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From Cell Biology to Tissue Engineering

Influence of three different histological methods on the morphology and morphometrical data in human testis

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Summary. Coagulant fixatives and paraffin embedding were widely used in the past for histomorphometrical evaluations of the human testis under physiological and pathological conditions. However, new methods are applied nowadays using better combinations of fixatives and plastic resins as embedding media, improving cell and tissue structural preservation. In an attempt to compare old and new data, the present study evaluated histomorphometrical data obtained from human testis after three different histological processing methods: Bouin/paraplast, glutaraldehyde/glycol methacrylate and glutaraldehyde/araldite. The morphometrical parameters were not affected by glutaraldehyde fixation after both resin embedding (methacrylate or araldite). On the other hand, Bouin/paraplast embedding lead to tissue shrinkage, which could give rise to misinterpretations on the measurements performed. Since some germ and somatic cells recognition do not depend upon high resolution techniques, counting of such cell types could be performed even using routine Bouin/paraplast protocols. Thus, the morphometrical analyses relying on cell recognition were not affected by the methods here applied, however, when metric measurements were applied, the obtained results could not be promptly compared. On the other hand, if the study requires confident spermatogonial identification for kinetics evaluation, glutaraldehyde/araldite processing is highly recommended.

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Introduction

The classical study of spermatogenesis in man developed by Clermont (1963) demonstrated that the seminiferous epithelium cycle was divided into VI stages, which were not established exclusively on the morphology of the acrosome, as for other species. In fact, the deviation from the typical cellular association in human presented germ cell generations in non-expected locations (i.e. germ cells from two different stages mixed and/or missing where they were expected). Subsequently, a series of studies developed by Clermont and colleagues (Clermont and Leblond, 1955; Heller and Clermont, 1964; Clermont, 1966a,b; Heller et al., 1969 and reviewed in Clermont, 1972) characterized human spermatogenesis with details, along with spermatogonial kinetics and renewal. Their results were so accurate that they are still applied as baseline studies for many researches. However, care should be taken when comparing morphological and morphometric results obtained today, considering that more advanced methods of tissue processing have been developed. In fact, it has been claimed that human spermatogenesis should be revised to reevaluate the cycle of the seminiferous epithelium in the light of new methodologies (Amann, 2008).

Fixatives and embedding media must be carefully chosen because of their different penetration and preservation abilities. While coagulant fixatives, such as

Bouin, cause tissue retraction and compromise detailed cytological observation, the non-coagulant fixatives, such as paraformaldehyde and glutaraldehyde show higher preservation capacity, since many water molecules remain bound to the tissue, resulting in higher structural stability (Bozzola and Russell, 1999). Additionally, the embedding medium is also a determinant step in tissue preservation influencing cell resolution and tissue retraction. For instance, resin embedding provides thinner sections with refined cytological details due to better tissue preservation, when compared to paraffin embedding. Ehmcke and Schlatt (2006) reported the difficulties in identifying spermatogonial types in non-human primates, since the distinction between A_{dark} and A_{pale} relies on the fixative, embedding and staining methods used to process the samples studied.

The studies developed by Clermont and colleagues in the 1960s used Zenker-formol and/or Clelands solution (modified Bouin) as fixatives, along with paraffin embedding, a combination that causes cell/tissue shrinkage and is not popular today. Such tissue processing had been used in the past to describe human spermatogenesis, as is currently known. Since new methods have been applied to evaluate the testes under different conditions, questions concerning the reliability of the morphological and/or morphometrical data obtained today and if they could be compared with the oldest ones are crucial. Therefore, the present study aimed to compare the morphological and morphometrical data of the human testis after aldehyde fixation and plastic resin embedding (glycol methacrylate or araldite) with those fixed with Bouin solution and paraplast embedding.

Materials and methods

Patients

Five patients (74.0±8.2 years-old) with advanced prostate cancer who underwent surgical orchiectomy were used. This project was approved by the Brazilian Ethics Committee (CONEP nº 189/2008) and the surgeries took place at Hospital das Clínicas (Universidade Federal de Minas Gerais, Brasil). All patients were previously informed and formally agreed to donate the organs.

Experimental design

Testicular fragments from each patient were processed as following: (a) Bouin fixation and paraplast embedding, (b) glutaraldehyde fixation and glycol methacrylate embedding and (c) glutaraldehyde fixation and araldite embedding.

Tissue processing

(a) Bouin fixation and paraplast embedding (BP) - Fixation in Bouin solution for 24h, dehydration in ethanol series, clarification in xylene and embedding in paraplast (Histosec, Merck). Histological sections (5 μm thickness) were stained with Harris' hematoxylin and eosin (Humason, 1979).

(b) Glutaraldehyde fixation and glycol methacrylate embedding (GGMA) - Fixation in 5% glutaraldehyde (biological grade, EMS) in phosphate buffer 0.05M, pH 7.3 for 24h, dehydration in ethanol series and embedding in glycol methacrylate resin (Historesin, Leica) (Chiarini-Garcia et al., 2011). Histological sections (3 μm thickness) were stained with toluidine blue/sodium borate.

(c) Glutaraldehyde fixation and araldite embedding (GA) - Fixation in 5% glutaraldehyde (biological grade, EMS) in phosphate buffer 0.05M, pH 7.3 for 24h, dehydration in ethanol series and embedding in araldite (EMS). Semi thin sections (1 μm thickness) were stained with toluidine blue/sodium borate (Chiarini-Garcia and Meistrich, 2008).

Morphometrical analysis

(a) Histopathological scores

Three different scores were used in order to evaluate spermatogenesis. One of them is currently used in the pathological anatomy laboratories, which divide the patients into five patterns of alterations of spermatogenesis: normal, hypospermatogenesis, germ cell arrest, Sertoli cell only and fibrosis (Levin, 1979; reviewed by McLachlan et al., 2007). The second was the Johnsen score (1970), which divides the spermatogenesis alterations into 10 scores, with more detailed information than the standard five scores, separating hypospermatogenesis and maturation arrest in subtypes. Finally the score of Bergmann (Bergmann et al., 1994; reviewed by Bergmann and Kliesch, 2010), which assesses changes in spermatogenesis in relation to the presence of elongated spermatids in testicular parenchyma. At least 100 cross-sectioned seminiferous tubules were scored per patient for each method.

(b) Tubular parameters

The seminiferous tubule diameter, the epithelium height and the tunica propria width were measured using 50 randomly chosen seminiferous tubule cross sections per patient. All measurements were made in digital micrographs using the software Image Pro-Express (Media Cybernetics), calibrated with a stage micrometer. The images were obtained with a digital Q-Color 3 camera coupled to the Olympus BX-51 microscope.

(c) Diameter of the nuclei/nucleoli of germ and somatic cells

Nuclear diameters (average of two perpendicular axes) were obtained from the cell types present in stage III (Heller and Clermont, 1964) of the seminiferous epithelium cycle (A_{dark} spermatogonia, A_{pale}

spermatogonia, preleptotene spermatocytes, pachytene spermatocytes and round spermatids - Sb1) as well as the nucleoli and nucleus of Sertoli and Leydig cells, respectively. The diameter of 10 nuclei/nucleoli from randomly chosen cells were measured for each patient using a graduated ruler fitted in a 10x eyepiece calibrated with a micrometer ruler, in a final magnification of 1000x.

(d) Spermatogenesis efficiency

To compare the spermatogenesis efficiency among the three different embedding procedures, germ cell nuclei (A_{dark} and A_{pale} spermatogonia, preleptotene and pachytene primary spermatocytes and round spermatid Sb1) and Sertoli cell nucleoli were counted in 30 round or nearly-round seminiferous tubules cross sections in stage III (Heller and Clermont, 1964) per patient. The obtained data were corrected by the thickness of the histological section using the formula of Abercrombie (1946), modified by Amann and Almquist (1962). Using the corrected cell counts, the following ratios were obtained as described previously by Melo et al. (2014): (1) mitotic index: number of spermatocytes at preleptotene divided by type A_{dark} spermatogonia, to determine the coefficient of spermatogonial efficiency mitosis; (2) meiotic index: number of round spermatids Sb1 divided by pachytene spermatocytes, to obtain the rate of germ cell loss during meiosis; (3) Sertoli cell efficiency: number of round spermatids Sb1 divided by Sertoli cell nucleoli, to estimate the number of sperm supported per each Sertoli cell and (4) the spermatogenesis efficiency: number of round spermatids divided by type A_{dark} spermatogonia at stage III, to

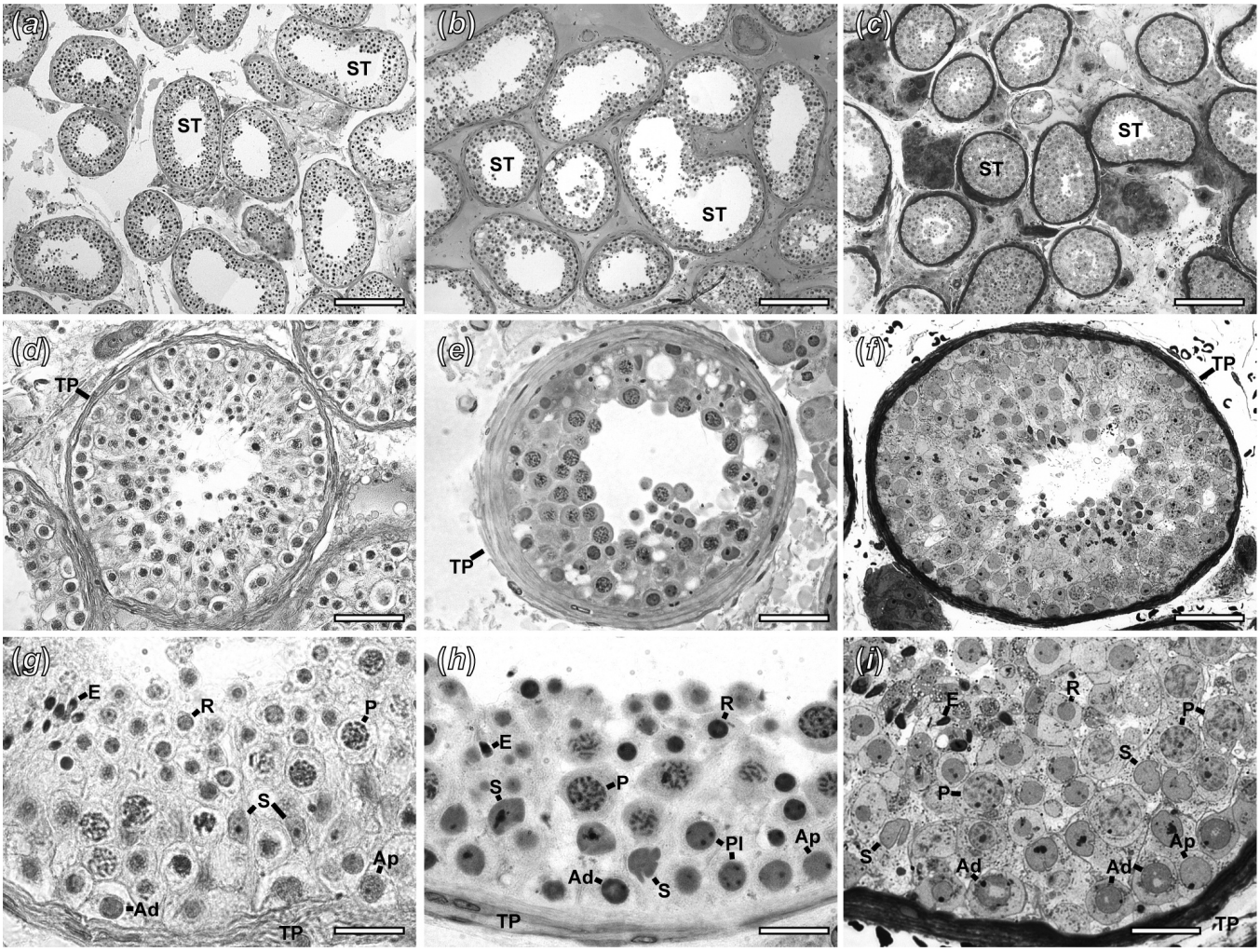

Fig. 1. Photomicrographs of human testicular parenchyma after Bouin/paraplast **(a, d, g)**, glutaraldehyde/glycol methacrylate **(b, e, g)** and glutaraldehyde/araldite **(c, f, i)** processing. ST, seminiferous tubules; TP, tunica propria; S, Sertoli cell; Ad, Adark spermatogonia; Ap, Apale spermatogonia; Pl, preleptotene spermatocyte; P, pachytene spermatocyte; R, round spermatid; E, elongating spermatid. Scale Bars: a-c, 200 μm; d-f,

determine the number of spermatids after mitotic and meiotic processes, estimating the overall rate of spermatogenesis.

(e) Number of spermatogonial mitosis and germ cell apoptosis

Spermatogonial mitosis, germ cell apoptosis, and Sertoli cell nucleoli number were determined in 10 round or nearly-round seminiferous tubule cross sections randomly chosen per patient. The number of mitosis and apoptosis was determined by 100 Sertoli cell nucleoli. Spermatogonial mitosis was considered as those dividing cells (late prophase, metaphase, anaphase and telophase) positioned in the basal compartment, near the tunica propria. Apoptosis was morphologically characterized according to the border blabbing of chromatin, which become condensed, and fragmentation of the nucleus, which was identified by their marked irregularity and basophilia.

Statistics

All variables measured were tested for normality prior to analyses, using the univariate procedure of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Comparisons among the experimental groups were done by ANOVA. In the event that significant treatment effects were established, multiple comparisons were performed using probability of differences adjusted by Tukey–Kramer (SAS, 2001) with $P<0.05$ being considered significant. In the tables and graphs, data are reported as means and S.E.M.

Results

Morphological findings

Tissue morphology was remarkably different among the technical procedures applied. Testicular fragments processed in Bouin/paraplast (BP) showed moderate empty spaces among seminiferous tubules due to shrinkage (Fig. 1a). At the cytological level, type A and B spermatogonia showed apparently empty cytoplasm and evident nuclear shrinkage (Fig. 1d,g). In addition, differentiation among spermatogonial subtypes (A_{dark}, A_{pale} and B) were not clearly distinguished due to the dark cell staining, which was associated with the section thickness, and to the low resolution and grainy aspect of the euchromatin (Fig. 1g). Besides, type A and B spermatogonia and preleptotene spermatocyte could not be easily distinguished due to their comparable nuclear round shape, similar euchromatin aspects and location on the basal compartment. Likewise, the acrosomal vesicle in round spermatids (Sa1, Sa2, Sb1 and Sb2) was hard to identify, compromising their distinction. Sertoli cell nuclei and nucleoli were not easily identified (Fig. 1g) and Leydig cells cytoplasm showed poor morphological resolution.

There was an improvement in the morphological preservation of the tissue fixed in glutaraldehyde and embedded in glycol methacrylate, observed by the less

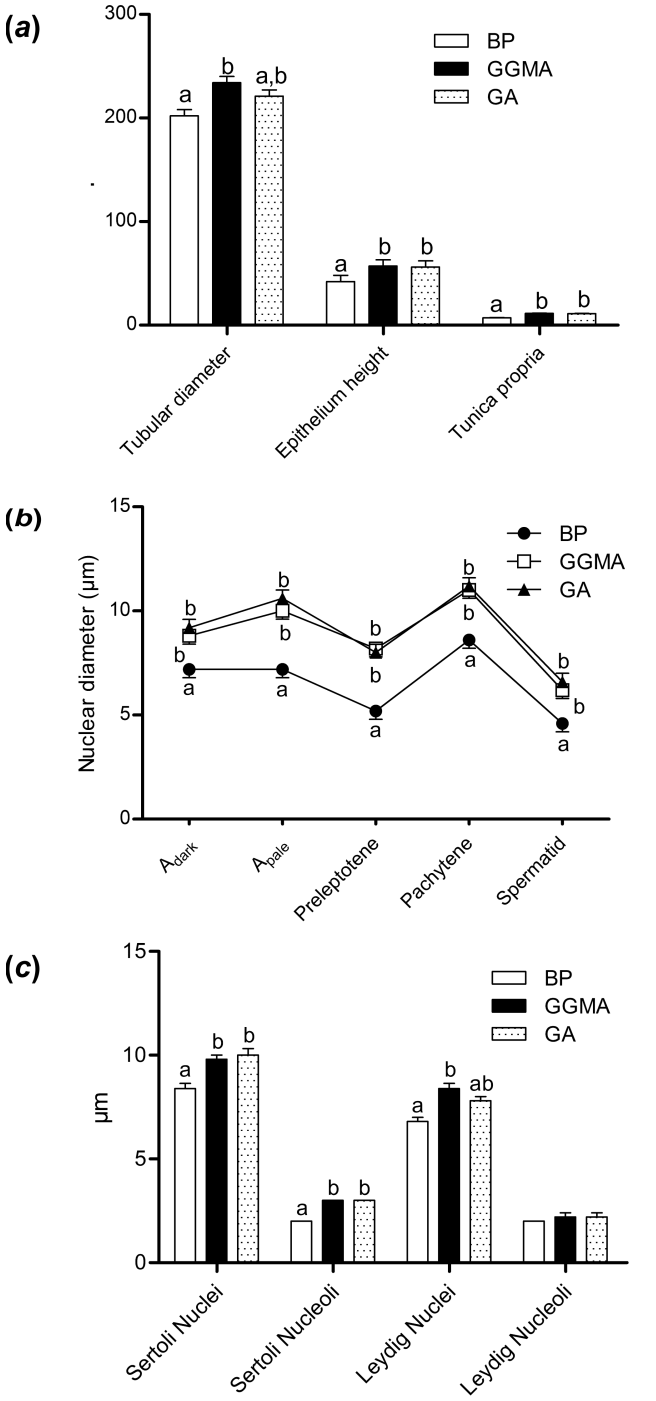

Fig. 2. Comparison of some testicular parameter measurements (μm) among the three different processing methods: **(a)** tubular parameters, **(b)** germ cell nuclear diameters and **(c)** nuclei/nucleoli measurements of Sertoli and Leydig cells.

pronounced testicular parenchyma shrinkage, which made the spaces between seminiferous tubules and interstitium less evident (Fig. 1b). The germ and somatic cell nuclei were larger and less grainy and the round spermatids' acrosomes were better preserved than those processed with BP (Fig. 1e,h). The differentiation of spermatogonial subtypes was easier, since the euchromatin patterns and nuclei shape were well preserved (Fig. 1h). Also, Sertoli cells showed conspicuous and characteristic nuclei/nucleoli with low cytoplasmic shrinkage. Leydig cells were well preserved showing evident cytoplasmic droplets.

The best preservation was observed after glutaraldehyde fixation and araldite embedding, resulting in less cell and tissue shrinkage (Fig. 1c,f,i). The spermatogonial subtypes, nuclear folds from Sertoli cells, and even the lipid droplets in the Leydig cell cytoplasm were easily identified. The acrosome was clearly visible which helped spermatid differentiation, allowing easy distinction of the seminiferous epithelium cycle stages (not shown).

Histopathological scores

Although cell resolution and tissue retraction were clearly different among the three protocols applied, they did not interfere with the score determination (Table 1). Even in Johnsen's score (Johnsen, 1970), which separates hypospermatogenesis and maturation arrest into detailed subtypes by dividing the testis in 10 histopathological classes, the scores obtained were similar.

Tubular parameters

Tubular diameter, epithelium height and tunica propria thickness were significantly reduced after BP embedding when compared with those embedded in GGMA and GA ($P<0.05$; Fig. 2a). On the other hand, both embedding plastic medium, glycol methacrylate and araldite, provided similar measurement of the tubular parameters.

Nuclei/nucleoli diameter of germ, Sertoli and Leydig cells

The nuclear diameter of all germ cells, spermatogonia (A_{dark} and A_{pale}), primary spermatocyte (preleptotene and pachytene) and spermatid (Sb1), were significantly reduced after BP processing when compared with GGMA and GA embeddings ($P<0.05$; Fig. 2b). Similar results were observed for Sertoli cell nuclei and nucleoli and Leydig cell nuclei ($P<0.05$; Fig. 2c). However, the same variables were not significantly different between GGMA and GA protocols.

Table 1. Histopathological scores used for clinical diagnosis of spermatogenesis status.

Fixative/Embedding	Histopathological score methods		
	Levin's (1979)	Johnsen's (1970)	Bergmann's (1994)
Bouin/paraplast	4.80±0.20	7.96±0.23	7.40±0.66
Glutaraldehyde/methacrylate	4.80±0.20	7.93±0.25	7.45±0.57
Glutaraldehyde/araldite	4.80±0.20	7.89±0.28	6.91±0.67

Data are expressed as mean ± SEM

Germ cell number and efficiency of spermatogenesis

Germ cell numbers were similar after the three protocols applied (Fig. 3a), demonstrating that even with some morphological alterations cell distinctions were still confidently realized. In addition, all spermatogenic indexes were also similar, regardless of the technical procedure. No differences were observed in the mitotic, meiotic, Sertoli cell efficiency and efficiency of spermatogenesis indexes, regardless of the technical procedure employed (Fig. 3b).

Germ cell apoptosis and spermatogonial mitosis

Even though the mitotic and apoptotic indexes presented numerically different values, they did not reach the level of significance, once the individual variations were very large (Fig. 3c).

Discussion

In an attempt to understand the physiological and pathological aspects of the human testis, studies developed up until now have applied various histomorphometric methods to evaluate several parameters of the testicular parenchyma. Qualitative and quantitative alterations of the testicular components have demonstrated that the instability detected in the cell populations can be related to positive or negative impacts that may be associated with testicular homeostasis. These findings have been used to support the diagnosis and clinical treatment of patients under andrological evaluation. However, the results of similar studies may not be suitable for comparison since researchers may have used different methods of histological processing. In fact, we demonstrated in the present study that different fixation and embedding methods originate statistically different results regarding linear measurements, which impairs direct comparison among groups. On the other hand, morphological data that takes into account only cell recognition and counting could be performed with some confidence.

The morphological aspects of the same cells may change depending on the fixative used (Chapin et al., 1984; Amaral et al., 2004). When the cell is preserved with coagulant fixatives such as Bouin solution, its cytoplasm and nucleus show a much more granular aspect, which negatively interferes with the observer's interpretation. This situation is directly associated with paraplast embedding and thickness of histological section. In fact, our results demonstrated that after BP

processing the morphology of most of the germ and somatic cells was remarkably changed, when compared with glutaraldehyde/plastic resins processing. However, this poor preservation still allowed cell recognition and, therefore, quantitative evaluations related to cell counting could be performed. Thus, data from studies of germ/somatic cells counting developed after GGMA or GA processing for better resolution of cytological details at light microscopy could be confidently compared with those obtained after BP processing. Nevertheless, recognition of either spermatogonia (A_{dark}, A_{pale}, B) or early primary spermatocytes (preleptotene) are not easily performed, demanding an experienced and trained examiner to avoid counting mistakes. After BP processing, confident recognition of such germ cell types became even more difficult, although doubts could still remain after GGMA processing. Studies developed in other species have demonstrated similar difficulties concerning spermatogonial recognition and counting after GGMA processing (Chiarini-Garcia et al., 2011; Melo et al., 2014). On the other hand, semithin sections made after GA processing provide better differentiation of the spermatogonial types in human (present study) and other species (Chiarini-Garcia and Russell, 2001; Chiarini-Garcia et al., 2003, 2009; Nascimento et al., 2008; Chiarini-Garcia and Meistrich, 2008; Schultz et al., 2010). Thus, in studies of spermatogonial biology after different insults or in infertile patients that require precise cell recognition to perform confident morphometric analysis, the combined use of glutaraldehyde and araldite is recommended (Chiarini-Garcia and Meistrich, 2008). The success of such protocol relies on the combined capacity of glutaraldehyde to cross-link proteins, and to some extent also lipids, carbohydrates, and nucleic acids, tying up cytological and nuclear components, along with the ability of araldite resin to provide a solid and stable medium for sectioning (Bozzola and Russell, 1999). Finally, sections of 1 μm thickness can be obtained from these blocks with well preserved tissues allowing high resolution evaluations of cytological details under light microscopy.

The present study confirms the remarkable retraction effects of BP processing on tissues and cells (Amaral et al., 2004). However, we demonstrated, for the first time, that the nuclear diameters of testicular germ and somatic cells do not suffer interference after glycol methacrylate and araldite embeddings. These findings showed that the numerical value of the nuclear diameter could be directly compared after these two histological resins processing without the use of correction factors. However, morphometric associations between both plastic resin methods and BP could only be made by means of a correction factor. We found that a 30% reduction must be applied for the nuclei diameter of germ cells, while for nuclei of somatic cells this reduction must be of 15%. The confident rearrangement of the obtained morphometrical data after different methodological procedures allows the use of many testis embedded in

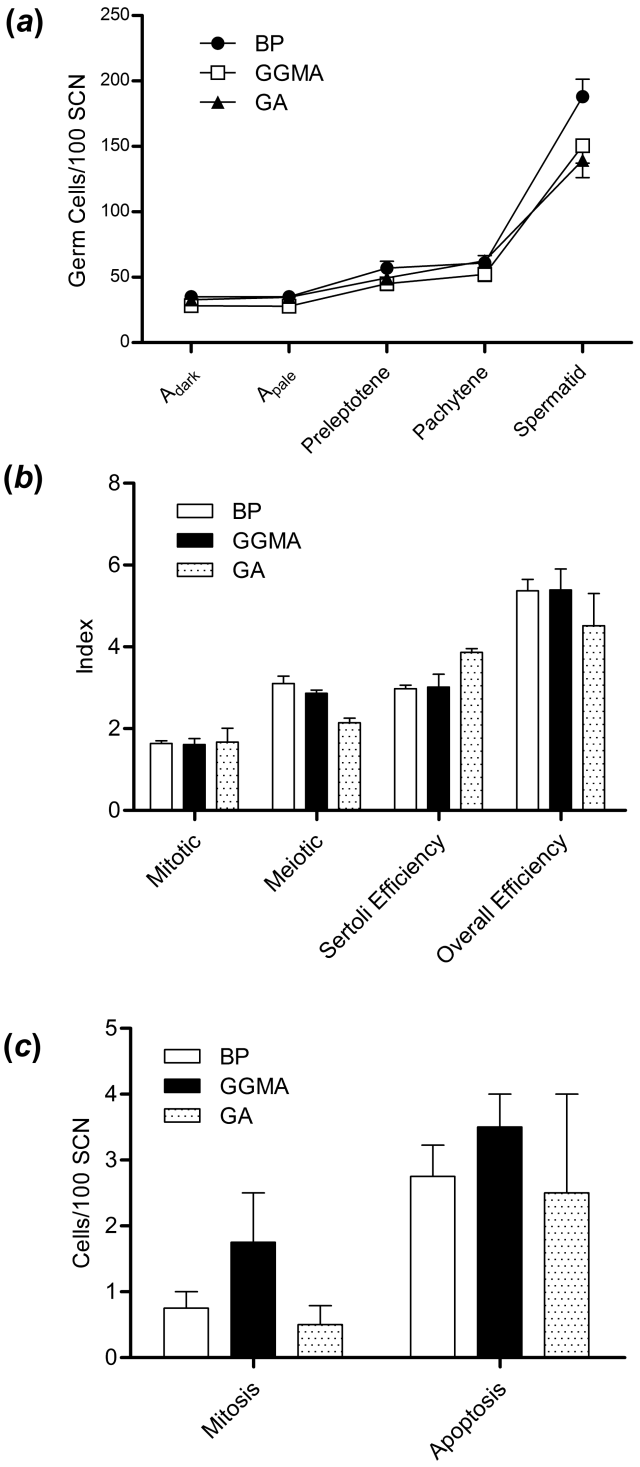

Fig. 3. Comparison of cell counting among the three different processing methods: **(a)** germ cells, **(b)** spermatogenesis index and **(c)** mitosis and apoptosis numbers.

paraffin and stocked in a bank of organs for scientific and clinical evaluations purposes, even if they have been maintained for a long time in hospitals and laboratories.

In our opinion, the similarity of the results found after evaluations of histopathological scores were due to the capacity to recognize germ cells, as discussed above, and also due to the homogeneous group of patients used in this study. The group consisted of five individuals with prostate cancer who did not undergo any treatment until the time of orchiectomy and showed well preserved spermatogenesis. We believe that the small differences between their spermatogenesis were not enough to detect the interference of processing techniques on the score. Our group has shown that the histological processing could interfere with the histopathological scores in azoospermic man and that such change was evident, especially in patients with fully compromised spermatogenesis, as in cases of maturation arrest and in Sertoli cell only phenotypes (unpublished data).

Taken together, our findings showed that testicular tissues fixed with glutaraldehyde and embedded in plastic resins are comparable for morphometric data and morphology. However, assessments of spermatogonial biology are only confident after GA processing. On the other hand, after BP processing, such morphometrical and morphological correlations cannot be made directly due to the high degree of tissue retraction and cellular granularity. Moreover, it was shown that germ cell counting did not differ between protocols, since most cells have been identified, even with loss of morphological quality. On the other hand, when linear measurements were compared, the differences were significantly reduced, demonstrating that tissue retraction interfered with the results, which invalidates the comparison without correction factors.

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