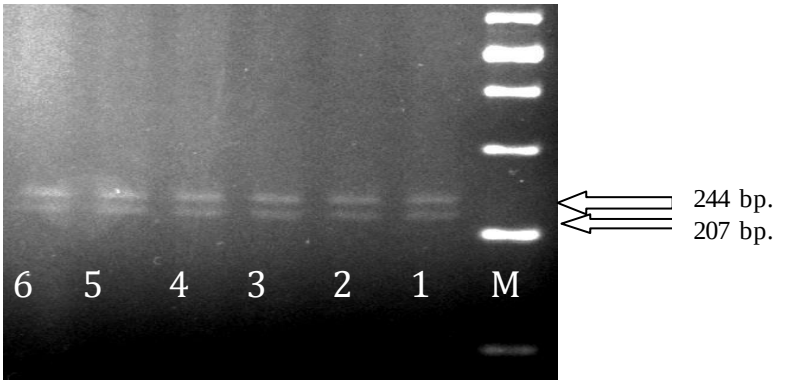

Fig. 4. Red safe-stained gel of RFLP products obtained after digestion of PIT-1 PCR with HinfI restriction enzyme visualized on 3% agarose gel. M: 100-bp ladder marker. Lanes 1-6 Homozygous BB genotypes showed two digested fragments at 244 and 207 bp.

Discussion

Semen evaluation is probably the most common procedure to evaluate male fertility in domestic animals (Rodríguez‐Martínez, 2003). In the present study, ejaculate volume in the Egyptian buffalo bulls was significantly ($P<0.01$) higher in adult than in young and old buffalo bulls. In accordance with our results, different studies proved that, ejaculate volume increased with increasing the age and then decreased in old buffalo bulls **(**Nordin *et al.*, 1990, Younis,1996 and Pant *et al.*, 2003**)** and bull (Ahmad *et al.*, 2011 and Bhakat *et al.*, 2011). Variation in semen volume reported by different studies might be due to differences in genetic information, age, and reproductive health of bulls, frequency of semen collection, nutrition, management and season (Nazir, 1988). Variations can also be due to skill of semen collector/attendant and temperature of artificial vagina. Moreover, Almquist (1978) attributed the increased volume and number of cells per ejaculate, with the age of the bull up to 4 - 5 years, to the increase in the size of the testes. In contrast to our findings, Ghosh (2004) stated that volume of semen had no significant differences between age groups especially at younger ages.

Individual motility percentage, in the different age groups, was significantly ($P<0.05$) higher in adult and young than old buffalo bulls. This finding was in agreement with Younis (1996) in Nili-Ravi buffalo bulls that attributed the lowering percentage in old bulls to senility changes. Similarly, Kiani *et al.* (2014) reported that the age had significant effect on sperm motility which is lower in old bulls than in young and adult bulls in Kundhi Buffalo. In contrast, Ghosh (2004) recorded that individual motility had no significant effect between age groups. Percentage of progressive forward motility is in normal range of fertile buffalo bulls and suitable for fertilization in the studied population. The

average percentage of individual motility, range from 65% to 80% in fertile bulls and buffalo bulls, depends on the age of the sires (Koonjaenak *et al*., 2007).

The live sperm percentage reported in the present study agreed with the recommendation for a normal fertile bull in different age groups (Dhami *et al*., 1998). Live sperm percentage in buffalo spermatozoa was about 65-75 % which was assessed by using eosin-nigrosin (Nordin *et al*., 1990 and Mahmoud *et al*., 2016). Also, the chromatin damage reported in the present study agreed with that recorded for fresh buffalo semen (Mahmoud *et al*., 2016), which is less than 1% with no significant differences between the bulls in different ages.

The PCR amplified fragment of PIT-1 gene was at the expected size, 451 bp. By digestion of the amplified PCR 451 bp fragment with Hinf1 restriction enzyme, all buffalo bulls were genotyped as BB with the predominance of B allele with no genetic variation in all studied buffalo bulls. Genotype of PIT-1/HinfI obtained in this study was similar to that reported previously in buffaloes and some cattle breeds with low variation or no polymorphism. The studies in Indonesian (Misrianti *et al*., 2011) and Egyptian buffaloes (Othman *et al*., 2011) indicated all animals genotyped as BB (100%) with the predominance of B allele with no polymorphism. On the other hand, different studies recorded polymorphisms in PIT-1/HinfI locus, Javanmard *et al*. (2005) reported low variation in Iranian river buffalo where BB genotype recorded as (0.80) while AA and AB was (0.10) with B allele (0.85) and A allele (0.15). Mukesh *et al*. (2008) revealed the predominance of BB genotype and B allele with a mean frequency of 0.881 and 0.937, respectively, in Indian native cattle (*Bos indicus*), while in Nagori cattle all the animals were homozygous for BB genotype and B allele (100%). Mattos *et al*. (2004) recorded high frequency of B allele (0.95) and BB genotype (0.90) and AB (0.10) in Brazilian Gyrsian cattle, with complete absence of AA genotype and concluded that the heterozygous bulls (AB) were superior for milk fat yield production. Jawasreh *et al*. (2009) showed that genotype frequencies of PIT-1/HinfI polymorphism in Jordanian native cattle population were BB (0.82) and AB (0.18) with the predominance of B allele (0.91). Renaville *et al*. (1997a) recorded three patterns of polymorphism with frequencies 2.2, 31.5, and 66.3% for AA, AB, and BB, respectively and showed a significant superiority of PIT-1A allele over the B allele for milk and protein yields compared to fat percentage in Italian Holstein-Friesian Bulls.

The polymorphisms of PIT-1 gene and its relationship with growth and carcass traits were confirmed by different studies in various breeds. Zhang *et al*. (2009) found a favorable positive effect of the B allele on growth trait in Germany Yellow × Quinchuan cross cattle. Renaville *et al*. (1997b) found a relationship of higher body weight at 7 months of age with B allele in Belgian Blue bulls. Kai *et al*. (2006) recorded the frequencies of alleles A and B were 0.465 and 0.535 respectively and found a significant effect of B allele on the growth performance traits from birth to yearling Nanyang cattle. Also, Selvaggi *et al*. (2011) recorded that the allelic frequencies at PIT-1/HinfI locus were A

and B representing 0.3 and 0.7 ratio respectively and a positive association of B allele with growth traits in Podolica young bulls was also observed. Moreover, the association between polymorphisms of PIT-1gene with growth and carcass traits in Hanwoo (Korean cattle) was reported on carcass weight (Han *et al*., 2010). In addition**,** Ribeca *et al*. (2014) found an association between PIT-1/HinfI polymorphism and carcass weight and carcass daily gain in double muscled Piemontese cattle. Also, Thomas *et al*. (2007) studied the associations of PIT-1/HinfI polymorphism with growth and carcass traits in two populations of Brangus bulls and detected a significant effect with intramuscular fat.

It may be concluded that semen collected from mature bulls had better quality than the semen of young and old bulls. Monomorphic pattern of the amplicon 451 bp in PIT-1HinfI locus in exon 6 was fixed in Egyptian buffalo with the predominance of B allele and BB genotype in such a high frequency (100%).

Declaration of interest: The authors declare that there is no conflict of interest.

Acknowledgements : This research was supported by National Research Centre, Cairo, Egypt. Project No (10060116).

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(*Received* 22/ 8/2016;
accepted 29/11/2016)

خصائص السائل المنوي والتنميط الجيني لجين عامل النسخ المحدد للنخامية (PIT1) في الجاموس باستخدام PCR-RFLP

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هدفت هذه الدراسة إلى تقييم السائل المنوي لطلائق الجاموس ودراسة الطرز الجينية المتعددة لجين عامل النسخ المحدد للنخامية (PIT1) وذلك كوسيلة لاختيار الطلائق ذات القيمة التربوية الجيدة. تمت الدراسة على ستين طلوقة جاموسي وقسمت الحيوانات إلى ثلاث مجموعات بناء على العمر .المجموعة الأولى مابين سنتين الى أقل من ثلاث سنوات وتضم ٣٥ طلوقة والمجموعة الثانية مابين ثلاث الى أقل من خمس سنوات وتضم ١٤ طلوقة والمجموعة الثالثة مابين خمس إلى ثمان سنوات وتضم ١١ طلوقة. تم تجميع ثلاث قذفات من السائل المنوي لكل طلوقة كل ١٥ يوم وفحصه مباشرة لحجم القذفة ونسبة الحيامن الحيه ونسبة الحركة الفردية الطبيعية وأيضا نسبة تكسير الكروماتين .تم حفظ السائل المنوي عند -٢٠°م حتى استخلاص الحمض النووى منه واستخدمت التقنيات المرتبطة بدراسة الدلائل الوراثية للجينات مثل تفاعلات البلمرة المتسلسل (PCR) و(RFLP). أثبتت النتائج أن عمر الحيوانات له تأثير معنوي حيث كانت الحيوانات الناضجة هي الأفضل في حجم القذفة ونسبة الحيامن الحية ونسبة الحركة الفردية الطبيعية بينما لم يؤثر العمر على نسبة تكسير الكروماتين. نتج عن استخدام تفاعل البلمرة المتسلسل لجين عامل النسخ المحدد للنخامية قطعة طولها ٤٥١ نيوكليتيدة مزدوجة، ونتج عن استخدام تقنية RFLP أن جميع الحيوانات تمتلك طرز جينى مماثل BB حيث تم قطع نتائج البلمرة المتسلسل باستخدام انزيم القطع (Hinf1) في كل الحيوانات بدون اختلافات وراثية وكان الأليل السائد هوB حيث فصلت القطعة ٤٥١ إلى قطعتين ٢٠٧ و ٢٤٤.

والخلاصة أن عمر الحيوان له تأثير معنوي على تقييم السائل المنوي. وجود نمط جيني واحد لجين عامل النسخ المحدد للنخامية في الجاموس المصري (BB) بدون اختلافات وراثية.

الكلمات الدالة :الجاموس -السائل المنوي - تفاعل البلمرة المتسلسل -الطرز الجينية المتعددة -PIT1 – PRL .