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EFFECT OF INDOMETHACIN ON COLLAGEN METABOLISM OF RAT FRACTURE CALLUS *IN VITRO*

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The collagen metabolism of callus tissue from closed, non-immobilized rat femoral fractures was studied *in vitro* after *in vivo* treatment with indomethacin 2 mg/kg/day for 6, 9 and 12 days after fracture. Total hydroxyproline synthesis and incorporation of hydroxyproline into collagen were found to be significantly increased after indomethacin treatment, whereas no significant difference was found regarding collagen resorption. The results suggest that the recently demonstrated inhibition of fracture healing by indomethacin is not brought about by an inhibition of collagen synthesis.

Key words: anti-inflammatory agents; bone; callus; fractures; indomethacin; rats

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Non-steroidal antiphlogistica are used for the treatment of a wide range of diseases. They are also used to reduce the acute inflammation following trauma. Most publications studying the effect of these drugs on fracture healing conclude that such treatments do not affect the healing process (Eschberger 1973, Nordenram & Bang 1970).

However, three recent publications report delayed fracture healing in experimental models after indomethacin treatment (Huusko et al. 1975, Sudmann 1975, Rø et al. 1976). The effect of antiphlogistic drugs on collagen outside bone has been studied by many authors and results are divergent (Aalto & Kulonen 1972, Famaey et al. 1975, Fukuhara & Tsurufuji 1969, Kulonen & Potila 1975, Lee 1968, Lee & Tong 1970, Morton & Malone 1972, Winter 1965). The divergent results may depend on the different doses administered (Aalto & Kulonen 1972, Kulonen & Potila 1975).

* Dr. Rø died in April 1976.

The present study was planned to investigate the effect of indomethacin administered *in vivo* on the *in vitro* metabolism of collagen of fracture callus tissue from rats. In the system used both collagen synthesis and resorption can be studied separately (Flanagen & Nichols 1969, Langeland 1975).

MATERIAL AND METHODS

Forty-two male rats of the Wister/Möllegaard strain were divided into two matched groups. One group received indomethacin 0.4 mg (Indocid suspension 0.4 mg/ml, Merch Sharp & Dohme, Haarlem, The Netherlands) by stomach tube once daily. The resultant dose of indomethacin was about 2 mg/kg/day. The other group serving as a control was given equivalent amounts of the vehicle. The first doses was given immediately after having fractured the animals' left femur (Rø et al. 1976).

The treatment was continued until sacrifice of the animal. The pair matched animals were divided into three series.

I. Twelve rats sacrificed 6 days after fracture and start of treatment. Initial weight 194.9 ± 4.7 g. At sacrifice the indomethacin treated animals weighed 222.8 ± 8.1 g and their controls 214.0 ± 14.4 g. The difference was not significant.
II. Twelve rats sacrificed 9 days after fracture and start of treatment. Initial weight 194.9 ± 4.7 g. At sacrifice the indomethacin treated animals weighed 234.3 ± 7.7 g and their controls 234.3 ± 8.9 g. The difference was not significant.
III. Eighteen rats sacrificed 12 days after fracture and start of treatment. Initial weight 197.4 ± 2.8 g. At sacrifice the indomethacin treated animals weighed 245.7 ± 8.5 g and their paired controls 251.1 ± 9.3. The difference was not significant.

The animals of series I and II, treated for 6 and 9 days, were, at the end of the experiment, anaesthetized with ether. Blood was collected from the vena cava for indomethacin analysis and after that the animal was bled to death.

The animals of series III, treated for 12 days, were, at end of the experiment, killed by a blow to the neck and decapitation.

In all animals the left hind limb with the fractured femur was exarticulated at the hip joint and immediately cooled to 0°–2°C. While still in the cold (0°–2°C) the callus tissue was carefully dissected free.

The 6- and 9-day-old callus was cut into 0.5 mm slices in a microtome while the 12-day-old callus was cut into pieces of 1–2 mm^3 by hand (Flanagan & Nichols 1969). The subsequent procedure, including the incubation, was as previously described (Borle & Nichols 1960, Flanagan & Nichols 1969, Langeland 1975). To the media were added proline 1.5 mM and Proline (U)-^{14}C (SA 290 mCi/mmol, from the Radiochemical Centre, Amersham) 1 μCi/ml.

The callus tissue was incubated in 25 ml ground glass stoppered Erlenmeyer flasks in an atmosphere of 95 per cent O_2/5 per cent CO_2 in a New Brunswick Incubator G 24 (New Brunswick, New Jersey, USA) at 100 oscillations/min and 37.5°C for 6 hours. 4 ml of medium was added to each flask.

The decanted medium was immediately chilled to 0°–2°C, then centrifuged at 5000 *g* for 10 minutes and hydrolyzed in equal amounts of 12 N HCL in sealed ampoules at 125°C for 4 hours.

The callus tissue was, immediately after incubation, chilled to 0°–2°C, washed in two changes of chilled Krebs-Ringer buffer and hydrolyzed in 6 N HCL (2 ml/100 g wet weight) in sealed ampoules at 125°C for 18 hours.

Analysis. The tissue hydrolysates were treated with humin precipitate (Prockop & Udenfriend 1960), centrifuged and the volume was adjusted. The amount of hydroxyproline was determined by the direct acid method of Firschein (Firschein & Shill 1966, Firschein 1969). To determine the specific activity of the hydroxyproline an exact amount of hydrolysate was evaporated *in vacuo*. The residue was dissolved in 250 μl aqua dest. and hydroxyproline was isolated using a Dowex 50 W-x8 200–400 mesh, 0.8 × 20 cm column, as described by Firschein (1969). The hydroxyproline fraction of the eluate was once again evaporated, dissolved and put on a TLC-plate (TLC-plates Woelm 20 × 20 cm, pre-coated silica gel, F 254/366) and eluated with phenol/water 4 : 1. The hydroxyproline fraction was identified with 0.2 per cent (w/v) ninhydrin in acetone using warm air to produce a pink colour. The portion of silica gel containing the hydroxyproline (3 mm stripe) was eluated in 2 ml H_2O and centrifuged for 10 minutes at 5000 *g*. Of the eluate 150 nl was used for hydroxyproline analysis according to the direct acid method of Firschein (Firschein & Shill 1966) while 1500 nl was added to 20 ml Unisolve R (Koch-Light) and counted in a Nuclear-Chicago Mark II Liquid Scintillation counter. Specific activity was calculated based on these results. Media from 1–3 hours and 4–6 hours were pooled, treated and analysed as previously described (Juva & Prockop 1966, Langeland 1975).

Calculations were done as described by Flanagan & Nichols (1969) and the statistical significance of differences was tested by the Wilcoxon test (Wilcoxon 1947) or the Wilcoxon-Van Eltern test for grouped data (Høyland & Walløe 1975).

RESULTS

The wet weight of the callus tumour is given in Figure 1. At 6 days there was no difference between indomethacin treated and untreated animals in this respect. However, comparing the grouped data of 6, 9 and 12 days the indomethacin treated animals had a higher weight of callus tumour than their controls. The same tendency can be seen when studying the total collagen content of callus tumour (Table 1) although these differences are insignificant. Both the total hydroxyproline synthesis (Figure 2) and the deposition of hydroxyproline into collagen of callus tissue (Figure 3) are significantly higher in the indomethacin treated animals. Studying the deposition of hydroxyproline into collagen this parameter is higher in the treated

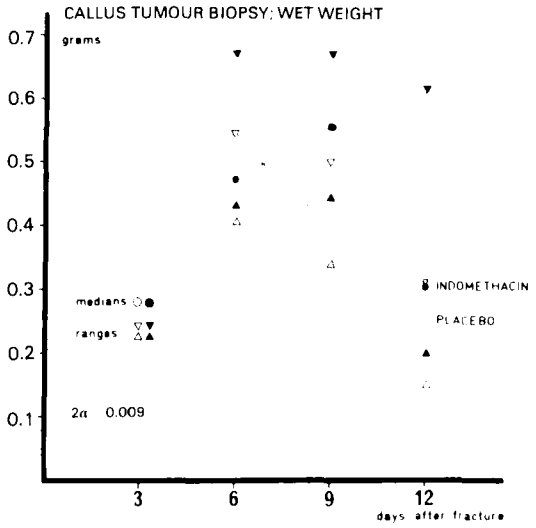

Figure 1. Wet weight of callus tumour. Medians and ranges are given. The difference between the grouped data is significant ($2\alpha = 0.009$).

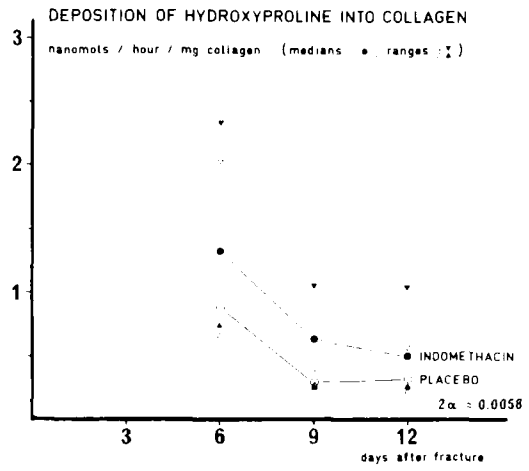

Figure 3. In vitro *deposition of hydroxyproline into collagen of callus tumour. The difference between the grouped data is significant ($2\alpha = 0.0058$).*

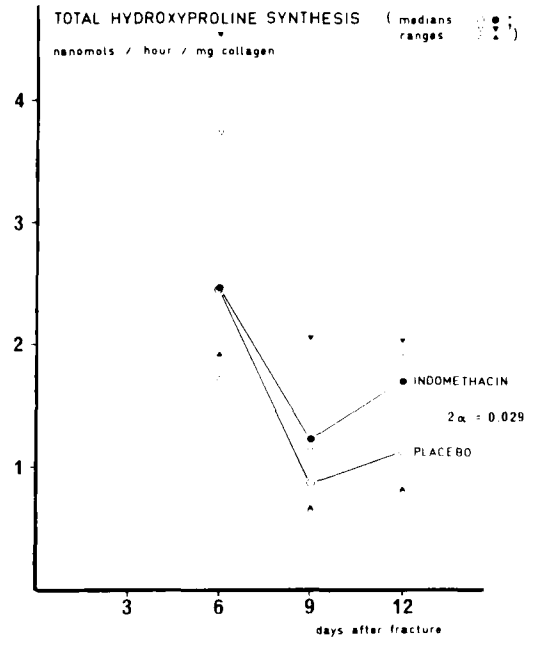

Figure 2. The total in vitro *synthesis of hydroxyproline in callus tumour. The difference between grouped data is significant ($2\alpha = 0.029$).*

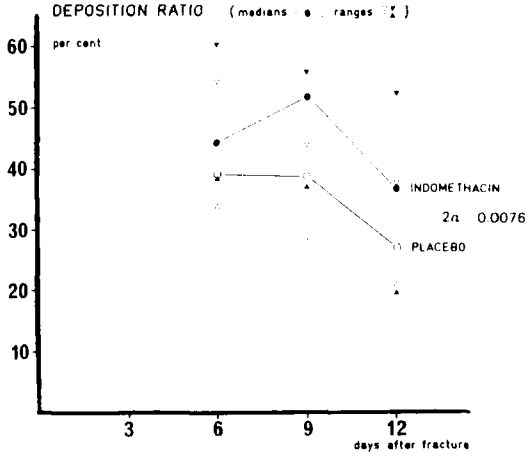

Figure 4. The ratio between synthesized collagen and collagen deposited in the callus tumour. The difference is significant ($2\alpha = 0.0076$).

animals throughout the experimental period, and so is the deposition ratio, i.e., the ratio: synthesis of collagen/deposition of collagen (Figure 4) (Flanagan & Nichols 1969).

The *in vitro* resorption of collagen is dealt with in Figure 5. For this parameter there were no significant differences between indomethacin and placebo treated animals.

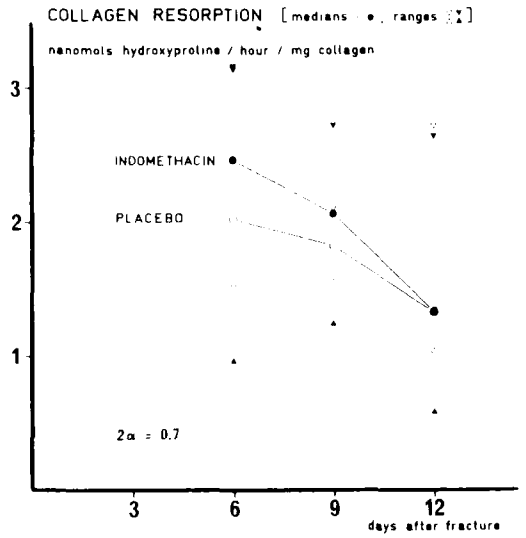

Figure 5. The in vitro *resorption of collagen (hydroxyproline) from incubated callus tissue. There was no significant difference between indomethacin and placebo treated animals ($2\alpha = 0.7$).*

DISCUSSION

In a previous study from our laboratory (Rø et al. 1976) an impaired mechanical strength of the same type of fractures in the same type of animals given the same amount of indomethacin was reported. Tensile strength, elastic stiffness and maximal bending moment were all impaired in the indomethacin treated animals. While fracture union was observed within 18 to 24 days in the control animals, the fractures of the indomethacin treated animals were still unstable at the end of the experiment 24 days after fracture. However, large cartilaginous collars around both fracture ends were found in these animals and a slit-like cavity was found between the ends. This slit was lined with fibrous tissue and fibrin on days 9 and 12. New bone formation was delayed as compared to the control animals.

The present study deals with the *in vitro* metabolism of the organic component of bone and callus, and it demonstrates clearly that the synthesis and deposition of collagen in callus is higher in the indomethacin treated animals than in their controls. This is consistent with the findings of Alto & Kulonen (1972) and Kulonen & Potila (1975) as far as low doses of indomethacin are concerned, and is to be expected if the primary effect of indomethacin is an inhibition of prostaglandin synthesis (Vane 1971, Raisz & Koolemans-Beynen 1974). However, others have reported inhibited collagen synthesis after indomethacin treatment (Winter 1965, Fukuhara & Tsurufuji 1969) and this might be more easily explained in relation to the impaired mechanical properties of the fractures.

Huusko et al. (1975) reported (from a study of tooth extraction wounds) that indomethacin had no effect on the epithelium and the subadjacent connective tissue while bone repair was retarded when treatment was given. This may be consistent with our findings.

As regards the *in vitro* results of the present study it may be argued that if there is a direct effect of indomethacin on bone cells, the drug may be washed away during preparation of the tissue for incubation. Thus the *in vitro* metabolism might take place without the influence of indomethacin. However, from Figure 1 and Table 1 it will be realized that callus tumour weight and

Table 1. Collagen content in callus tumour in mg. Medians and ranges are given. Differences are insignificant ($2\alpha = 0.058$)

Days after fracture	*n*	Placebo	*n*	Indomethacin
6	6	10.0 (6.9–13.3)	6	11.0 (6.9–21.0)
9	6	17.1 (13.8–18.9)	6	19.0 (13.9–20.2)
12	9	13.6 (8.2–16.0)	9	14.8 (8.8–20.5)

collagen content in callus tumour, both reflecting the *in vivo* metabolism, were higher in the indomethacin treated rats than in the controls. Thus both the *in vitro* and *in vivo* experiments indicate that indomethacin treatment produces a higher rate of collagen synthesis in callus.

The present study suggests that the recently demonstrated impaired mechanical strength of rat femur fractures is not caused by an inhibition of collagen synthesis. It seems that indomethacin treatment produces a collagen of lower quality and some observations might indicate that indomethacin may interfere with the transformation of immature connective tissue to calcified callus and bone. This should be investigated further, as should the possibility that the greater instability of the fractures of indomethacin treated animals may be responsible for the larger callus tumour and at least some of the increased collagen synthesis.

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