Investigation into a new softening agent for use on formalin-fixed, paraffin wax-embedded tissue

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ABSTRACT

The use of tissue softening agents to improve microtomy of keratotic tissues is employed widely. Many of these softeners contain hazardous constituents such as phenol. In this study, the use of non-ionic surfactants or non-toxic ingredients are investigated with the aim of creating a new softening agent. The new agent should be more effective in facilitating the sectioning of hardened tissue while reducing toxicity and complications associated with sectioning hard tissue compared to a commercially available phenol-based formulation. Four formulations are compared against the commercial product for their capability to section routinely processed paraffin-embedded tissue under standard operating procedure parameters. The trial formulations were shown to be fast acting and enabled improved serial sectioning of hard keratotic tissue in nearly all the cases tested. There was no evidence of adverse staining using either tinctorial or immunohistochemical methods. The new formulations had advantages over the commercially available solutions, improving on the number and quality of sections attainable from the tissue blocks, as well as offering a composition less toxic than phenol-based products.

KEY WORDS: Bone.
Histopathology.
Keratosis.
Microtomy.
Skin.
Tissue softener.

Introduction

Heavily keratotic or generally hardened tissues have posed difficulties for successful microtomy for many years. The majority of reagents used to soften tissue blocks have arisen from very early work performed in the first half of the last century.[1–5] Many of these substances contained hazardous constituents such as phenol,[5] and this remains a common constituent of commercial histological tissue softening agents.

Other more intriguing publications reported the use of fabric conditioner as a softener, and in a few cases to reconstitute mummified tissue prior to paraffin-wax embedding with the objective of rehydrating tissue.[6–9] In addition, many reagents reported to facilitate tissue softening have never been investigated adequately and have gained transient popularity as a result of word of mouth rather than proven and substantiated fact. In reality, comparative studies looking at several proposed agents have never truly been performed.

A compounding problem is the fact that some form of objective evaluation is difficult to substantiate unless a controlled appraisal process is performed in which all trial reagents and commercially available reagents can be evaluated on the same tissue block and then compared in an objective fashion. These reagents should also be compared to an existing product sold for the specific purpose of softening tissue for sectioning. The important indicators of performance need to be established and tabulated in exactly the same manner for all the trial reagents and then compared to the commercially available product.

The objective of the present study is to produce a new product which would be a more effective tissue softener that does not pose a health and safety risk to biomedical scientist staff and would be an improvement on currently available products. Existing commercially available sources of tissue softening agent are expensive and many contain hazardous reagents such as phenol. In the authors' experience, these agents do not provide the answer in terms of the adequate sectioning of a surprising number of hardened cutaneous tissue conditions.

A rapidly acting softener is required which offers sufficient penetration to allow multiple sections to be cut, and subsequent staining should not be affected. Scent is also a consideration as it is hoped that the product could mask unpleasant smells (e.g., cysts generally contain unpleasant smelling degenerative tissue) to ensure section cutting is not an unpleasant task.

Materials and methods

A blind study was conducted, testing two trial formulations at their prepared concentrations and at a dilution of 50%, and also a commercially available product anonymised alphabetically and tested at random to ensure the test participants were unaware of the composition. The trial

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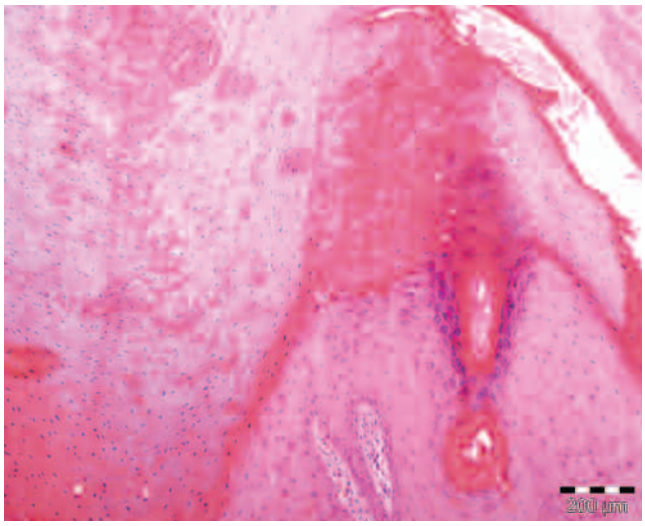

Fig. 1. Cutaneous horn following softening with trial sample B, showing uniform and undisrupted epidermal layers (haematoxylin and eosin [H&E] stain, original magnification x20).

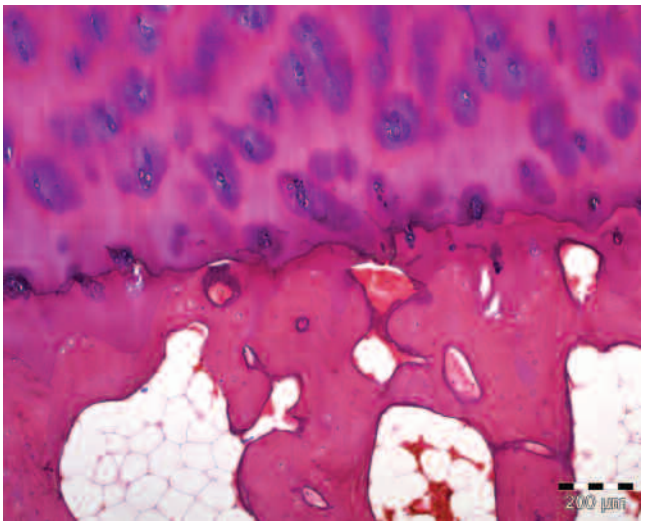

Fig. 2. Decalcified tissue from a femoral head, demonstrating the bone and cartilage interphase, following softening with trial sample B. Note there is no shrinkage or retraction of the interphase (H&E stain, original magnification x20).

participants were informed that a placebo may be included in an attempt to avoid bias.

Four samples were prepared by CellPath (samples A, B, C and D), created from the two original formulations used at two concentrations. A well-known commercially available tissue softener (sample E) was also assessed in comparison to the four trial formulations on the same histological blocks.

All test histological blocks involved in the study were chosen for their difficulty of sectioning from reserve tissue used for control purposes from the primary author's archives. These included two cases of cutaneous horn, one case of idiopathic scrotal calcification, two cases of keloid, one case of decalcified femur tissue, one case of decalcified osteoid tissue formation in cutaneous scalp tissue, two cases of basal cell carcinoma with focal calcification, two cases of verucca from the sole of the foot and one case of a fungal infection from the sole of the foot.

All tissue had been fixed in 10% neutral buffered formalin for 12 hours and processed using a routine 17-hour schedule on an enclosed tissue processing machine (Leica TP 10/50). In order to evaluate the tissue samples in a standardised fashion, the following protocol was devised and applied in all cases. From the first study,[9] it was felt that the softening effect should be achieved in most cases within 30 min.

Sectioning protocol

- Having exposed the full face of the paraffin block, tissue blocks were immersed in softening agent sample A for 30 minutes at room temperature.
- Tissue blocks were removed, rinsed in tap water and excess softener wiped from the surface. The tissue block was then placed on a cold plate for 3–5 min before sectioning.
- Tissue blocks were sectioned on a Leica rotary

Table 1. Overall factors and performance indicators used to evaluate the softening agents.

Sample	Section cutting	Section scores/ knife marks	Section ribboning	H&E and tinctorial staining	Immuno-cytochemistry	Overall performance	Additional comments
A	All except one block	Only on cases with focal calcification	Excellent quality of ribboning (20–30 sections on average)	Not affected	Not affected	Excellent	Very dense keratinised tissue block may require longer treatment
B	As above	As above	As above	As above	As above	Excellent	As above
C	As above	As Above	Section ribboning achieved in most but less than in A and B	As above	As above	Very good	As above
D	As above	As Above	As for C	As above	As above	Very good	As above
Commercial product	Three blocks not sectioned	Some cases of keratin distortion	Much less section ribboning achieved (average 2–3 sections)	As above	As above	Least successful softening agent	As above

Samples colours and scents were not popular. Further evaluation was deemed necessary.

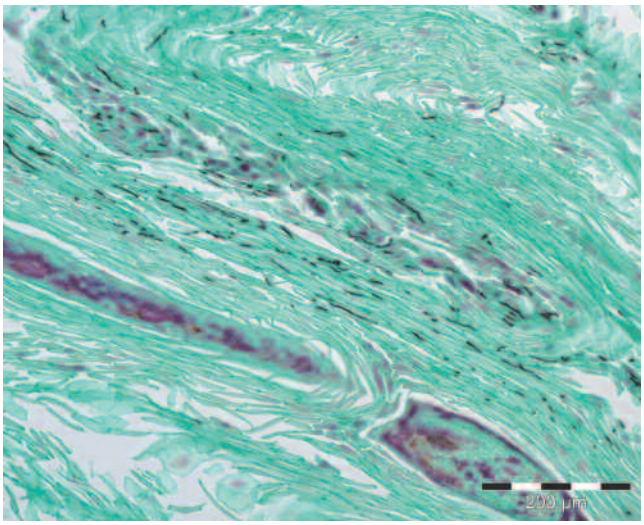

Fig. 3. Fungal hyphae from the sole of the foot, following softening in the commercial softener (Grocott stain, original magnification x20).

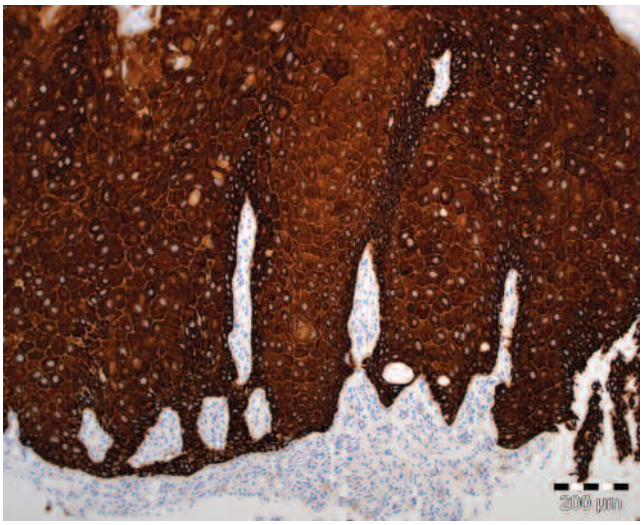

Fig. 4. Cutaneous horn, following softening in trial sample C. Note the uniform immunolabelling throughout the keratin layers (MNF116 immunohistochemical labelling, original magnification x20).

microtome (RM 2125) using a new disposable blade at 4 µm. All staff performing sectioning completed a table for each block assessed (Table 1). The parameters for optimisation were set as follows: section cutting easy to perform or not, sections could be serialised or not, scores or knife marks appeared or not. In addition, the biomedical science staff members were asked to comment on which of the samples they preferred in terms of colour and fragrance. All sections were mounted on Super Frost Plus slides (VWR).

- Once observations were complete (Table 1), the paraffin block was trimmed 150 µm or until the tissue became hard to section. The blocks were then immersed in sample B and the procedure was repeated, using a new portion of the disposable blade. Blocks were then placed in trial formulations C and D, followed by the commercially available softener, and the same assessments were performed.

Haematoxylin and eosin (H&E)-stained sections were then prepared for all the test samples (Figs. 1 and 2). In addition, special stains and immunohistochemistry were performed on the reserve sections to ascertain if staining quality was compromised. Special staining included periodic acid Schiff (PAS) and Grocott (Fig. 3) and immunohistochemistry for the pan-cytokeratin antibody MNF116 (Fig. 4; Dako M 0821). Immunohistochemistry involved the use of the Ventana Benchmark XT automated enclosed immunostaining machine. All slides were then reviewed by the authors to assess staining quality.

Results

Table 1 shows that all test samples enabled sectioning of all but one of the tissue blocks. The tissue that did not soften adequately to enable sectioning within 30 min was a heavily keratinised piece of nail. The commercial tissue softening agent failed to enable sectioning in three of the tissue samples.

Softening agents A–D not only permitted tissue sectioning but also allowed serial sections to be cut from almost all the cases in the study, suggesting that the test agents penetrated the paraffin block to some depth. This was not the case with the commercially available softening reagent, which was able to produce a ribbon of perhaps two or three sections on average. The only difference in performance between the four tested samples (A–D) was that more serial sections (average: 25 sections) could be cut using solutions A and B, while for samples C and D this figure was slightly lower.

Following discussions with CellPath, it was confirmed that samples A and B contained higher concentrations of the identified active ingredient than did samples C and D. All test samples out-performed the commercial softening fluid and no placebos were provided in any of the test samples.

Discussion

The results of the study reported here indicate that the four trial samples performed better to soften tissue and enable adequate microtomy, compared to the existing commercially available histological product, and serial sectioning of many of the test blocks was achieved with the test samples. In some cases, 50–60 sections could be produced, indicating that softening was more than superficial. Only one tissue sample, a piece of dense nail tissue, resisted sectioning following immersion in any of the test samples or in the commercially available softener.

Using the commercially available histological softening agent, three blocks could not be sectioned following a 30 min immersion. It is possible that a longer immersion period might enable sectioning in some cases as it appears to be an issue of penetration of the softener into the block; however, this was beyond the objectives set in the present study.

In terms of performance, there were marginal differences between the more concentrated trial formulations (samples A and B) and the diluted versions (samples C and D); however, success in serial sectioning seemed to correlate with the concentrated solutions.

Juddering or chattering on sectioning was not noted with the test samples. Scoring and knife marks were apparent in sections cut from just a few tissue blocks, but could be attributed to focal or more widespread calcification present within the tissues.

Staining performance appeared not to be compromised with routine tinctorial or immunohistochemical methods. None of the colours or fragrances associated with the test samples were found to be agreeable, and this information was relayed to CellPath for further evaluation.

Hardened keratotic tissues (e.g., nail tissue) are composed of amino acid side chains rich in cysteine bonds. Cysteine is an amino acid that contains a thiol (-SH) group. In a protein structure, two cysteine units can link together via the thiol groups, effectively producing a disulphur bridge. In terms of functionality, this crosslinking will stiffen the protein structure.

The proposed rationale for the softening effect is that the active ingredient in the test samples breaks this bond, linking to the exposed thiol groups. Once the crosslinks have been broken, the tissue structure becomes 'softened'. In addition, the test samples contained a lubricant to assist microtomy, thus reducing the impact of chattering or juddering; an effect commonly seen in unsoftened tissue material on sectioning.

The present study has enabled final adjustments to be made to the production of a new product for use as a softening agent. It will be available commercially from CellPath and will be called CellSoft. The new agent has been produced following a systematic approach to identify the desirable active ingredients and to refine these ingredients to optimise effectiveness. Further evaluations could include more detailed trials to establish its effect on a wider range of tissue types. However, the product proved ineffective on undecalcified tissue, but further modification to incorporate a decalcifying agent should enable this objective to be met.

In conclusion, this study demonstrates a clear advantage to the use of the trial formulations over a leading commercial histological tissue softening agent. In addition, the trial formulations pose only minor health and safety risks compared to phenol-based softening agents. The new product will be CE-marked and will therefore satisfy recommended guidelines for the use of reagents in a laboratory setting. □

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