**Testicular cytology of alpaca: Comparison between impressed and smeared slides**

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**A R T I C L E   I N F O**

Article history:
Received 7 April 2010
Received in revised form 9 March 2011
Accepted 11 March 2011
Available online 23 March 2011

**Keywords:**
Testicular cytology
Alpaca

**A B S T R A C T**

Testicular fine needle aspiration (TFNA) has proven to be a simple and minimally invasive procedure, which allows assessments of cytological parameters of seminiferous epithelium/tubules more accurately in a short time. Though this technique does not cause negative effects on sperm quality or any damage to testicular tissue, its use is very limited in male animal infertility diagnostics. Report on the use of this technique in South American Camelids (SAC) is very limited. Therefore, the aim of this study was to evaluate the efficacy of TFNA for identification of different testicular cells and cell indices, and their correlation with that of impression cytology. A total of 98 slides were prepared from testes of six adult alpaca males, collected immediately after slaughter. Aspiration samples were performed by inserting a fine butterfly needle (21 G) connected to a 50 ml syringe into a testicle and multiple plane aspirations were carried out to obtain the materials destined to the smear. Three different imprints on slides were taken from each testicle. All slides were air-dried, stained with modified May–Grunwald–Giemsa (MGG) stain and then examined under light microscope with 1000× magnifications. Spermatogenic cells such as, spermatogonia (Sg), primary spermatocytes, secondary spermatocytes, early spermatids (ab), late spermatids (cd) and spermatozooa, and Sertoli cells were counted. The spermatozoa percentage was expressed as spermatocit index (SI) and the number of Sertoli cells, counted apart, was expressed as sertoli cell index (SEI). There was no any significant difference between the spermatogenic cell parameters obtained from the two types of slides, but SEI were significantly different in two types of smears. The results of the study provide support for the use of TFNA as a useful minimally invasive modality to identify different spermatogenetic cell classes in alpaca. Moreover, the possibility to standardize this method might provide a greater impulse to the clinical diagnostics of SAC male infertility.

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1. **Introduction**

The reproductive system of the male cameld presents several anatomical and physiological peculiarities (Tibary and Vaughan, 2006) and they are necessarily bred at older ages than most domestic species. Testicular growth in these species is slow and maximum size is not reached until 3 years of age (Bravo and Johnson, 1994). Though sperm production starts as early as 10–12 months of age in a few male alpacas (Smith et al., 1994), male alpacas do not reach full maturity until 5 years of age. Therefore, proper clinical examination and reproductive analysis of male alpacas are necessary for evaluation of breeding soundness and infertility, and several techniques such as testicular palpation, ultrasonography of reproductive organs and testicular biopsy have been performed during sire selection.
(Tibary and Vaughan, 2006). Generally, testicular biopsy has been used to evaluate and classify males with varying degrees of testicular failure (Papic et al., 1988) and it provides a definitive assessment of seminiferous tubule and interstitial cellular architecture (Gottschalk-Sabag et al., 1993; Turek et al., 1997). Among different biopsy techniques, TFNA has gained increasing popularity as a painless, minimally invasive and cost-effective procedure, which can provide a more representative sample of the testis in much shorter time as compared to open biopsy. TFNA has proved to be useful diagnostic tool for male infertility (Mahajan et al., 1999), testicular tumors as well as in non neoplastic and inflammatory conditions of testes (Al-Jitawi et al., 1997). In addition to its usefulness and accuracy in diagnosis of the state of spermatogenesis in the infertile male, TFNA offers many advantages over open testicular biopsy as this technique avoids the problems of post-operative haemorrhage, fibrosis, adhesions, and risk of development of anti-sperm antibodies resulting from the breach of blood–testis barrier during testicular biopsy (Agarwal et al., 2004). Moreover, in assisted reproduction technique (ART), TFNA has gained wide acceptance in Intra Cytoplasmic Sperm Injection (ICSI) for the management of azoospermic patient (Aridogan et al., 2003). Though this effective technique is widely used in man, there are few reports on TFNA in domestic animals, such as, in dog (Dahlbom et al., 1997; Romagnoli et al., 2009), bull (Chapwanya et al., 2008) and stallion (Leme and Papa, 2000). Moreover, report on the use of this technique in male alpacas is very limited and standardization of this technique may have great impact on assessment of male alpacas for breeding suitability. Therefore, the purpose of this study was to compare percentage variability of different testicular cells obtained from two slide types (TFNA and impression/IMPRINT smears) to assess the effectiveness of TFNA in male alpacas for qualitative and quantitative identification of testicular cells.

2. Materials and methods

Testes from six alpaca males were collected immediately after slaughter. A fine butterfly (21 G) needle connected to a 50 ml syringe was inserted into a testicle and multiple plane aspirations were carried out to obtain materials destined to the smear from different areas to obviate sampling errors. Five slides were prepared from aspiration of each testicle. After making aspirated smears, all testes were incised to make impression slides. The impression slides were taken into consideration as a simulating procedure of testicular biopsy and three different imprints on slides were taken from each testicle. All slides (n = 96; 60 TFNA and 36 IMPRINT smears obtained from twelve testes) were air-dried, stained with modified May–Grünewald–Giemsa (MGG) (Forestà, 1993). Briefly, smears were immersed into May–Grünewald stain for 4 min, rinsed, then immersed into Giemsa (diluted 1:2 in distilled water) for 10 min. Then the extra stain was rinsed and samples were air-dried. Two types of cytological smears were examined separately by four observers previously trained, under light microscope with 100 × magnification. An adequate and informative TFNA specimen was defined by observing several clusters or cords of cells that contained 10 or more cells, and for interpretation at least 200 well-dispersed spermatogenic cells and Sertoli cells were counted from different clusters on each slide. Spermatogonia (Sg), primary spermatocytes (I), secondary spermatocytes (II), early spermatids (ab), late spermatids (cd), spermatozoa (S) and Sertoli cells (SC) were identified and counted. The percentage of spermatozoa were expressed as the spermatic index (SI – the number of spermatozoa/the number of total spermatogenic cells), and the percentage of the Sertoli cells were expressed as Sertoli cell index (SEI – the number of Sertoli cell/the number of total spermatogenic cells). Cellular findings from two different smears were examined cautiously to determine the relationship between the histological diagnosis, SI and SEI, the results were given as mean ± S.E.M.

Statistical analysis was performed using GLM statistical software SIGMASTAT 2.03. ANOVA was carried out to analyze significant variation among cellular types of two smears where slides types, testicles and observer considered as independent variables and cells types considered as dependent variables.

3. Results

In this study, it was possible to obtain enough material from all testes to identify and quantify the spermatogenic cells and Sertoli cells with fine needle aspiration. In TFNA smears, different types of spermatogenic cells were scattered over the slide and normal cell-to-cell contacts were less in most cases in contrast to impression smears (Fig. 1a and b). The cytological appearance of spermatogenic cells in an air-dried MGG stained smear showed various transitional forms, from spermatogonia to sper-
spermatogonia, characterized by diminution of nuclear size and condensation of chromatin. Various cell types were identified by their distinctive morphology and criteria described by Schenck and Schill (1988). Spermatogonia were not frequently found. They had a naked, round or oval nucleus with finely threaded chromatin pattern (Fig. 2b) and cytoplasm was scanty. Primary spermatocytes were easy to distinguish because of their larger nucleus, which had thread-like appearance of the chromatin and an eccentric nucleolus. Cytoplasm was scanty and more basophilic than that of spermatogonia (Fig. 2a and b). Secondary spermatocytes were seldom observed in cytological smears. Their centrally placed round nucleus contained fine and granular chromatin, and was surrounded by slightly basophilic cytoplasm. Their nuclear size was approximately half that of primary spermatocytes. Early spermatids were small and had a round or triangular nucleus, often eccentrically located, showing uniformly fine granular chromatin without nucleoli. Late spermatids or spermatids cd had a smaller, elongated darker nucleus with uniformly darker chromatin and the cytoplasm was mostly intact (Fig. 2a and b). Spermatocytes were clearly visible and most of them appeared without a tail (Fig. 2a and b). Sertoli cells were the most uniform type of cells in both aspirates and impression smears. Cytoplasm was often difficult to discern, and nucleus was round and slightly granular with a clearly visible, darkly stained nucleolus (Fig. 2a and b). Leydig cells were not detected or, possibly, not recognised.

Quantitative analysis of testicular cytology was performed. The percentages (mean ± S.E.M.) of various spermatogenic and Sertoli cells were shown in Table 1. Considering the cells individually, there was not any significant difference among percentage of spermatogenic cells in two smears types. Whereas sertoli cell index was significantly higher in aspirations smears than that of imprint smears. Considering the numerosity of cells, early spermatids were the most numerous, followed by spermatocytes, primary spermatocytes, late spermatids, Sertoli cells, spermatagonia and secondary spermatocytes (Table 1) in impression smears and Sertoli cells were the most numerous, followed by early spermatids, spermatocytes, primary spermatocytes, late spermatids, spermatagonia and secondary spermatocytes (Table 1) in aspiration smears. Moreover, no statistical differences were observed in cell values of right and left testes or in cell values obtained by different observers.

### Table 1

<table>
<thead>
<tr>
<th>Cell types and cell index</th>
<th>Right testicle</th>
<th>Left testicle</th>
<th>Normal testicular cell parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TFNA</td>
<td>IMPRINT</td>
<td>TFNA</td>
</tr>
<tr>
<td>Sg</td>
<td>7.64 ± 5.87</td>
<td>8.82 ± 5.76</td>
<td>9.04 ± 8.48</td>
</tr>
<tr>
<td>I</td>
<td>14.31 ± 7.77</td>
<td>17.31 ± 8.39</td>
<td>16.19 ± 5.58</td>
</tr>
<tr>
<td>II</td>
<td>2.54 ± 2.85</td>
<td>1.61 ± 1.61</td>
<td>3.08 ± 1.80</td>
</tr>
<tr>
<td>ab</td>
<td>36.03 ± 8.84</td>
<td>47.60 ± 9.19</td>
<td>38.87 ± 12.01</td>
</tr>
<tr>
<td>cd</td>
<td>12.20 ± 3.97</td>
<td>9.76 ± 4.01</td>
<td>12.20 ± 7.46</td>
</tr>
<tr>
<td>Sl</td>
<td>27.28 ± 13.84</td>
<td>14.90 ± 7.17</td>
<td>20.63 ± 14.74</td>
</tr>
<tr>
<td>SEI</td>
<td>39.31 ± 27.17a</td>
<td>12.19 ± 6.81b</td>
<td>45.75 ± 24.79a</td>
</tr>
</tbody>
</table>

Sg – spermatogonium, I – primary spermatocyte, II – secondary spermatocyte, ab – early spermatid, cd – late spermatid, Sl – spermatocyte index, SEI – Sertoli cell index. a, b indicate significant difference (P>0.05).


b Foresta (1993).
Different cytological patterns indicating normal and abnormal spermatogenic process like normal spermatogenesis, hypospermatogenesis, early maturation arrest, late maturation arrest, germ cell aplasia and sertoli cell syndrome can easily be identified and interpreted by TFNA (Foresta and Varotto, 1992; Qublan et al., 2002). In this experiment, various testicular cells were identified by their distinctive morphology and ell morphology in alpaca testes was generally similar to that described in human testicular cytology. The accuracy of fine needle aspiration cytology was determined by comparing the TFNA findings with that of histological findings obtained from impression or imprint slides. A touch imprint technique has been described as an adjunct of testicular biopsy for the immediate interpretation of testicular cytological findings (Abdelmoneim et al., 2004). Moreover, it has been established that testicular cytology (using either TFNA or imprints) can be more precise than histologic surgical biopsy to assess the spermatogenic status of the tests (Düsmez et al., 2001; Amin et al., 2006). Percentages from spermatogonia to late spermatids were increased showing a normal evolution of the spermatogenic process in both smears. No statistical differences were observed between the right and left testis. This finding corresponds with the findings of Batra et al. (1999) and Santos et al. (2010) who did not observe differences between bilateral aspirates of testes in man and dog respectively. Considering the cells individually in aspiration smears, Sertoli cells were the most numerous, followed by early spermatids in TFNA. In contrast, early spermatid number was higher in impression smears. There is no report on the cytology of Alpaca testes to compare this finding. But quantitative analysis of cytological smears in men with normal fertility revealed that the late spermatids were in greater numbers than other cells, followed by spermatogonia, early spermatids, primary spermatocytes, secondary spermatocytes and spermatogonia (Foresta and Varotto, 1992). It may be a normal cytological finding for alpaca testes which needs a further confirmation. The number of secondary spermatocytes was very small. This finding is in agreement with Pâpic et al. (1988) who stated that secondary spermatocytes were seldom obtained in this type of cytological examination and the small number might be due to their short life span. Leydig cell was not observed in our study. Aspiration of interstitial tissue is difficult and hence Leydig cells are usually not visualized in the cytological smears (Mahajan et al., 1999, Dahlborn et al., 1997). Occasionally, the chromatin threads of the primary spermatocytes were dispersed in the background of aspiration smear. We found that the cords like chromatin pattern of primary spermatocytes varied in thickness. Similar finding was observed by Santos et al. (2010) who described this as an indication of different differentiation process. The spermiogenesis (golgi phase, cap phase, acrosomal phase, and maturation phase) could also be identified in smears by observing different morphology of late spermatids.

SI demonstrates the efficacy of the spermiogenesis and progressively decreases in hypospermatogenesis, maturation arrest, and “Sertoli cell–only” syndromes (Foresta and Varotto, 1992). In alpacas, SI value was lower than that reported in stallion (31.5 ± 8.5: Leme and Papa, 2000) and men (34.8 ± 13.3: Foresta, 1993). In our study, the spermatogenic cells parameters from aspiration smears correlated well with the impression cytology with an exception in SEI. In contrast, no differences in morphology or ratios have been observed between TFNA and imprint cytology in man (Düsmez et al., 2000). The relationship between spermatogenic and sertoli cell numbers or SEI has been studied as an indicator of tubular germ cell potential or the spermatogenesis efficiency (Griswold, 1995; Blanchard and Johnson, 1997). In both testes, there were significant differences (P > 0.05) between SEI obtained from two methods. The exact cause of the difference between sertoli cell numbers of two smear types is unknown. It might be a result of technical process of preparing aspiration smears, which could be attributable to the usual counting technique. We observed that ruptured cells resulting in naked nuclei, cytoplasmic fragments, and streaming nuclear material were frequent in TFNA smears. The more matured spermatogenic cells are preserved very well in aspiration smears (Schenck and Schill, 1988).

This study proves that TFNA can evaluate all classically defined histological types. Moreover, by collecting samples from different areas of testes, TFNA may provide much testicular tissues to detect spermatogenesis more precisely. Results of this study also provide support for the use of TFNA as a useful minimally invasive, reliable, simple modality for evaluation of spermatogenic activity. However, further study should be concentrated on standardization of qualitative and quantitative findings from aspiration smears considering age, breeding activities and pathological influences (e.g. oligospernia, azospernia, etc.), which could help for the assessment of testicular cellular function in determining breeding soundness of male alpacas more effectively.

References


