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Fixation of human detrusor smooth muscle cells: role of osmolarity and magnesium ions on the ultrastructural morphology

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Abstract Magnesium ions added to fixatives for processing to Transmission Electron Microscopy (TEM) have been claimed to cause relaxation of detrusor smooth muscle cells [1]. This should facilitate the morphologic evaluation of the tissue. However, magnesium ions are osmotically active and their addition may cause the fixative to become hypertonic to the tissue. To ascertain whether the presence of magnesium ions causes significant changes compared to those found where the osmolarity is raised without the presence of magnesium, human detrusor specimens were fixed in glutaraldehyde to which increasing amounts of $MgCl_2$ or NaCl were added in different concentrations. With the addition of increasing amounts of $MgCl_2$ and NaCl, the osmolarity of the fixative increased, causing significant changes in the morphology and morphometry of the tissue. The intercellular distances increased, the cells shrank and the shape of the cells changed from smooth and rounded to spiky and angulated. With regard to its muscle-relaxing effect, it was not possible to distinguish the specimens fixed in magnesium-containing fixatives from those without. In this study it was not possible to prove any relaxing effect of magnesium ions added to the fixative. On the contrary the magnesium ions caused an increase in the osmolarity, with significant changes in both the morphometry and the morphology of the human detrusor smooth muscle cells.

Key words Detrusor · Fixation · Magnesium · Osmolarity · Smooth muscle cells · Ultrastructure

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Recent electron microscopic studies on the morphology of the human detrusor smooth muscle cells have revealed interesting correlations between the urodynamic parameters and morphological features [3]. Widening of intercellular spaces, branching ends of the muscle cell profiles and protrusion junctions [2] (Fig. 3) have been ultrastructurally found as diagnostic markers for bladder outlet obstruction and detrusor instability, respectively [3]. In these studies the specimens were fixed in 2.5% or 4% glutaraldehyde in Millonig's phosphate buffer modified by the addition of Mg^{2+} [1] to prevent contraction of the detrusor smooth muscle cells [1]. However, addition of osmotically active ions, such as Mg^{2+}, increases the osmolarity of the fixative and may thus alter the quality of the fixative and therefore also the morphology of the tissue.

Different fixatives and buffer systems used for electron microscopy each have their characteristics, but it is generally accepted that pH and tonicity of the solutions should be held as close to the physiological level as possible [10, 14, 15]. In a study on rat kidneys the osmolarity of the fixative was shown to alter the ultrastructural morphology significantly, whereas the choice of buffer system was of only minor importance [10].

In order to study detrusor morphology in a prospective series of patients with different voiding disorders, we found it of importance to investigate to what extent magnesium ions and/or varying osmolarity of the fixative may influence the ultrastructure of detrusor smooth muscle cells.

Materials and methods

Biopsies from the detrusor muscle were obtained from: (1) 17 men undergoing a transurethral resection of their prostate (TURP) because of bladder outlet obstruction, (2) 7 men undergoing urodynamic investigation because of lower urinary tract symptoms (LUTS) and (3) 2 men admitted for cystoprostatectomy because of invasive bladder cancer.

Study of the role of magnesium ions

From the 17 patients undergoing TURP, 1 detrusor biopsy was obtained transurethrally from the right bladder wall with forceps (Storz 27072A, Karl Storz, Germany). The biopsy was immediately sectioned into three equal parts – each fixed overnight by immersion in one of the following fixatives: (a) 2.5% glutaraldehyde with cacodylate buffer in a 0.1 M solution (our routine fixative), (b) 2.5% glutaraldehyde with cacodylate buffer with addition of $MgCl_2$ in a 0.1 M solution and (c) 2.5% glutaraldehyde with cacodylate buffer with addition of $MgCl_2$ in a 0.2 M solution [the amount of $MgCl_2$ corresponds to that used in [1] (personal communication)].

From the seven patients admitted for urodynamic investigation, two detrusor biopsies were obtained from the anterior bladder wall by the ultrasound guided technique described previously [7]. One biopsy was fixed in fixative (a) and the other was fixed in fixative (c).

Study of the role of osmolarity

Five detrusor biopsies were obtained from a tumor-free area in each of two cystectomy specimens. The biopsies were fixed overnight by immersion in one of the following fixatives: (1) 2.5% glutaraldehyde with cacodylate buffer in a 0.1 M solution [the same as fixative (a)], (2) 2.5% glutaraldehyde with cacodylate buffer in a 0.1 M solution with addition of NaCl in a 0.05 M solution, (3) 2.5% glutaraldehyde with cacodylate buffer in a 0.1 M solution with addition of NaCl in a 0.1 M solution, (4) 2.5% glutaraldehyde with cacodylate buffer in a 0.1 M solution with addition of NaCl in a 0.2 M solution and (5) 2.5% glutaraldehyde with cacodylate buffer in a 0.1 M solution with addition of NaCl in a 0.3 M solution.

The amount of NaCl in each of the five fixatives was chosen in order to obtain an osmolarity of the fixatives as close as possible to the osmolarity of fixatives (a), (b) and (c). After primary fixation all the above-mentioned biopsies were treated identically: postfixation in 2% osmium tetroxide for 1 h and embedded in Epon resin. Semithin (1 μm) survey sections were stained with toluidine blue. Ultrathin sections were mounted on 150-mesh copper grids and stained with uranyl acetate/lead citrate before examination under a Philips EM 208 transmission electron microscope.

The osmolarity of all fixatives used in the study was measured in an Osmomat 030 (Bie & Berntsen).

Light microscopy

Survey sections were subjected to *morphological evaluation* including recording of uniformity in size and shape of the detrusor muscle cells and the intercellular distances.

Transmission electron microscopy

For each specimen detrusor ultrastructure was documented in randomly obtained photomicrographs at an original magnification of ×2500. The photomicrographs were subjected to semiquantitative morphologic as well as morphometric analysis.

Morphological evaluation

The photomicrographs were examined semiquantitatively for the presence of degenerative changes (vacuolization of the sarcoplasm, irregular scattering of dense bodies and the presence of cell debris in the intercellular space), bizarre muscle cell profiles such as branching ends, the presence of protrusion junctions and finally the presence of elastin in the intercellular space. The semiquantitative grading was: the feature not present in any micrographs from one specimen, 0; present in less than one-third of the micrographs from one specimen, +; present in one-third to two-thirds of the micrographs from one specimen, ++; present in more than two-thirds of the micrographs from one specimen, +++.

Morphometric analyses included the measurement of intercellular distances, cell thickness and nuclear-to-cytoplasmic ratios of muscle cells and were performed according to the methods described previously [11, 12]. Shortly, a transparent grid with horizontal lines 10 mm apart was placed on the photomicrographs. One hundred and fifty intercellular distances from each specimen were measured as the shortest distance between two adjacent muscle cells where the sarcolemma of the cells crossed one of the lines on the grid. Forty cell:nucleus ratios were measured in each biopsy. Muscle cell profiles containing nucleus were used for that purpose. The shortest diameter of the cell was measured through the center of the nucleus. The diameter of the nucleus was measured at the same position and a cytoplasmic/nucleus ratio was calculated. Semiquantitative as well as morphometric analyses were performed blinded.

All patients signed a written informed consent form and the study was approved by the local Ethics Committee.

The morphometric data were analysed statistically using Microsoft Excel computer software. This included the means, medians, standard deviations, variances and a one-way analysis of variance test (ANOVA). The biopsies were compared for each patient.

Results

Study of the role of magnesium ions

Of the 17 men undergoing TURP (group 1), 12 were excluded since 1 or more of the specimens did not contain detrusor muscle on survey sections. All of the seven men admitted for urodynamic investigation (group 2) were included. Below groups 1 and 2 are considered as one group. A total of 12 men 53–84 years of age (median 71 years) entered this part of the study.

Light microscopy

The survey sections showed no difference between the specimens fixed in fixatives (a) and (b) from each patient. Specimens fixed in fixative (c) revealed evident differences from the other two as the intercellular distances had clearly increased and the shape of the muscle cells was angular with finger-like protrusions (Fig. 1a,b).

Electron microscopy

A total of 1024 photomicrographs, 17–46 (median 37) per specimen, were examined and analyzed. One hundred and fifty intercellular distances, 40 cell-to-nucleus ratios and 40 shortest cell diameters were measured and analyzed for each specimen.

By *semiquantitative analysis* the detrusor muscle cells fixed in fixatives (a) and (b) were smooth, uniform in size and shape and seemed to fit into each other (Fig. 4a). The intercellular junctions were mostly of the intermediate type [2] (Fig. 2), but a few protrusion junctions [2] (Fig. 3) were also observed. Except for a slightly wider intercellular distance in the specimens fixed in fixative

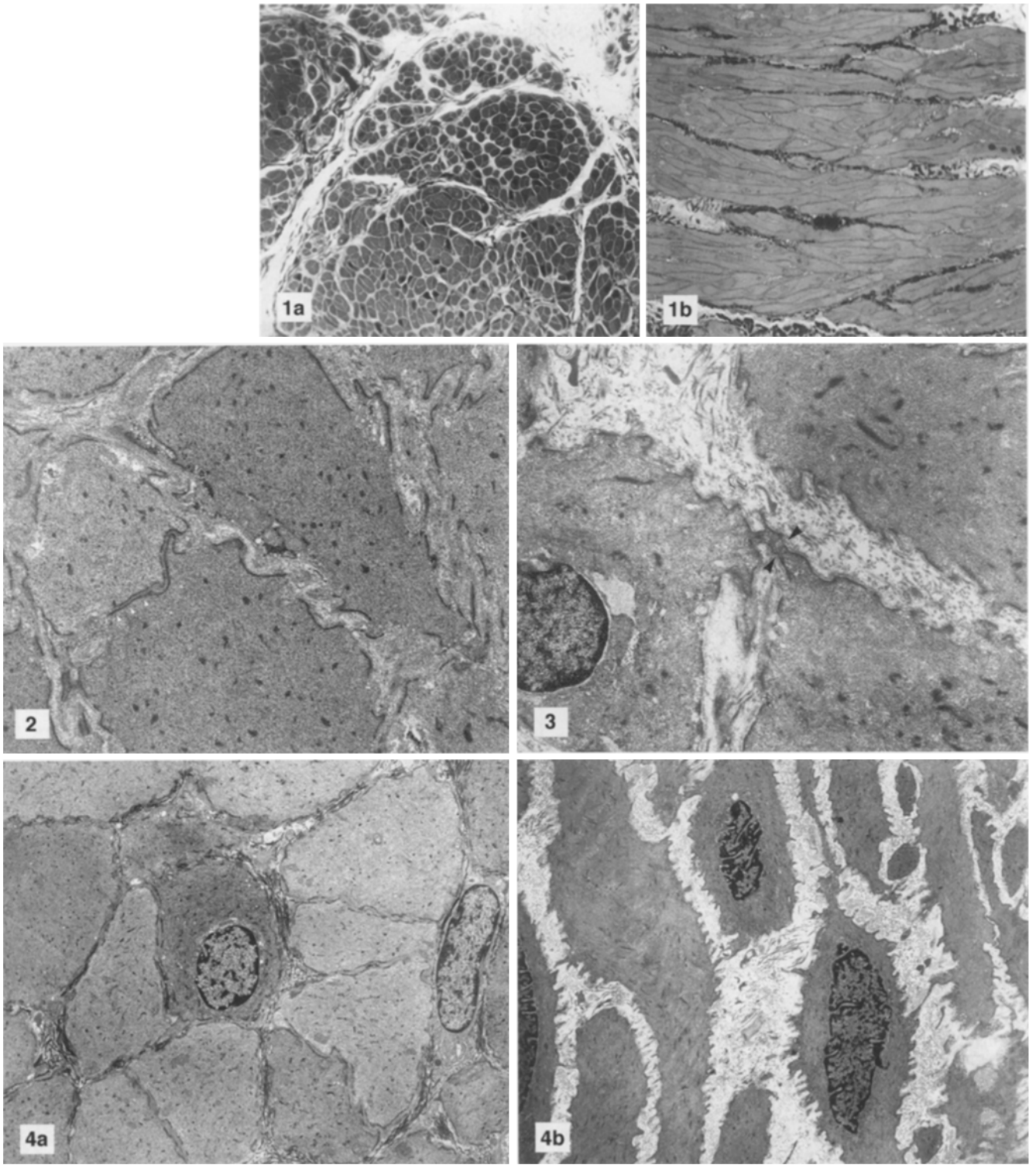

Fig. 1a,b Survey section of detrusor specimens fixed in **a** fixative (a) and **b** fixative (c). Toluidine blue. Original magnification ×600

Fig. 2 Intermediate junction between two detrusor smooth muscle cells (*arrowheads*). Original magnification ×4000

Fig. 3 Protrusion junction between two detrusor smooth muscle cells (*arrowheads*). Original magnification ×6300

Fig. 4a,b Two detrusor specimens from the same patient fixed in **a** fixative (a) and **b** fixative (c). Original magnification ×2500

(b), it was not possible to distinguish these specimens from those fixed in fixative (a).

Specimens fixed in fixative (c) revealed a disorganized pattern (Fig. 4b). Muscle cells were angular with finger-like protrusions, uneven in size and shape and the distance between the muscle cells had increased compared to the specimens fixed in fixatives (a) and (b). The intercellular junctions were almost entirely protrusion

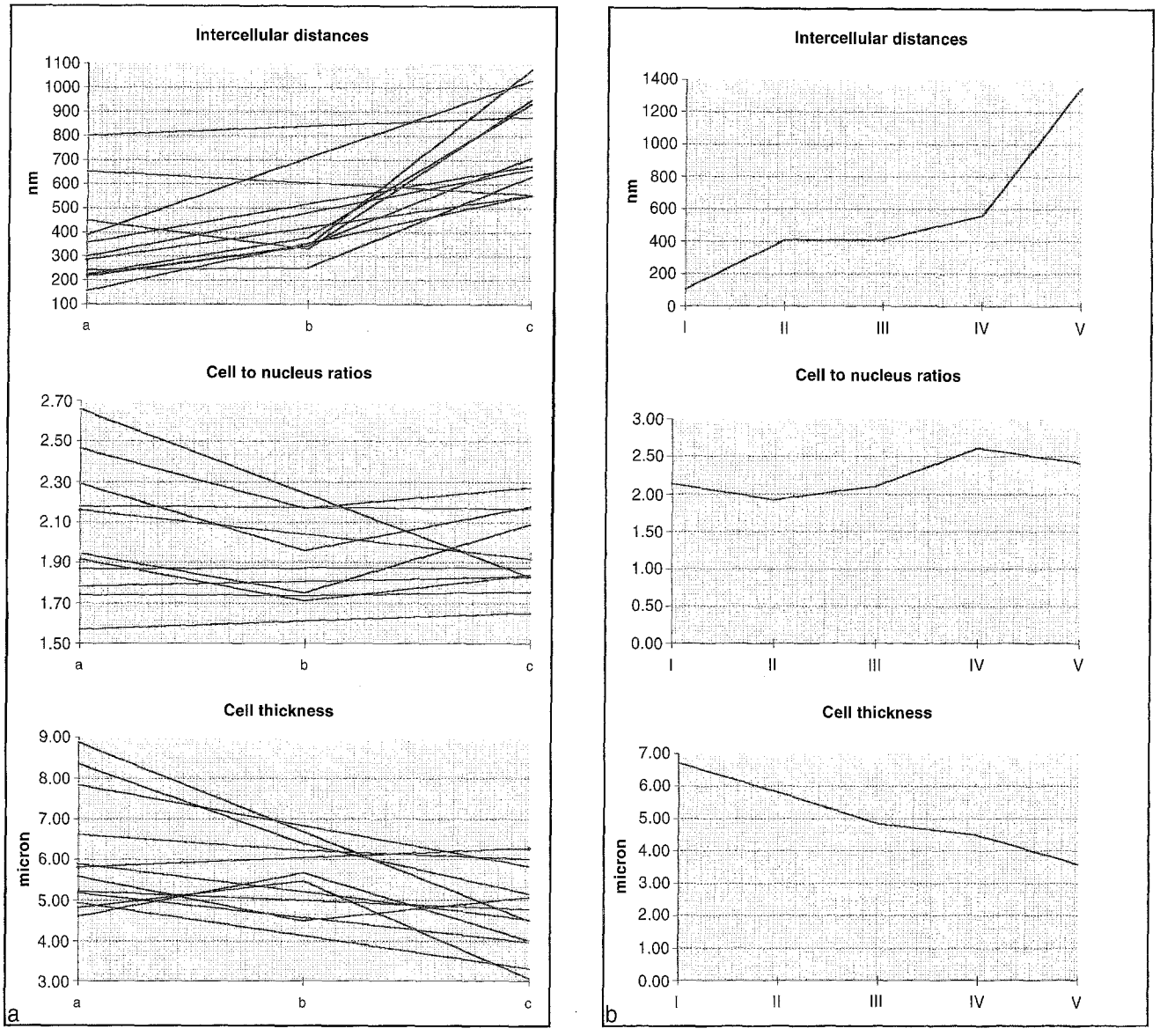

Fig. 5 a Schematic drawings from the 12 patients in the magnesium study of the geometric meanvalues of the intercellular distances (*upper*), the cell-to-nucleus ratios (*middle*) and the cell thickness (*lower*). The values are very similar in some of the patients, which makes the curves more or less confluent. **b** Schematic drawings from the patient in the osmolarity study of the geometric mean values of intercellular distances (*upper*), the cell-to-nucleus ratios (*middle*) and the cell thickness (*lower*)

junctions (Fig. 4b). The cell membranes were more electron dense and the caveolae were more prominent (Fig. 4b). With regard to degenerative changes and the presence of elastin in the intercellular spaces, no differences between the three fixatives were recorded.

Morphometry

It became obvious from the histograms that the measured data were positively skewed and a log transformation was therefore performed. From the log-transformed data the means and standard deviations were calculated. The back-transformed means, the geometric means, are shown in Fig. 5a. A one-way analysis of variance (ANOVA) was performed for each patient.

Intercellular distances showed statistically significant increases ($P < 0.05$) from fixative (a) to (c) in 10 of the 12 patients (Fig. 5a, upper). In one patient the intercellular distances increased but not significantly so. In another patient the intercellular distances decreased significantly from fixative (a) to (c). The morphology of specimen (a) from this patient revealed large smooth muscle cells with very wide intercellular distances. In the five patients where fixative (b) was included there was a statistically significant increase ($P < 0.05$) in intercellular distances from fixative (a) to (b) in three of the patients.

Cell-to-nucleus ratios showed no statistically significant difference ($P > 0.05$) between fixative (a) and (c) in

9 of the 12 patients (Fig. 5a, middle). In one patient the cell-to-nucleus ratios increased significantly and in two other patients the ratios decreased significantly.

In those five patients where fixative (b) was included, there was no statistically significant difference ($P > 0.05$) between fixatives (a) and (b) in four of the patients. *Cell thickness* showed a statistically significant decrease ($P < 0.05$) from fixative (a) to (c) in 10 of the 12 patients (Fig. 5a, lower). In two patients the thickness decreased but not significantly so. In those five patients where fixative (b) was included there was a statistically significant decrease ($P < 0.05$) in the cell thickness in three of the patients.

Study of the role of osmolarity

Two men aged 64 and 68 years entered this part of the study (group 3). A total of 277 photomicrographs, 48–63 (median 55) per specimen from the first patient, were examined and analyzed as described for magnesium. Only a small number of photomicrographs were obtained from the second patient in order to confirm the findings from the first patient.

Light microscopy

The specimens fixed in fixatives (1)–(3) showed similar appearances and it was not possible to distinguish one from the other. The specimens fixed in fixatives (4) and (5) were different since intercellular distances had increased and the detrusor muscle cells were angular, an appearance similar to that found in specimens fixed in fixative (c) from the previous study.

Electron microscopy

Specimens fixed in fixatives (1)–(3) showed a morphology similar to that seen in specimens fixed in fixatives (a) and (b) (Fig. 6a). The only difference between the specimens fixed in the three fixatives was a slight increase in the intercellular distances between fixatives (1), (2) and (3). The specimens fixed in fixatives (4) and (5) were different from (1) to (3) but similar to specimens fixed in fixative (c) from the previous study. The intercellular distances had increased, the detrusor muscle cells were different in shape and size, they were angular or rather spiky with finger-like protrusions and they seemed shrunken. Caveolae were more prominent (Fig. 6b).

Morphometric analysis

The histograms showed a positive skewness and a log transformation was performed. The means and standard deviations were calculated and the back-transformed means, the geometric means, are shown in Fig. 5b.

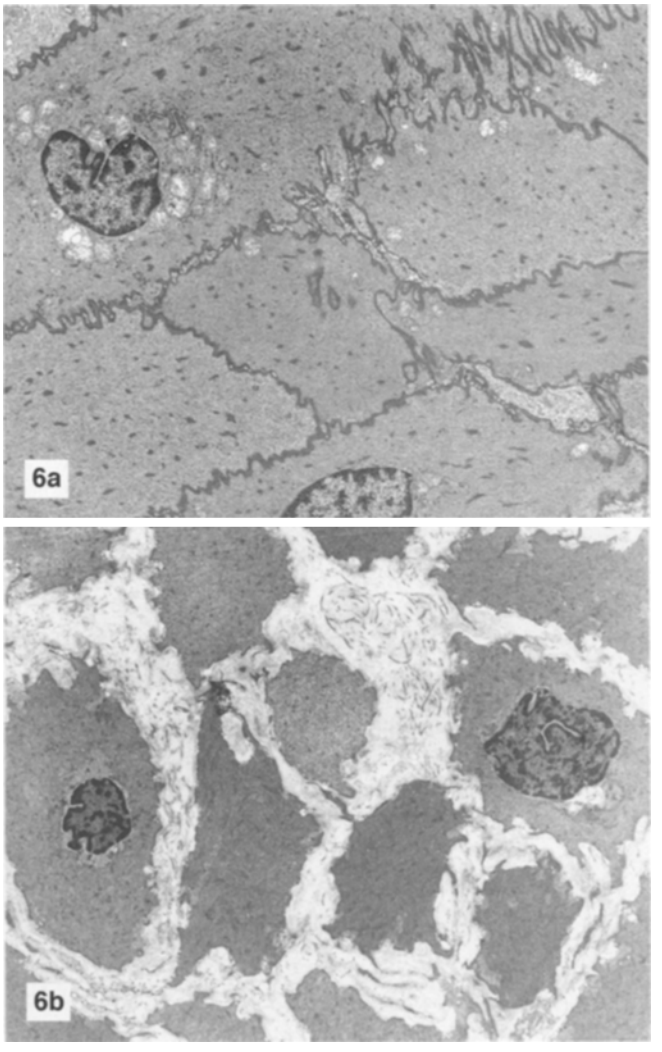

Fig. 6a,b Two detrusor specimens from the same patient fixed in **a** fixative (1) and **b** fixative (5). Original magnification ×2500

Intercellular distances showed statistically significant increases ($P < 0.05$) from fixative (1) to fixative (5). Between fixatives (1) and (2) there was a statistically significant increase but not between fixatives (2) and (3) (Fig. 5b, upper).

Cell-to-nucleus ratios showed statistically significant differences ($P < 0.05$) between the five fixatives. Between fixatives (1) and (2) and between fixatives (2) and (3) there was no statistically significant difference ($P > 0.05$) (Fig. 5b, middle).

Cell thickness decreased significantly ($P < 0.05$) from fixative (1) to fixative (5). From fixative (1) to (2) and from fixative (2) to (3) a significantly decrease in cell diameter was also observed (Fig. 5b, lower).

Osmolarity

The osmolarity of the fixatives is shown in Table 1.

Table 1 Total osmolarity of the different fixatives (mOsm)

Fixative no.	Fixative	Total osmolarity of fixative (mOsm)
(a) = (1)	2.5% glutaraldehyde with cacodylate buffer	452
(b)	2.5% glutaraldehyde with cacodylate buffer and 0.1 M $MgCl_2$	715
(c)	2.5% glutaraldehyde with cacodylate buffer and 0.2 M $MgCl_2$	947
(2)	2.5% glutaraldehyde with cacodylate buffer and 0.05 M NaCl	599
(3)	2.5% glutaraldehyde with cacodylate buffer and 0.1 M NaCl	686
(4)	2.5% glutaraldehyde with cacodylate buffer and 0.2 M NaCl	883
(5)	2.5% glutaraldehyde with cacodylate buffer and 0.3 M NaCl	1033

Discussion

Optimal preservation of tissue can be obtained by in vivo perfusion of fixative [4, 15]. For ethical and practical reasons this is impossible in human tissue. Immersion fixation of small ($< 1\text{-mm}^3$) tissue blocks immediately after their removal is often the only alternative. Study of smooth muscle cells from the human detrusor may be influenced not only by the autolytic artifacts caused by hypoxia and shrinking artifacts caused by the properties of individual solutions for preparation, but also by the degree of relaxation/contraction of the individual muscle cells. It has been claimed that addition of magnesium ions to the fixative for the processing of human detrusor to TEM causes relaxation of the smooth muscle cells [1]. However, the role of magnesium in the contractile activity of smooth muscle cells is debatable. In vitro studies by Ikebe et al. [8] on skinned smooth muscle fibers showed a relatively high (up to 40 mM) requirement of $MgCl_2$ for contraction. This was supported by Richard and Ruegg [13]. It also seems that the magnesium requirement for contraction is much higher for smooth muscle cells than for striated muscle [5]. On the other hand, $MgCl_2$ is recommended as a relaxant of highly motile organisms and for those that contract when exposed to fixative. Magnesium salts are added to the water containing the organisms until the appropriate anesthesia has been attained [9]. It is emphasized that the organisms should only be anesthetized and not killed by the anesthetic and that fixation should follow immediately after narcotization.

In the study by Elbadawi et al. [3] as well as in our study, magnesium was added to the fixative. From a practical standpoint this differs from the above-mentioned studies but theoretically magnesium could still exert its effect on dying cells with more or less passive cell membranes. However, we could not identify any relaxing effect of magnesium. Instead the smooth muscle cells shrank and were retracted from each other, which can probably be attributed to the hypertonicity of the fixative since this shrinking phenomenon was observed both with and without magnesium (Figs. 4b, 6b). Shrinking artifacts may play a role in the interpretation of previous publications [1–3], but new studies on the detrusor using isotonic fixatives are necessary.

For most mammalian tissues the recommended total osmolarity of fixatives ranges from 500 to 700 mOsm [6], which is hypertonic to human tissue (blood plasma is 300 mOsm [15]). In a study on rat kidneys the osmolarity of the fixative was raised to 650 mOsm (rat serum is 300–320 mOsm) by addition of NaCl. Upon electron microscopic examination the tissue appeared shrunken compared to the tissue fixed in isotonic (354 mOsm) or slightly hypertonic (408 mOsm) fixatives [10]. It was concluded that the observed structural differences were related to the total amount of osmotically active ions or molecules in the fixative vehicle and not to the ionic strength of the glutaraldehyde [10]. The choice of buffer system was also of minor importance at least in fixation of rat kidney tissue as none of the components in the different solutions exerted any specific effect on the cells [10].

The osmolarity of our routine fixative [fixative (a)] was 452 mOsm, which is slightly hypertonic to human tissue. The osmolarity of fixative (c) which corresponds to the fixative used in a prior ultrastructural/urodynamic study [3] (personal communication), was 947 mOsm. The osmolarity of fixatives (4) and (5) was 883 and 1033 mOsm, respectively. In specimens fixed in these three fixatives [(c), (4) and (5)], we observed a significant shrinking and retraction of cells as compared to specimens fixed in fixative (a). This was confirmed morphometrically (Fig. 5b). Even light microscopically it was possible to distinguish specimens fixed in fixatives (c), (4) and (5) from those fixed in fixatives with lower osmolarity. In specimens fixed in fixatives (b), (2) and (3), where the osmolarity ranged from 599 to 715 mOsm, only a slight increase in the intercellular distances was observed but we could not identify any shrinking of the cells. By the morphometric analysis we found a statistically significant difference in the intercellular distances and the cell diameters in about 50% of the cases. This could be due to the fact that the osmotically active ions (Mg^{2+} and Na^{+}) start exerting their effect at this osmolarity.

The cell-to-nucleus ratios did not change significantly in most specimens. This might be explained by a simultaneous shrinking of nucleus and cytoplasm to the same degree, suggesting that the cell-to-nucleus ratio remains almost the same no matter how high the osmolarity of the fixative is.

The purpose of fixation is to keep the morphology as close to the living state as possible but in certain cases an intended unphysiological treatment of the tissue could be relevant in order to visualize some special elements of the

tissue. This study showed the importance of keeping the total osmolarity of the fixative at a physiological level. *Our routine fixative*, which is the routine fixative in many places, fulfils this requirement. Furthermore, in our study we were unable to see any relaxing effect of magnesium and we do not find it advisable to add magnesium to the fixative due to its osmotically active effect.

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