

Polarization microscopy of picosirius red stained sections: A useful method for qualitative evaluation of intestinal wall collagen

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Summary. Collagen pattern in healing anastomosis of intestinal wall was compared with its normal pattern in the submucosal layer. Polarization colours were recorded for thin (0.8 μm or less) and thick (1.6-2.4 μm) collagen fibres. The polarization colours of thick collagen fibres in the anastomotic site were more greenish-yellow and yellow than those in normal intestine which were more yellowish-orange and orange. These findings indicate that the collagen in the anastomotic site 4 days after operation is less packed than the collagen of normal rat intestine. Examination of the polarization colours of Picosirius red-stained sections is a useful procedure to follow healing of anastomotic sites or diagnosis of collagen pathology in different pathologic conditions in the intestinal wall.

Key words: Anastomosis, Collagen, Picosirius-red, Polarizing microscopy, Intestinal wall

Introduction

The amount and structure of collagen in the submucosal layer of the intestinal wall determines the bowel wall strength and integrity (Hendriks and Mastboom, 1990). Picosirius red stain of deparaffinized tissue sections followed by polarization microscopy selectively demonstrates collagen fibres (Constantine and Mowry, 1968; Junquiera et al., 1979).

The polarization colours differ according to the fibre thickness (Junquiera et al., 1982; Szendroi et al., 1984). Thin fibres usually show green to yellowish-green polarization colours, thick fibres show polarization colours of longer wavelengths mainly yellow through

yellowish-orange, orange to red (Junquiera et al., 1982; Szendroi et al., 1984; Dayan et al., 1989). In addition, packing of collagen molecules in fibres also plays an important role in the pattern of polarization colours of Picosirius red-stained collagen. Tightly packed and presumably, better aligned collagen molecules had polarization colours of longer wavelengths (Dayan et al., 1989). Thus, examination of collagen fibres of known thickness in Picosirius red-stained sections by polarizing microscopy can serve as a procedure for differentiating procollagens, intermediates and other non-tightly packed collagen fibres from normal tightly packed fibres (Hiss et al., 1988; Trau et al., 1991; Dayan et al., 1993).

The aim of this study is to show that this method can be useful to evaluate the collagen pattern and structure in physiologic and pathologic processes of the intestinal wall. Normal intestinal wall collagen structure was histochemically established and compared with healing suture intestinal anastomosis.

Materials and methods

Animal model

A midline laparotomy was performed in 20 male Wistar rats, weighing 400-450 g, following intra-peritoneal pentothal anaesthesia, 15/mg/kg. Ten rats served to determine the normal collagen structure in the ileum of rats. At the time of laparotomy, a segment of intact ileum 15 cm from the ileocecal junction was excised and fixed in 10% buffered formalin. Rats were then killed with an overdose of pentothal.

In 10 rats, an antimesenteric enterotomy was performed, 15 cm from the ileocecal junction, followed by its closure with one layer 5/0 Dexon sutures. After completion of the operative procedure, 100 mg of cefamezine were injected into the peritoneal cavity and the abdomen was closed with 4/0 Dexon sutures. Post-

operatively, the rats were maintained on a dextrose solution for 3 days before resuming their normal diet. On the fourth post-operative day, rats were killed with an overdose of pentothal. The abdomen was opened and the anastomotic site identified. A segment of the ileum, including the anastomosis, was resected and similarly processed.

Histopathologic studies

After fixation of 24 h, the tissue specimens were dehydrated and double embedded in paraffin. Sections, 5 μm thick, were stained with hematoxylin and eosin for routine microscopy. In addition, sections were stained by a modified Picrosirius red procedure as previously described (Dayan et al., 1989). Briefly, after deparaffination and hydration to distilled water, the sections were incubated for 1 h at room temperature in 0.1% (W/V) Sirius red in saturated picric acid solution. Sections were then incubated 30 min in 1% acetic acid, rinsed with distilled water and stained with Mayer's hematoxylin for 5 min. This was followed by differentiation in 1% HCl in 70% alcohol alkalization with running tap water, dehydration and mounting with merckoglass. The sections were examined by polarization microscopy using an Olympus BH-2 microscope equipped with polarized filters. Polarization colours were determined separately for thin ($p < 0.8 \mu\text{m}$) and thick (1.6-2.4 μm) fibres. Fibre thickness was determined with the aid of a previously calibrated ocular

micrometer using an oil immersion x100 objective. At least 50 fibres were examined in each tissue sample in different sections.

Results

General examination of the intestinal wall stained with Picrosirius red showed a bright red staining of the collagen fibres in the submucosal layer (Fig. 1A). This was not observed in the other mesenchymal structures of the intestinal wall. Examination of the same sections with crossed-polarizing filters (Fig. 1B) revealed that the predominant polarization colours in the submucosal layer were yellow and yellowish-orange. Most of the thin fibres in normal submucosal collagen were green and greenish-yellow ($60 \pm 9.4\%$) and the remainder were yellow and yellowish-orange ($40 \pm 4.3\%$) (Table 1). The polarization colours of the thick fibres were about $34 \pm 4.7\%$ green and greenish-yellow, and $66 \pm 9.4\%$ yellow and yellowish-orange.

Table 1. Fibres of known thickness (thin= 0.8 μm , thick= 1.6-2.4 μm) with different polarization colours (in % mean $\pm$ SD).

	NORMAL INTESTINE		ANASTOMOTIC SITE	
	Thin	Thick	Thin	Thick
Green and greenish-yellow	60 $\pm$ 9.4	34 $\pm$ 4.7	70 $\pm$ 4.6	66 $\pm$ 12.4
Yellow and yellowish-orange	40 $\pm$ 4.3	66 $\pm$ 9.4	30 $\pm$ 4.7	34 $\pm$ 4.7

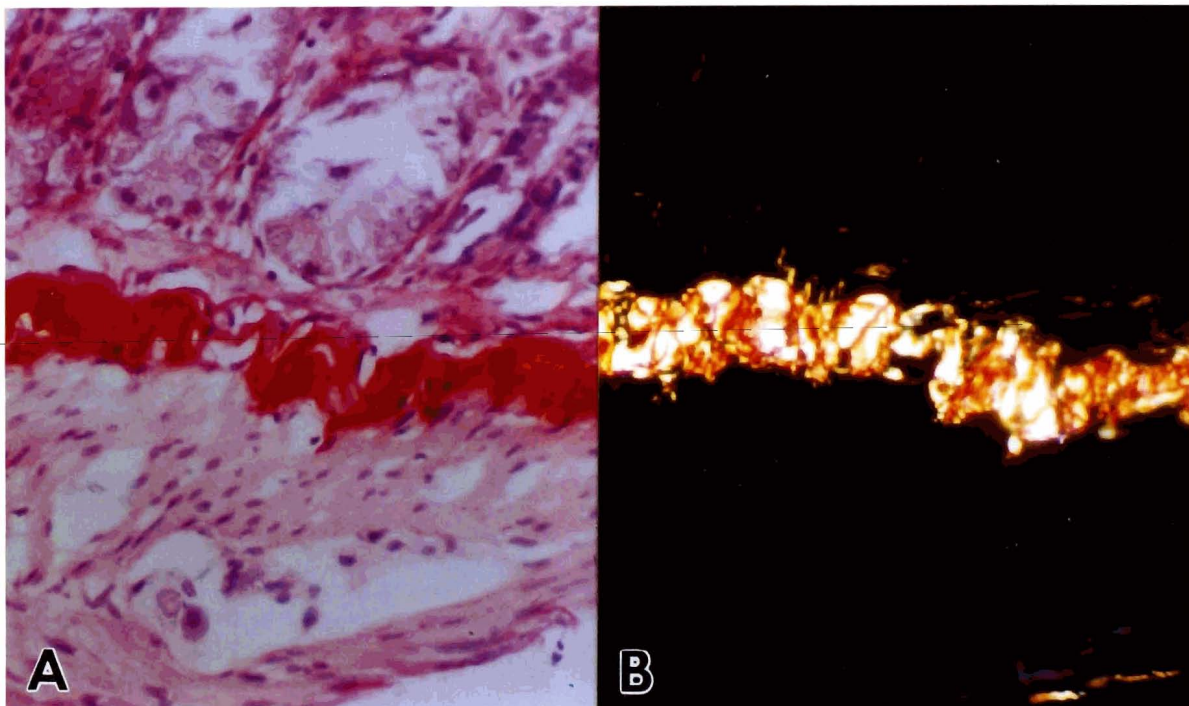


Fig. 1. A. Photomicrograph of a Picrosirius red-stained section of normal intestinal wall. x 200. B. Same photomicrograph as A seen under crossed-polars and showing yellow-orange to orange appearance of submucosal collagen. x 200

Four days after intestinal anastomosis, most of the connective tissue at the anastomotic site was loose and formed by very thin and delicate fibres with a bright red colour when stained with Picrosirius red (Fig. 2A). Examination of this wounded area with cross-polarizing filters showed a green to greenish-yellow colour of the fibres (Fig. 2B) in contrast to the normal submucosal collagen which was yellow to yellowish-orange (Fig. 1B). When the polarization colours were recorded by examination of the fibre with known thickness, $70 \pm 4.6\%$ of the thin fibres were green and greenish-yellow and almost the same percentage ($66 \pm 12.4\%$) was for the thick fibres. Only a small percentage of thin and thick fibres ($30 \pm 4.7\%$ and $34 \pm 4.7\%$, respectively) showed polarization colours of yellow and yellowish-orange range.

Discussion

The healing process of intestinal anastomosis still presents an interesting problematic issue in gastro-intestinal surgery. Studies conducted on experimental intestinal anastomosis healing (Hendriks and Mastboom, 1990) focused on the quantitative description of the anastomotic repair by mainly using a mechanical parameter (i.e., measure of breaking strength) (Jonsson et al., 1983; Blomquist et al., 1984) and biochemical parameters (i.e., collagen content and concentration) (Hendriks et al., 1985; Jonsson et al., 1988). Since no

distinct correlation has been demonstrated between the development of mechanical strength or occurrence of leakage and collagen levels in the healing anastomosis, it was strongly suggested to investigate the quality of anastomotic collagen.

This study shows that staining of anastomotic sites with Picrosirius red and examination of the sections with polarizing microscopy can serve as a useful method to follow the healing process of the intestinal wall. The existence of numerous green and greenish-yellow fibres in the anastomotic sites 4 days after wounding, compared with normal intestinal wall collagen, are consistent with the results of a previous study on experimental wound healing in mice skin which showed the same pattern of polarization colours 1 week after the experimental wound (Hiss et al., 1988). Since it is well-known from previous studies that there is a positive relationship between fibre thickness and polarization colours (Junquiera et al., 1982; Szendroi et al., 1984; Dayan et al., 1989), one can conclude that the nature of the collagen fibres in the anastomotic site 4 days after wounding, is not yet similar to the nature of the collagen fibre of the normal intestinal wall.

Furthermore, examination of fibres of known thickness (thin and thick) can suggest the nature of collagen fibres packing in different physiologic and pathologic processes (Dayan et al., 1989). In a previous study (Trau et al., 1991), collagen of collagenous connective tissue nevi in skin was less well-

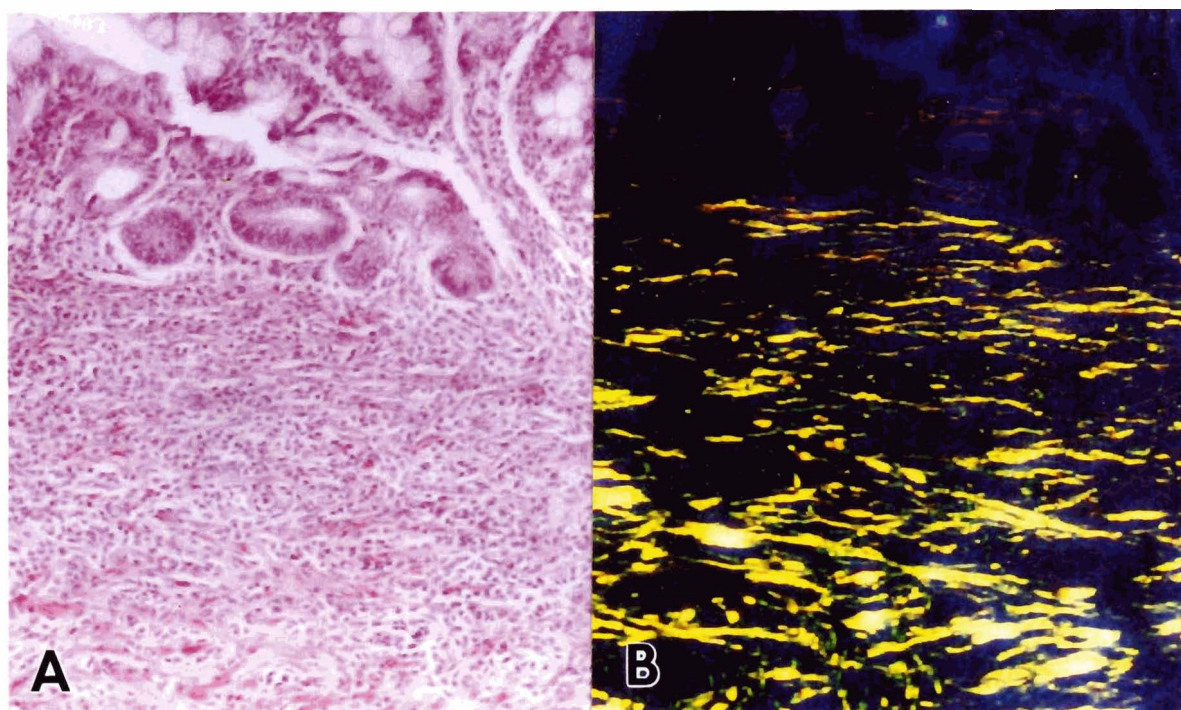


Fig. 2. **A.** Photomicrograph of a Picrosirius red-stained section of anastomotic site in the rat intestinal wall 4 days post-operatively. $\times 200$. **B.** Same photomicrograph as A seen under crossed-polars showing the greenish-yellow and yellow appearance of the collagen fibres in the anastomotic site. $\times 200$

packed than the normal skin collagen since they showed mostly polarization colours of green and greenish-yellow range. Polarization colours of Picrosirius red-stained thin and thick collagen fibres in anastomotic sites of intestinal wall 4 days after wounding, were mostly green and greenish-yellow, suggesting that the collagen fibres were less packed than in the normal intestinal wall. These findings may explain the vulnerability of the anastomosis on the first post-operative week. Moreover, these findings suggest that this method can be used to investigate the quality of collagen fibres in the anastomotic sites and also in different pathologic conditions in the intestinal wall. Further studies are now in progress.

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