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INFLUENCE OF FIXATION, EMBEDDING AND SECTION MOUNTING ON STEREOLOGICAL ESTIMATES OF CANCER CELL MEAN VOLUME-WEIGHTED NUCLEAR VOLUME

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ABSTRACT

Objective malignancy grading of tumours by stereological estimation of the mean volume-weighted volume of cancer cell nuclei ($\overline{v}_V$(nuc)) might be clinically useful. Prior to introduction in routine pathology, however, the effect of variations in tissue processing should be investigated. In the current study, the effect on $\overline{v}_V$(nuc) was assessed in 27 breast cancers regarding variations in formalin fixation and embedding medium, whereas the influence of the mounting procedure was studied in 14 bladder tumours. For comparison, changes in gross dimensions of tumour specimens due to embedding were assessed in tissue from 5 breast cancers. Estimates of $\overline{v}_V$(nuc) were stable regarding the duration and acidity of formalin fixation, but $\overline{v}_V$(nuc) was, on average, 13% larger in methacrylate than in paraffin ($2p=0.004$). Mounting of paraffin sections directly from water provided a mean $\overline{v}_V$(nuc) about 30% larger than standard mounting from xylene or mounting from ethanol ($2p\leq0.02$). The overall mean linear shrinkage of tissue bars in paraffin was 5%, whereas, in methacrylate, tissue bars swelled by 2% ($2p\geq0.10$). The dynamic changes in the dimensions of tissue were, however, dramatic during paraffin embedding (up to 15%), whereas methacrylate embedded tissue was much more stable (up to 5% change). Thus, for reproducible assessments of $\overline{v}_V$(nuc), tissue processing should be standardized with respect to the type of embedding medium and the section mounting procedure. As paraffin embedding leads to great dynamic changes in tissue dimensions, the process is, most likely, sensitive, and should be strictly controlled. Mounting of paraffin sections from ethanol could replace routine mounting from the potentially toxic xylene without affecting estimates of $\overline{v}_V$(nuc).

Key words: malignancy grade, nuclear size, quantitative histopathology, tissue processing.

INTRODUCTION

Previous studies have shown the prognostic significance and feasibility of the stereologically estimated cancer cell mean volume-weighted nuclear size in several solid tumours (see, Sørensen (1992)). The unbiased technique of Point Sampled Intercepts (Gundersen and Jensen, 1985) seems well suited for objective malignancy grading, as it is

applicable on routine sections, efficient, and associated with an excellent observer reproducibility (Baak et al., 1994).

It is well-known, however, that variations occur in the routine processing of tissue for histological examination, especially among different laboratories. Thus, to ensure validity and accuracy of estimates of the mean nuclear volume, the influence from variations in tissue processing should be clarified, and a standard introduced for sensitive steps of the process.

A related problem concerns modifications to the processing protocol. For example, the need for replacement of potentially toxic compounds with less harmful ones has lead to the suggested replacement of xylene with non-toxic substances in the paraffin-block clearing process (Rasmussen et al., 1992). Also mounting of Haematoxylin-Eosin (H.-E.) stained sections directly from the water bath has been advocated, avoiding xylene clearing of sections in the mounting process (Nøhr, 1990). The impact of these changes on quantitative estimates of histological structures has, however, not been assessed to date.

The present study summarizes the results of previous investigations of tissue processing (Ladekarl, 1994; Ladekarl and Svanholm, submitted) with regard to estimates of nuclear volume. For comparison with changes in this parameter, the gross dimensions of cancer tissue were measured during processing of paraffin and methacrylate embedded sections.

MATERIALS AND METHODS

Several 2-mm-thick tissue bars were cut from 2-mm-thick slices of 27 breast tumors and allocated to varying processing schedules. The influence of the acidity of fixative was studied by allocating tissue from 5 tumours to fixation in formalin for 3 days at pH 5.0, 6.0, 7.0, 7.4 and 8.0, respectively; the influence of fixation duration was investigated by allocating tissue from 5 tumours to fixation in formalin at pH 7.4 for 1 day, 3 days and 3 months, respectively. Finally, the influence of the embedding medium was investigated by allocating tissue from 17 tumours to embedding in Paraplast® paraffin and in Technovite® hydroxyethyl-methacrylate, respectively. Before metacrylate embedding, tissue was embedded in 5% agar and dehydrated in increasing concentrations of ethanol (70-90-99%). Measurements of the volume-weighted cancer cell mean nuclear volume, $\overline{v}_V(nuc)$, were performed on H.-E. stained sections using Point Sampled Intercepts (Gundersen and Jensen, 1985) in a vertical design (Baddeley et al., 1986). On average, 137 nuclei (range, 88-312) were measured in fields of vision sampled from the whole tumour using a systematic random sampling scheme (Ladekarl (1994)).

In 5 randomly selected breast cancers, the gross dimensional changes were assessed of tissue bars (cut at 2 x 2 mm and approximately 10 mm in length), allocated to embedding in paraffin and methacrylate, respectively. The exact length of formalin-fixed bars was measured before embedding, on the cut surface of the block, and on the final histological slide.

To investigate the influence of changes in the process of clearing for section mounting, tumour chips from 14 bladder tumours were embedded in paraffin. Consecutive, 5 μm-thick sections were allocated to H.-E. staining followed by mounting, either 1) directly from the water bath, 2) after washing in water and ethanol dehydration, or, 3) according to routine, after washing in water, ethanol dehydration and xylene clearing (Ladekarl and Svanholm (submitted)). The $\overline{v}_V(nuc)$ of bladder tumours was estimated in fields of vision sampled systematically randomly from the tumour areas of the sections, assuming isotropy of nuclei in chips. On average, 105 nuclei (range, 60-146) were measured per tumour.

RESULTS

As shown in Table 1, no significant differences were observed in mean $\bar{v}_V$(nuc) with regard to variables of fixation, whereas estimates of $\bar{v}_V$(nuc) obtained in methacrylate were, on average, 13% larger than those obtained in paraffin. Mounting of sections from water gave estimates of $\bar{v}_V$(nuc) about 30% higher than those obtained after mounting from ethanol or xylene, whereas no significant difference in mean $\bar{v}_V$(nuc) was found among sections mounted from ethanol or from xylene.

Table 1. Estimates of $\bar{v}_V$ **(nuc)** *in tissue processed at variable fixation duration, fixative acidity, embedding medium and mounting procedure.*

Variables of processing	Mean $\bar{v}_V$ **(nuc)** (μm^3)	Difference#	CE (CV)	2p-value
Fixation duration				
1 day	674*	-	(0.21)	-
3 days	617	-8%	0.76	0.26
3 months	674	0%	-	1.00
Acidity of fixative				
pH 5.0	402	-7%	0.74	0.25
pH 6.0	383	-12%	0.77	0.27
pH 7.0	387	-11%	0.86	0.31
pH 7.4	433*	-	(0.18)	-
pH 8.0	383	-12%	0.60	0.17
Embedding medium				
Methacrylate	418*	-	(0.29)	-
Paraffin	363	-13%	0.30	0.004
Mounting from§				
Water	459	31%	0.40	0.02
Ethanol	367	5%	1.02	0.67
Xylene	350*	-	(0.47)	-

* mean $\bar{v}_V$(nuc) of "standard" processed tissue.
§ geometric means indicated.
# relative difference between mean $\bar{v}_V$(nuc) of the current processing and that of the "standard"; 2p-values are the results of Student's paired t-tests. The coefficient of error (CE) is shown of *differences* between values of the current processing and the "standard". For "standard" processing, the coefficient of variation (CV) is given.

As illustrated in Fig. 1, the mean tumour tissue bar length, measured on the cut surface of paraffin blocks, was reduced by 15% as compared with the fixed, non-embedded bars (2p=0.001). A swelling occurred, however, during the final steps of processing, in that a 5%, non-significant total shrinkage resulted of the histological section (2p=0.16). In methacrylate, the bars shrank 3% with embedding (2p=0.02), and swelled during the last steps of the processing, resulting in a final 2%, non-significant total swelling (2p=0.10).

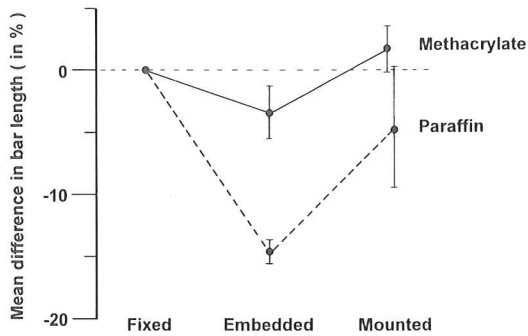

Fig. 1. Tissue bar length before and after embedding, and after mounting. Mean and CE of the difference in bar length relative to the length of fixed, unembedded tumour bars is indicated.

DISCUSSION

From theoretical and experimental data summarized elsewhere, it can be concluded that estimates of $\bar{v}_V$(nuc) are relatively stable to most variables in tissue processing (Ladekarl, 1994; Ladekarl and Svanholm, submitted). However, as indicated by the present study, the type of embedding medium and mounting procedure should be standardized. Most likely, measurements carried out in paraffin sections are prone to influence from laboratory variations, as paraffin embedded tissue changes dramatically in dimension during processing. In agreement, Boonstra et al. (1983) reported a 13% linear shrinkage of formalin fixed tissue during paraffin embedding, but, in contrast to our results, they found only minor variations in dimensions during the final steps of processing. Unfortunately, no details of laboratory procedure were provided, and, as indicated by the significant effect on nuclear size of mounting procedure found in the present study, even minor modifications in the final steps of the paraffin processing may result in great dimensional changes.

It has been suggested that nuclei might be less influenced by the shrinkage induced by paraffin embedding than other tissue components, because when compared with methacrylate embedded tissue, nuclear profile density estimates are reduced relatively more than are estimates of $\bar{v}_V$(nuc) (Ladekarl, 1994). This theory was supported by the present finding of a smaller relative reduction in $\bar{v}_V$(nuc) (of 13%) as compared to that of whole tumour bars (7% in length, corresponding to a volume reduction of 23%).

In conclusion, compared with methacrylate, paraffin embedding results in a non-negligible shrinkage of cancer cell nuclei, whereas the $\bar{v}_V$(nuc) is stable to variations in the duration of fixation and to variations in the acidity of fixative. Quantitative variables obtained in paraffin sections seem relatively sensitive to variations in tissue processing. Thus, using paraffin for embedding, tissue processing should be highly standardized. With respect to estimates of $\bar{v}_V$(nuc), mounting of H.-E. stained paraffin sections from ethanol could replace routine mounting from xylene, avoiding the potential health hazards of this organic solvent.

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