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ORIGINAL ARTICLE

The validity of testicular aspirate cytology and DNA image-analysis of the aspirate in the assessment of infertile men

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KEYWORDS

Cytological examination;
DNA image analysis;
Testicular fine;
Needle aspirate

ABBREVIATIONS

H&E, haematoxylin and eosin; FNA, fine-needle aspiration; LH,

Abstract ***Objective:*** To assess the possibility of using cytological examination and DNA image-analysis of testicular fine-needle aspirates instead of open surgical biopsy in the investigation of infertile men, as testicular biopsy has long been used for investigating infertility but the interpretation of histological slides is usually subjective.

Patients and methods: Thirty-three men (aged 22–36 years) were evaluated for infertility and underwent both open biopsy and fine-needle aspiration of their testes. Subsequently, the needle aspirates were assessed histopathologically and cytologically, and by DNA image cytometry. The percentages of haploid, diploid and tetraploid cells were determined for each patient.

Results: The cases were divided into four categories: (1) Complete spermatogenesis, with a DNA pattern of 1n > 2n > 4n; (2) Maturation arrest, with a DNA

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luteinising hormone; SCO, Sertoli-cell only

pattern of 2n > 4n with no haploid cells; (3) Sertoli cell-only syndrome, with a DNA pattern of only 2n, with no haploid or tetraploid cells; (4) Hypospermatogenesis, with a variable DNA pattern, i.e. mild with 1n > 2n, moderate with 2n > 1n > 4n, and marked where the DNA pattern was 2n > 4n > 1n. From the cytological and DNA image-analysis of the aspirate a diagnosis was possible that had a strong correlation with the histological diagnosis of the same case. From image analysis we could exclude interstitial cells, Sertoli cells and sperms on the static image, and differentiate between spermatozoa and spermatids based on morphological characteristics in the cytological smear. This technique can therefore be used to quantitatively determine the percentages of various cell types within the seminiferous tubules. By coupling image ploidy analysis and cytological examination of a cytological smear, spermatogenesis can be assessed accurately.

Conclusion: Image cytometry could be used to exclude interstitial cells, Sertoli cells and sperms on the static image and so produce an accurate assessment of spermatogenesis. A combination of ploidy and cell morphology characteristics in cytological smears provides an accurate, reproducible and easily used alternative to open testicular biopsy.

Introduction

In cases of infertility due to azoospermia, a finding identified in ≈10% of men evaluated for infertility, paternity can be achieved with spermatozoa retrieved from the testis in combination with *in vitro* fertilisation and intracytoplasmic sperm injection [1]. There are many clinical features, including testicular size, serum FSH, history of ejaculated sperms or testicular biopsy histology, that can predict whether sperm can be retrieved from the testis or not [2]. However, among these variables, successful sperm retrieval is most closely related to the findings of testicular biopsy [3].

However, the histological evaluation of open testicular biopsy specimens in male infertility is open to subjective interpretation [4] and has been restricted by its qualitative rather than quantitative nature [5]. Open biopsy also has its drawbacks, i.e. it requires a skin incision and some surgical expertise, and it is relatively time-consuming and painful. Moreover, in paraffin sections of a testicular biopsy the mature intact spermatozoa are not clearly identified, so it might not be possible to differentiate between maturation arrest at the late spermatid stage and normal spermatogenesis [6]. Therefore, there is a need for a procedure that can accurately diagnose the changes in testicular tissue. This is very important for planning the patient for assisted-reproduction programmes or the surgical correction of ductal obstruction.

Several other quantitative methods were devised but never gained popularity [7,8]. Testicular fine-needle aspiration (FNA) can replace open biopsy as a minimally invasive diagnostic method for assessing spermatogenesis [9–11]. FNA has several advantages over open biopsy. First, by using different punctures, each from a different area of the testis, more representative samples are obtained than by biopsy. Second, the biopsy procedure causes many artefacts in histological sections, which has not been seen in samples from FNA. Also, as with FNA of other organs, it is a quick, inexpensive and less-invasive method. However, testicular biopsy is more invasive, painful and requires operating-room facilities. Moreover, testicular open biopsies have side-effects like haematoma, inflammation and especially permanent devascularisation of the testis, resulting in testicular atrophy. The reasons that testicular biopsy has not become popular and developed into a routine investigation might be the fear of 'needle trauma' and local tumour implantation. However, as in the present study, there were no reported complications such as haemorrhage or trauma from the FNA procedure.

Ploidy can be analysed by several methods, the first being flow cytometry, which has been used in an attempt to quantify cell types, but has the significant disadvantage of not being able to distinguish between specific types of haploid cells, the spermatozoa and spermatids. Further limitations of flow cytometry include the total use of the tissue, precluding its further use, the need for relatively many cells, inconsistent reproducibility, and lack of user discrimination of nuclear artefacts [12–14].

Image analysis is a method for quantifying microscopic images of a cell by digital conversion and computer analysis of the DNA content and morphological features of the cell. Image analysis of the testis is particularly appealing because of the actively dividing cell populations and the small amount of material typically available for examination [15].

In the present study we evaluated the correlation between the results of DNA image analysis of testicular FNA cytological smears and the findings of routine

microscopic examination of cytological smears, with histological slides from open-biopsy specimens of the same testes in the same patients.

Patients and methods

In a prospective study we assessed 33 infertile men with azoospermia, from August 2009 to August 2010, who presented with infertility of at least a 1-year duration. Patients who attended the Infertility Clinic were included in the study. The procedure was carried out regardless of the size of the testis or the hormonal profile. All selected patients had a complete history taken, a physical examination, genital examination, a minimum of two semen analyses on two occasions conducted according to WHO guidelines [16] and a standard hormonal assessment of luteinising hormone (LH), FSH, testosterone and prolactin. The men had no previous hormonal treatment, previous irradiation, history of cryptorchidism or any inguinal surgery.

Procedures

Open biopsies and needle aspirates were taken simultaneously, with the patient under general anaesthesia, from the same testis. The needle aspirates were taken with a 23-G needle and 10 mL syringe attached to a holder (three punctures from the upper, middle and lower poles of each testis). By using different punctures, each from a different area of the testis, more representative sampling is obtained than by biopsy.

The cell material was placed on two glass slides. The first was allowed to air dry for 24 h, stained with May Grunwald–Giemsa stain and later examined cytologically. The other smear was prepared for image DNA analysis.

Open biopsy specimens were fixed in Bouin's solution and processed routinely using paraffin embedding. Haematoxylin and eosin (H&E)-stained sections from these cases were evaluated. Based on standard qualitative interpretation, on H&E-stained sections, biopsies were classified into normal spermatogenesis, hypospermatogenesis, spermatogenic arrest, or Sertoli cell-only (SCO) syndrome [17].

Interpretation of the cytological preparation

Two types of cells are found in the testis, Sertoli cells and spermatogenic cells. The Sertoli cells have round vesicular nuclei with a fine granular chromatin and large nucleolus. The cytoplasm is abundant, pale and vacuolated, with poorly delineated borders. The spermatogenic cells show transitional forms, from spermatogonia, spermatocyte and spermatid, to spermatozoa, characterized by a diminution of nuclear size and the condensation of chromatin. The spermatogonia have the largest nuclei, followed by the primary and secondary spermatocytes and the spermatids. Spermatozoa have an oval nucleus with a tail opposite to the acrosome. In all spermatogenic cells the cytoplasm is basophilic and well delineated. An adequate FNA cytological smear was defined as one that contained $\geqslant 100$ clusters of $\geqslant 20$ cells. Patients were classified according to the most mature spermatogenic cells present in the testis, regardless of the number of aspirates in which they appeared, and were thus classified accordingly into four groups; (1) patients with normal spermatogenesis (smears showed all stages of spermatogenesis and a considerable number of mature sperms); (2) hypospermatogenesis (fewer spermatogenic cells and a few sperms in the smears); (3) maturation arrest (no sperm in the smears); (4) SCO (only Sertoli cells and no spermatogenic cells) [18].

For image analysis we used Feulgen-stained cytological smears from the FNA sample, stained using standard techniques [9,15,19]. Images were analysed for the DNA index and ploidy histogram type using an image-analysis system (CAS-200 software. Becton Dickinson Co., Immunocytometry Systems, CA, USA) using a ×100 magnification objective (numerical aperture 1.30). The nuclear integrated optical density was computed on 256 densimetric levels; this measures the amount of absorbent material (nuclear DNA content). The DNA index is generated from the integrated optical density, the value of which is 1.00 in the case of a normal G0–G1 diploid population. A special filter suitable for testicular cytological smears was designed using measurements of tens of thousands of cells obtained from the default automated image analysis filter, which provided individual cellular area, shape, DNA content and density. For the quantitative DNA analysis, each slide was calibrated using Sertoli cells (diploid) as control cells. The smears were viewed under oil immersion (10×100) and 200 nuclei from each smear were analysed for DNA content, randomly, to ensure that each nucleus was measured only once. The mean DNA content of the analysed cells was analysed statistically using the image-analysis quantitative ploidy analysis software. The cells were analysed by image analysis and classified as haploid (1n; cells which have half of the normal amount of DNA, i.e. spermatids and spermatozoa), diploid (2n; cells with the normal amount of DNA, i.e. spermatogonia, primary spermatocytes, Sertoli cells and Leydig cells); and tetraploid (4n; cells with double the amount of DNA, i.e. cells at the G2/M phase) on the measured DNA contents (perceived as the optical density). The results were divided into four categories, i.e. complete spermatogenesis, hypospermatogenesis, maturation arrest and SCO syndrome. No attempt was made to take a microscopic biopsy because FNA is simpler and the microscopic biopsy has a poor yield.

The results of the histological examination, FNA cytology and image analysis were correlated for each patient using Fisher's exact probability test, and the chi-square test was used to determine the difference among the three methods.

Results

In all, 33 patients (aged 22–36 years) had diagnostic testicular biopsies for histological, cytological and DNA image analysis. All samples had enough material for evaluation. Of the 33 specimens examined histologically, six showed normal spermatogenesis, 10 showed hypospermatogenesis, eight complete spermatogenic arrest and nine had the SCO syndrome. Of the 33 smears examined cytologically, six showed normal spermatogenesis (abundant sperms, Fig. 1), 14 showed hypospermatogenesis (a few sperms per smear, Fig. 2), six showed spermatogenic arrest (all spermatogenic cells and no sperms, Fig. 3) and seven had SCO (no spermatogenic cells, no sperm, only Sertoli cells, Fig. 4). Of the 33 smears analysed by image cytometry analysis, five were normal (1n > 2n > 4n pattern, Fig. 5), 11 showed hypospermatogenesis (2n > 1n > 4n pattern, Fig. 6a–c), eight had spermatogenic arrest (2n > 4n pattern, Fig. 7) and nine had SCO (1n = 0, Fig. 8). The group with hypospermatogenesis was subclassified according to the histogram into mild (four), moderate (four) and severe (three) according to the number of haploid cells; the

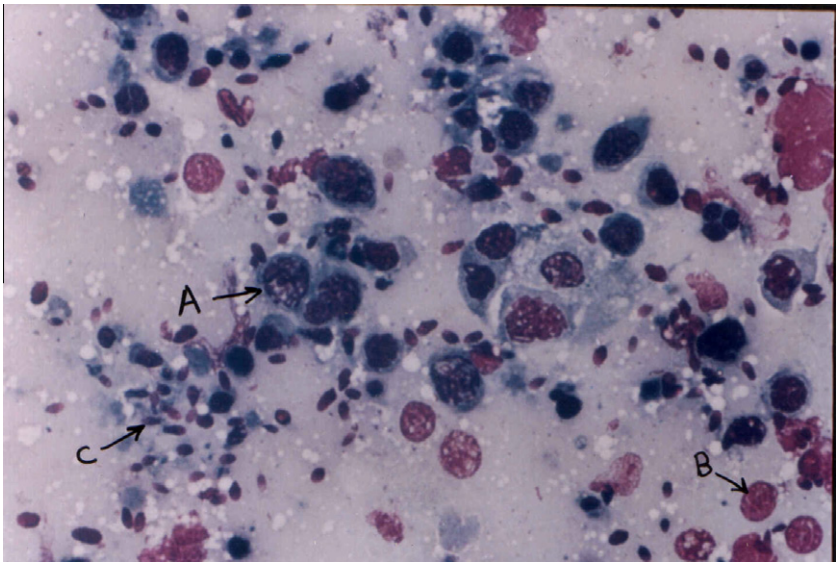

Figure 1 Active spermatogenesis in a case of obstructive azoospermia, with several typical spermatocytes (A), Sertoli cells (B) and spermatozoa (C) visible. Testis FNA, Giemsa stain × 600.

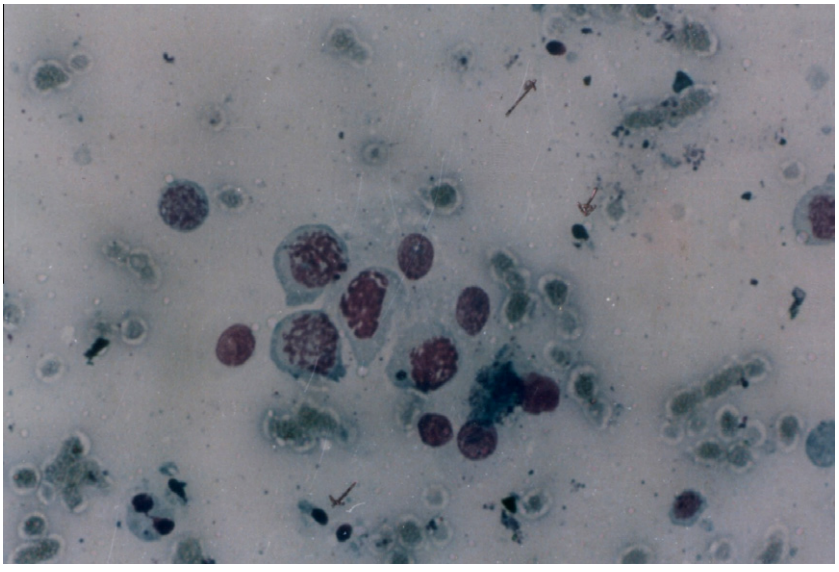

Figure 2 Hypospermatogenesis with a few scattered spermatozoa. Testis FNA, Giemsa stain × 600.

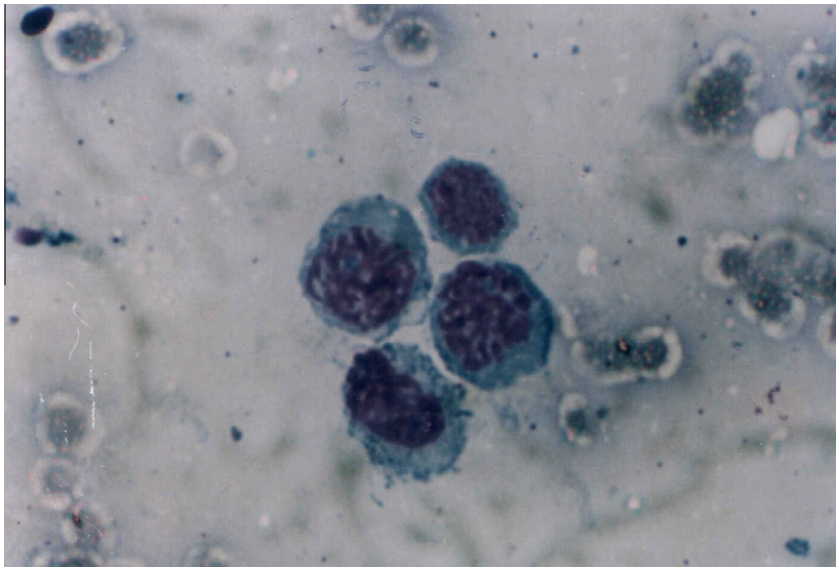

Figure 3 Arrest of spermatogenesis at the primary spermatocyte stage. The cell has a round nucleus, deeply stained, threaded chromatin, and an eccentric nucleolus. The cytoplasm is scanty and basophilic. Testis FNA, Giemsa stain × 600.

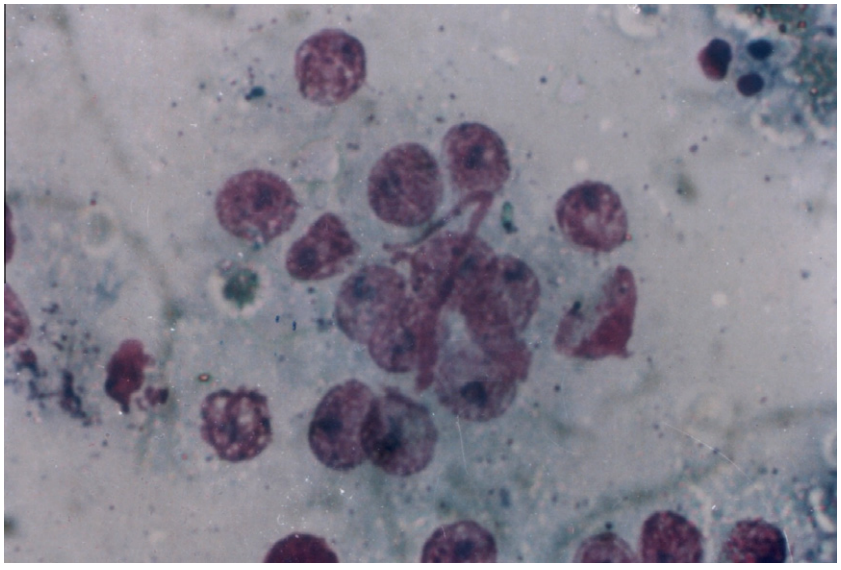

Figure 4 SCO syndrome; only Sertoli cells are shown in the smear with no other cells from the spermatocytic series. Cells have a round or kidney-shaped nucleus with an eccentric nucleolus. The cytoplasm is very large, pale or slightly basophilic, with a triangular or elongated shape and ill-defined borders. Testis FNA, Giemsa stain × 600.

DNA pattern was 1n ⩾ 2n in mild hypospermatogenesis (Fig. 6a), 2n > 1n in moderate (Fig. 6b) and 2n > 4n > 1n in severe hypospermatogenesis (Fig. 6c; Table 1).

Comparing the findings of the cytological smears with those of the histological examination, there was a good correlation, using Fisher's exact probability test ($P = 0.001$) in cases of normal spermatogenesis, while four of 14 cytological smears diagnosed as hypospermatogenesis showed spermatogenic arrest in two patients and germ cell aplasia in another two on histological examination.

There was also a strong correlation between the findings of the image cytometry histograms and the results of the histological examination ($P = 0.009$) in cases of spermatogenic arrest and SCO syndrome, while one of 11 diagnosed as hypospermatogenesis showed normal spermatogenesis on histological examination. The dif-

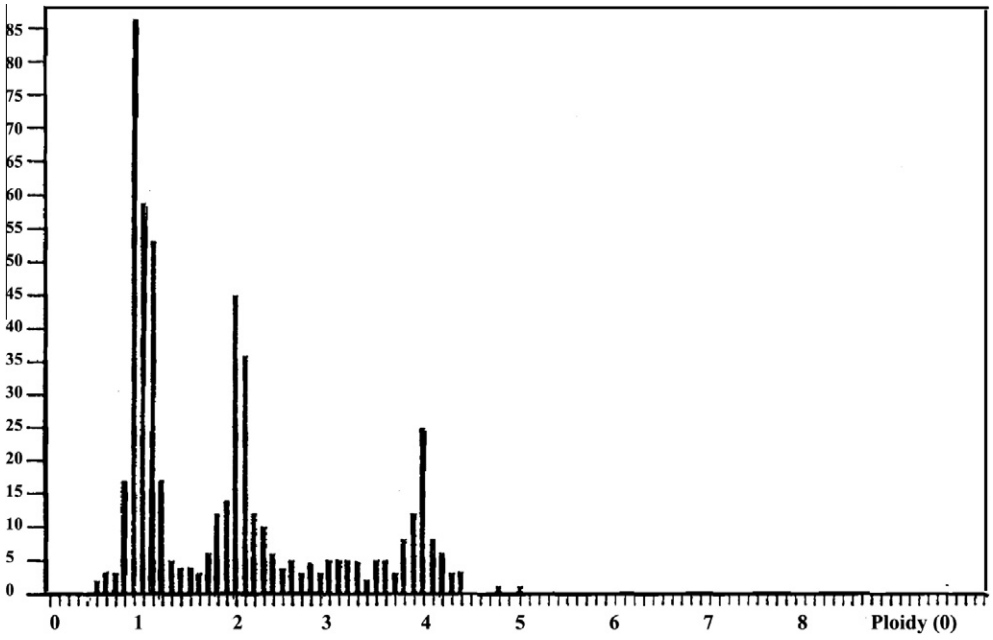

Figure 5 An image-cytometry histogram showing three peaks in an obstructive azoospermic case. There is a predominant haploid peak denoting active spermatogenesis (1n > 2n > 4n).

ference between the results of the histological examination, cytological smears and image cytometry histograms was statistically insignificant ($P = 0.35$).

Discussion

The recently updated European Association of Urology guidelines (2011) [20] recommended that a testicular biopsy is the best procedure not only to distinguish between obstructive azoospermia and unobstructive azoospermia, but also to predict the possibility of sperm retrieval in patients with the latter condition. The present study is the first to be reported in Egyptian patients, and compared to Western studies.

Until recently, open testicular biopsy was the standard method for ascertaining the cause of azoospermia. However, the evaluation of a testicular biopsy specimen and aspirate in male infertility has been hampered by the qualitative rather than quantitative nature of routine histological and cytological techniques, and the subjectivity in interpretation, resulting in a wide inter-observer discrepancy in the interpretation of samples from routine testicular biopsies taken to assess azoospermia. This wide inter-observer discrepancy was attributed to many factors, i.e. a poorly prepared biopsy, insufficient size of the biopsy, and that no particular method for evaluating a biopsy is uniformly applied and serves as a standard procedure for pathologists [4]. Because it is important to evaluate testicular spermatogenesis in azoospermic patients, we correlated the results of DNA image analysis of testicular cytological smears and the findings of routine microscopic examination of FNA cytological smears with histological slides from open biopsy specimens of the same testes.

FNA has emerged as a simple, minimally invasive, reliable and relatively well-tolerated alternative to open testicular biopsy for investigating azoospermic patients [21]. In the present study, of 33 specimens examined histologically, six showed normal spermatogenesis and 10 showed hypospermatogenesis. The procedure was carried out regardless of the size of the testis or the hormonal profile, so patients with azoospermia had normal spermatogenesis or hypospermatogenesis due to a secondary obstructive cause, and not primary testicular failure, i.e., eight with complete spermatogenic arrest and nine with SCO syndrome. Of the 33 smears examined cytologically, six showed normal spermatogenesis, 14 had hypospermatogenesis, six spermatogenic arrest and seven SCO. Of the 33 smears analysed by image cytometry analysis five were normal, 11 showed hypospermatogenesis, eight spermatogenic arrest and nine SCO.

In the present study, the FNA-derived stained cytological smears showed a good correlation with the histological findings of the open testicular biopsy. Our results agree with those of previous studies comparing FNA and open testicular biopsy [22–24]. In four of the present patients diagnosed with hypospermatogenesis by FNA, the histological diagnosis might suggest no active spermatogenesis, i.e. spermatogenic arrest in two and SCO in two. This finding could be explained in that active spermatogenesis might be focal or limited to some seminiferous tubules, and an open testicular biopsy (which is a single-site biopsy) might miss these areas, while FNA

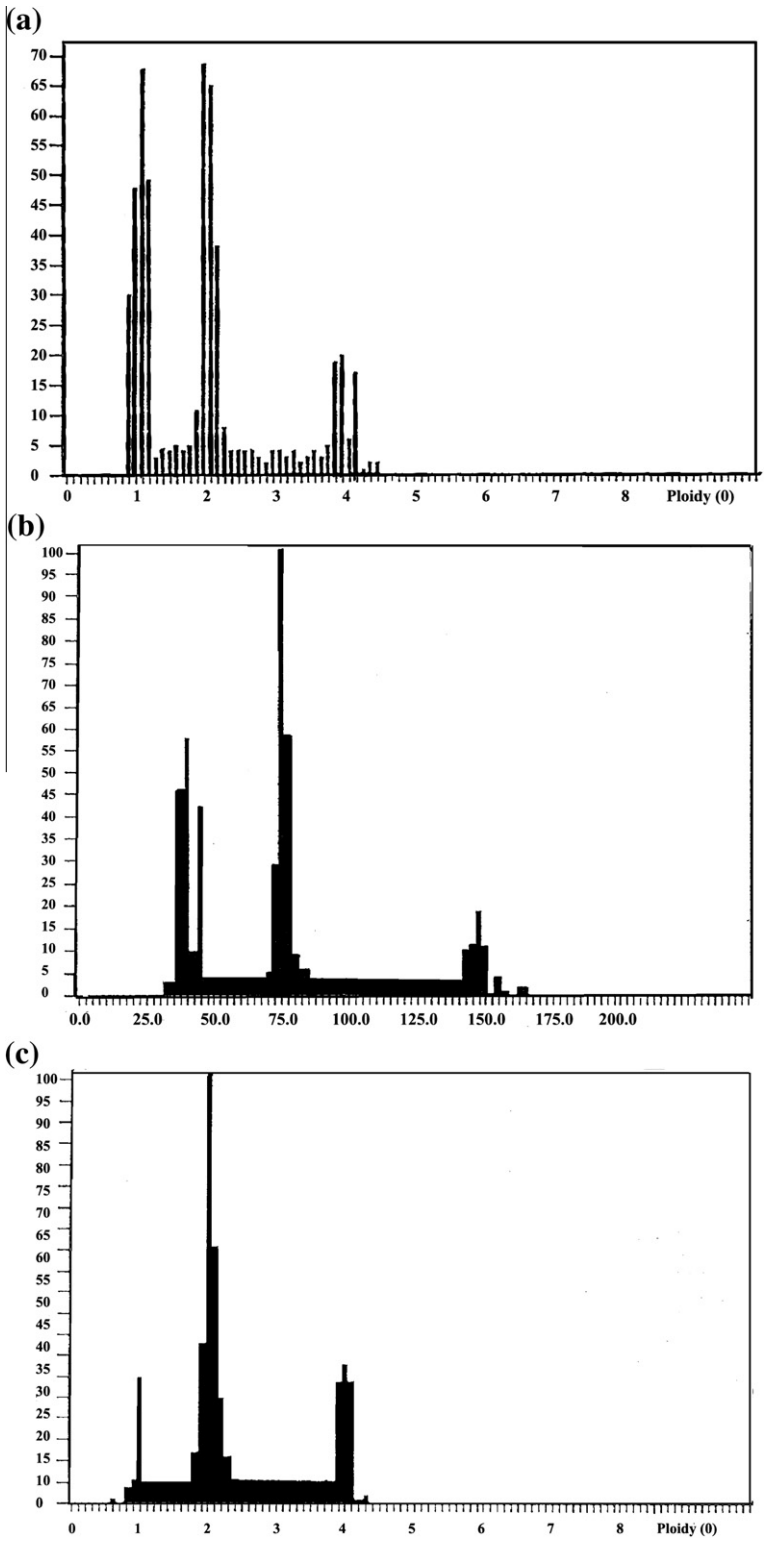

Figure 6 An image-cytometry histogram showing three peaks with; nearly equal diploid and haploid peaks denoting less active spermatogenesis in a case of mild hypospermatogenesis (1n $\geqslant$ 2n); b, with a predominant diploid peak denoting moderate hypospermatogenesis (2n > 1n > 4n); c, a large diploid peak, small tetraploid, and a much smaller haploid peak in a case with marked hypospermatogenesis (2n > 4n > 1n).

can be taken from the upper, middle and lower poles of each testis, and can precisely evaluate testicular spermatogenesis [25,26].

The advantages of image analysis are many, e.g. the ability of the operator to select the cells to be measured for ploidy, and the ability to distinguish between sper-

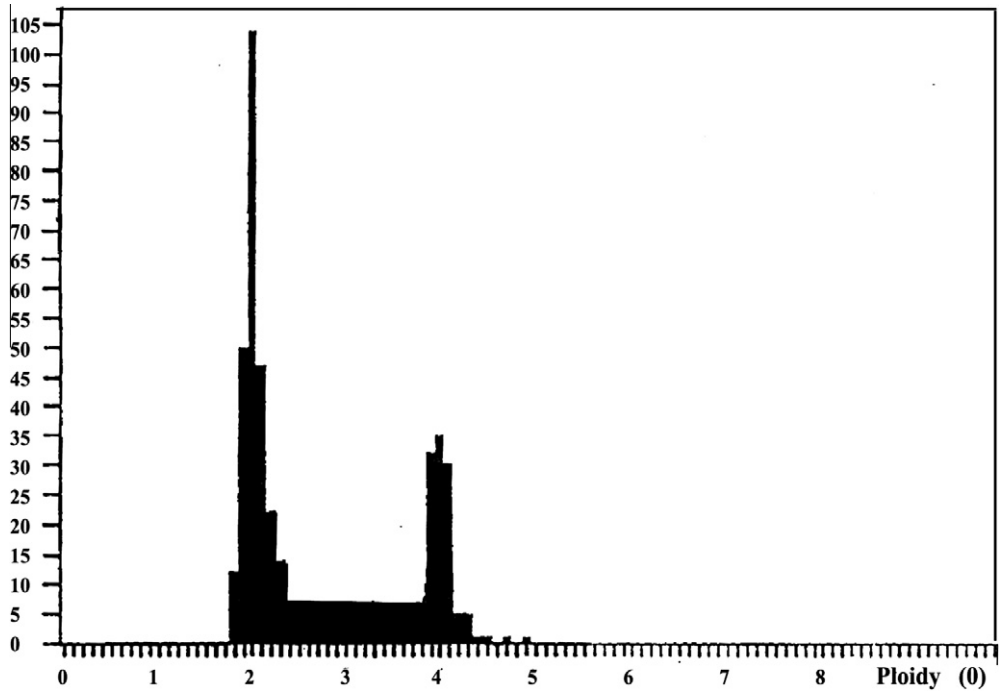

Figure 7 An image-cytometry histogram showing two peaks, a diploid and tetraploid peak in a case of complete maturation arrest (2n > 4n).

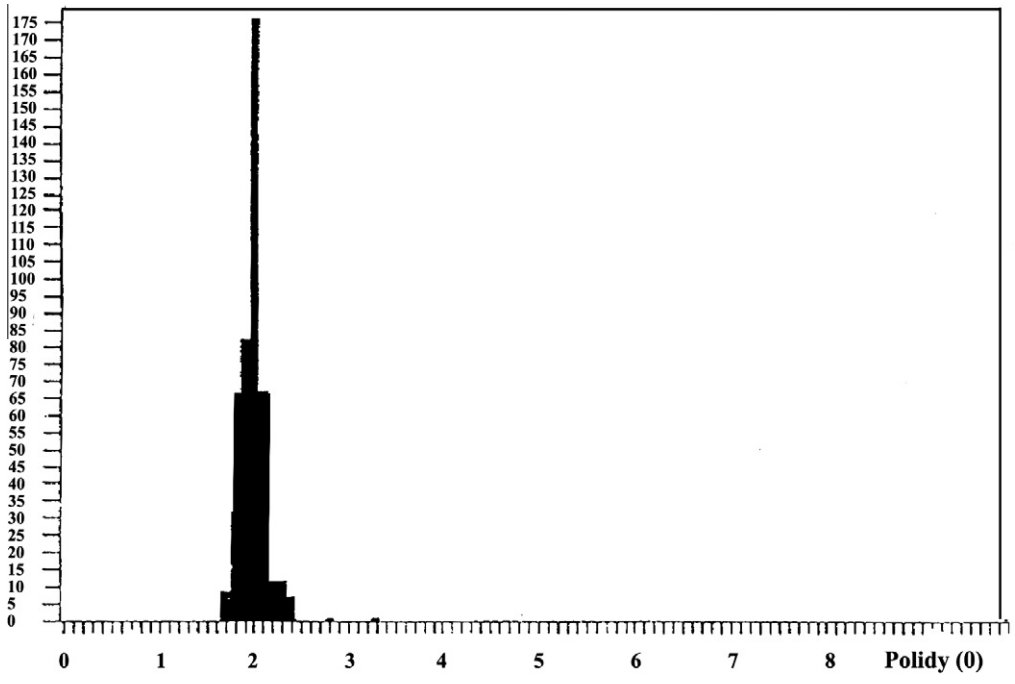

Figure 8 An image-cytometry histogram showing only one diploid peak in a case of the SCO syndrome. Only one type of cell is present in the smear (2n).

matids and spermatozoa, a distinction which is usually difficult in routine paraffin sections [6]. It can also be applied to cytological smears [27] and tissue embedded in paraffin blocks [15] after homogenisation of paraffin sections. Image analysis is widely used as a method to evaluate the ploidy of cells, especially of malignant cells. The technique using image analysis requires only an air-dried smear, which is prepared immediately with the routine smears from the testicular FNA. These can be prepared easily, even in outpatient clinics. There is no time limit on when these smears must be stained or analysed. In addition, the smears remain intact after analy-

Table 1 The histological, cytological and DNA image analysis results of the 33 patients.

Category	Open biopsy	FNA cytology	DNA image analysis
Normal spermatogenesis	6	6	5
Hypospermatogenesis	10	14	11
Spermatogenic arrest	8	6	8
SCO syndrome	9	7	9

sis, so that image analysis can always be repeated and reviewed.

In the present study there was an excellent correlation between DNA image analysis of FNA cytology and the histological diagnosis in cases of spermatogenic arrest and SCO syndrome, while one of the 11 histograms that suggested hypospermatogenesis was from a patient who had normal spermatogenesis on histological examination. However, this difference was statistically insignificant. These results are in agreement with previously published studies [15,27].

Although DNA image analysis cannot provide vital information on the architecture of the testis, it is useful in providing an objective and quantitative assessment of the degree of spermatogenesis.

Our data showed that a cytological examination coupled with DNA image cytometry of the FNA cytological smears can evaluate accurately the classically defined histological types, and might have the potential to replace testicular biopsy in the assessment of spermatogenesis in infertile patients with azoospermia. The value of FNA in sperm retrieval is also confirmed [28]. In centres like ours, where cryopreservation is not available, patients with azoospermia can be evaluated using FNA cytology.

The histological difference between late maturation arrest and normal spermatogenesis can be difficult to discern. We used other clinical features, including a physical examination, semen analysis, and hormone evaluation, to differentiate between the two. Therefore, patients with numerous spermatids on histology but with no clinical evidence of ejaculated spermatozoa or confirmed obstruction, were deemed to have late maturation arrest. As the aspiration of interstitial tissue is difficult, Leydig cells were detected in only two patients.

It was also suggested that testicular tissue from men with idiopathic azoospermia undergoing intracytoplasmic sperm injection should be evaluated histologically for carcinoma *in situ* of the testis, especially in countries where the testicular malignancy rate is high.

In conclusion, image cytometry can be used to exclude interstitial cells, Sertoli cells and sperm on the static image, and so produce an accurate assessment of spermatogenesis. The combination of ploidy and cell morphology characteristics in cytological smears represents a feasible, accurate and reproducible alternative to open testicular biopsy for evaluating the testes of infertile men.

Conflict of interest

None.

Funding

No funding.

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