Effect of tetracyclines on collagen biosynthesis in the dental pulp

VELI-JUKKA UITTO

Department of Medical Chemistry, University of Helsinki, Finland

Uitto, V.-J. Effect of tetracyclines on collagen biosynthesis in the dental pulp. *Acta Odont. Scand.* 33, 279—285, 1975.

The mechanism by which tetracyclines affect the formation of dentin was studied by measuring the biosynthesis of collagen in the pulp. The material consisted of 160 rabbit pulps and 108 rat pulps. Collagen synthesis was determined by incubating pulps with [^{14}C]proline and measuring the formation of non-dialyzable [^{14}C]hydroxyproline. The activity of protocollagen proline hydroxylase was measured as the conversion of [^{14}C]proline to [^{14}C]hydroxyproline in a protocollagen substrate by the supernatant of a pulp homogenate. In *in vitro* experiments, oxytetracycline or demethylchlortetracycline inhibited collagen synthesis. Also, the activity of protocollagen proline hydroxylase extracted from rabbit pulps was decreased in the presence of tetracyclines. In both cases the inhibition was related to the concentration of tetracyclines and the inhibition could be preveted by addition of ferrous iron to the incubation. In *in vivo* studies, injections of demethylchlortetracycline to rats inhibited collagen synthesis measured *in vitro* and the activity of protocollagen proline hydroxylase in the incisor pulps. It was concluded that this effect may be specific to collagen synthesis and the effect may be through chelation of ferrous iron, a cofactor of protocollagen proline hydroxylase. Consequently, the mineralization disturbances in developing teeth during tetracycline therapy may be partly due to a decreased formation of the organic matrix of dentin.

Key-words: Collagen biosynthesis; dental pulp; tetracyclines

Veli-Jukka Uitto, The Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10, SF-00170 Helsinki 17, Finland

The first report of the affinity of tetracycline in skeletal tissues was described by *André* (1956). After injection of tritium labelled tetracycline to mice he observed by radioautographic methods the localization of the drug in bone, dentin, cementum and the dental pulp. Since then several authors have reported that tetracyclines have inhibitory effects on the growth and mineralization of the calcified tissues, (*Bevelander*, 1964; *Saxén*, 1966 a, b; *Kaitila et al.*, 1970). In accordance, tetracycline administration during the tooth development is often associated with reduced mineralization of enamel and dentin (*Bevelander et al.*, 1961) as well as with discoloration and hypoplasia of the teeth observed clinically (*Wallman & Hilton*, 1962; *Hakala & Mäkelä*, 1963; *Witkop & Wolf*, 1963; *Hamp*, 1967).

The main causal mechanism of these deleterious effects has been shown to be the formation of stable tetracyclinecalcium complexes and the subsequent inhibition

Received for publication, October 4, 1974.

of two critical phases of mineralization, i.e., nucleation and crystal growth (*Caswell & Hutchison*, 1971; *Kaitila*, 1971). An additional mechanism in the inhibition of skeletal growth by tetracyclines is thought to be a suppression of the formation of the organic matrix which is mainly composed of collagen (*Bennet et al.*, 1967; *Halme et al.*, 1969). Since collagen of dentin is produced by odontoblasts of the dental pulp, it is possible that the deleterious effects of tetracyclines on developing teeth are partly explained by the inhibition of collagen synthesis in the pulpal cells. Differences in the potency of the inhibitory action have been reported between the different members of the tetracycline group antibiotics. Oxytetracycline has been shown to have less harmful effects while demethylchlortetracycline seems to be one of the most potent inhibitors of mineralization (*Ibsen et al.*, 1965; *Saxén*, 1966 a; *Bridges et al.*, 1969; *Yen & Shaw*, 1972). The experiments here were designed to elucidate the effects of tetracyclines on collagen synthesis and protocollagen proline hydroxylase activity in the dental pulp *in vivo* and *in vitro*.

MATERIAL AND METHODS

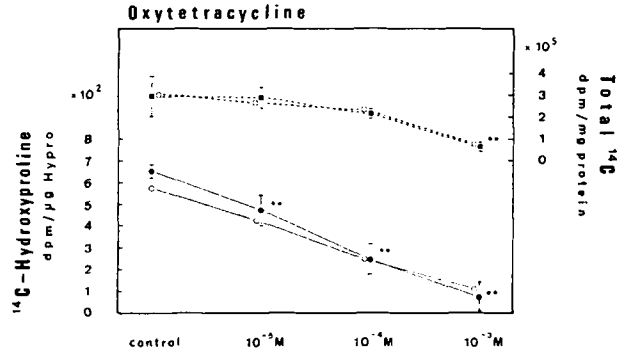
Assay conditions for collagen synthesis and protocollagen proline hydroxylase activity

The methods used in the present experiment have earlier been described in detail (*Uitto & Antila*, 1971).

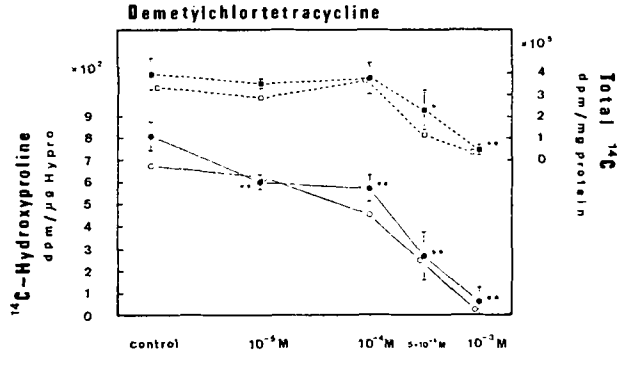
Pulpal collagen synthesis was determined by incubating whole pulps from rabbit or rat teeth for 6 hours in a medium containing [^{14}C]proline, and thereafter measuring the activity of [^{14}C]hydroxyproline by the method of *Juva* and *Prockop* (1966). Total ^{14}C-radioactivity in the nondialyzable fraction from the same samples served as an index for the total protein synthesis (*Prockop & Ebert*, 1963). The activity of protocollagen proline hydroxylase was studied by the method of *Kivirikko* and *Prockop* (1967). The pulps were homogenized and aliquots of the 15 000 x g supernatant containing 0.3 to 0.6 mg protein were incubated with a biologically prepared [^{14}C]proline-labelled protocollagen substrate. The enzyme activity is expressed as radioactivity (disintegrations/minute) of [^{14}C]hydroxyproline synthesized.

Studies in vitro. 160 pulps obtained from 11 male albino rabbits were included in these studies. After killing the animals the samples containing two molar pulps or one incisor pulp were placed as rapidly as possible in incubation with [^{14}C]proline to which oxytetracycline-HCl (Orimycin®, Orion, Helsinki) or demethylchlortetracycline-HCl (Ledermycin®, Lederle, Wayne) was added in 0.05 ml of the medium at molar concentrations of 10^{-5}, 5×10^{-4}, 10^{-4} or 10^{-3}. The hydroxylation of [^{14}C]proline by pulpal protocollagen proline hydroxylase was studied at different concentrations of ferrous iron in the presence of oxytetracycline or demethylchlortetracycline.

Studies in vivo. 27 Sprague-Dawley rats weighing from 195 to 230 g and maintained on a standard laboratory diet were used. In both experiments the rats were divided into three groups. A subcutaneous injection of 60 mg/kg body weight of oxytetracycline or demethylchlortetracycline dissolved in saline was given on 5 successive days. The rats in the control group were injected with saline only. Each group included 5 rats in the experiment for collagen synthesis and 4 rats in the experiment for protocollagen proline hydroxylase. After the experimental period the rats were killed by decapitation, the



1 a.



1 b.

Fig. 1. Effect of different concentrations of oxytetracycline and demetylchlortetracycline on the incorporation of [^{14}C]proline and the synthesis of collagen in rabbit dental pulp in vitro. Each sample contained 2 pulps which were incubated for 6 hours at 37 C in 1 ml of phosphate-free Krebs-Ringer solution containing 20 mM HEPES-buffer, pH 7.4, 20 mM glucose, 0.05 µg/ml ampicillin and 1 µCi [^{14}C]proline.
●——● : [^{14}C]Hydroxyproline
■- - -■: Total ^{14}C
Solid symbols: molar pulps, mean ± S.D. of 4 parallel determinations. Open symbols: incisor pulps, mean of 2 parallel determinations.

Fig. 1 a: oxytetracycline.
Fig. 1 b: demetylchlortetracycline.
*The difference from control value significant at level $p < 0.05$
**$p < 0.01$

incisors were removed from the surrounding bone and the pulps were immediately used for the experiments.

RESULTS

Effect of tetracyclines on the incorporation of [^{14}C]proline and synthesis of collagen in rabbit pulp in vitro

Oxytetracycline and demethylchlortetracycline significantly inhibited collagen synthesis at a concentration of 10^{-5} M while protein synthesis in general, indicated by total ^{14}C incorporation, was not inhibited at concentrations of less than 10^{-4} M (Fig. 1). No clear differences were found in the responses between molar and incisor pulps (Fig. 1). The addition of ferrous iron to the incubation medium at a concentration of 0.1 mM prevented the inhibitory action of tetracyclines on collagen synthesis (Table I).

Effect of tetracyclines on the hydroxylation of protocollagen by protocollagen proline hydroxylase from rabbit pulp

Oxytetracycline and demethylchlortetracycline inhibited the iron-dependent hydroxylation of protocollagen. The inhibition was concentration-dependent and of the same order of magnitude with both tetracyclines (Table II).

Effect of administration of tetracyclines on collagen synthesis and protocollagen proline hydroxylase activity in rat incisor pulps

Injection of 60 mg/kg/day of demethylchlortetracycline for 5 days to rats in-

Table I. *Effect of oxytetracycline and demetylchlortetracycline on the incorporation of* [^{14}C]*proline and the synthesis of* [^{14}C]*hydroxyproline in rabbit molar pulp in vitro*

Sample	Fe^{++} conc. mM	[^{14}C]Hypro dpm/μg Hypro	% of control	^{14}C total activity dpm/mg protein	% of control
Control	—	1 232	100	310 500	100
Oxy TC 10^{-4}M	—	660	54	219 000	71
Demetylchlor TC 10^{-4}M	—	552	45	207 600	67
Control	0.1	775	100	293 800	100
Oxy TC 10^{-4}M	0.1	688	89	252 900	86
Demetylchlor TC 10^{-4}M	0.1	640	83	283 000	96

Each sample contained 2 molar pulps which were incubated for 6 hours at 37 C with 1 μCi [^{14}C]-proline. Values are averages from 3 parallel determinations.

Table II. *Effect of oxytetracycline and demetylchlortetracycline on the hydroxylation of* [^{14}C]*proline labelled protocollagen by protocollagen proline hydroxylase from rabbit dental pulp at different concentrations of ferrous iron*

Sample	Fe^{++} conc. mM	[^{14}C]Hypro formed dpm/mg protein	% of control
Experiment I			
Control	0.1	6,900	100
Oxy TC 10^{-4}M	0.1	4,700	68
Demetylchlor TC 10^{-4}M	0.1	4,100	60
Control	0.01	4 790	100
Oxy TC 10^{-4}M	0.01	230	5
Demetylchlor TC 10^{-4}M	0.01	110	2
Experiment II*			
Control	0.001	6 310	100
Oxy TC 10^{-4}M	0.001	2 690	42
Demetylchlor TC 10^{-4}M	0.001	2 920	46

Aliquots of the 15 000 x g supernatant from molar pulps were incubated for 60 min at 37 C with [^{14}C]- proline-labelled protocollagen. The values are averages from 2 parallel determinations.
*Because of variations in the substrate activity of the various protocollagen preparations, the values for [^{14}C]hydroxyproline synthesis are comparable only within the same experiment.

hibited significantly the in vitro synthesis of collagen in pulps (Table III). The total incorporation of [^{14}C]proline was not inhibited in the same samples (Table III). Protocollagen proline hydroxylase activity of pulps was decreased significantly in rats treated with demethylchlortetracycline (Table IV). The administration of oxytetracycline did not affect collagen synthesis or protocollagen proline hydroxylase activity in rat pulps (Tables III and IV).

Table III. *Effect of administration of oxytetracycline or demetylchlortetracycline on the capacity of rat incisor pulps to synthesize collagen in vitro*

Group	[^{14}C]Hypro dpm/μg Hypro $\bar{x}$	S.D.[1]	^{14}C total activity dpm/mg protein $\bar{x}$	S.D.
Control	1 245	351	512 000	45 200
Oxy TC	1 277	494	607 000	121 800
Demetyl-chlor TC	846	124[2]	591 000	100 000

Rats were injected with tetracyclines 60 mg/kg for 5 days. Each sample contained 4 incisor pulps. Incubation conditions same as in Table 1.
[1]) Values from 5 parallel determinations.
[2]) The difference from control value significant at level $p < 0.05$.

Table IV. *Effect of administration of oxytetracycline or demetylchlortetracycline on protocollagen proline hydroxylase activity in rat incisor pulps*

Group	Enzyme activity[1] $\bar{x}$[2]	S.D.
Control	10 350	1 409
Oxy TC	8 640	720
Demetylchlor TC	5 200	1 950[3]

Rats were injected with tetracyclines 60 mg/kg for 5 days.
[1]) Enzyme activity is expressed as radioactivity (disintegrations/min) of [^{14}C]hydroxyproline synthesized in an aliquot of 50 000 disintegrations/min [^{14}C]proline-labelled protocollagen substrate per mg protein of the 15 000 x g supernatant of the pulp homogenate.
[2]) Values from 4 parallel determinations.
[3]) The difference from control value significant at level $p < 0.05$.

DISCUSSION

One of the unique features in the biosynthesis of collagen is the hydroxylation of proline residues in a polypeptide precursor of collagen, called protocollagen (*Grant & Prockop*, 1972). The intracellular synthesis of hydroxyproline is required for the formation and stabilization of the typical triple-helical conformation of collagen in which the molecule is secreted as a transport form from the cell (*Uitto & Prockop*, 1974). The hydroxylation of proline is catalyzed by the enzyme protocollagen proline hydroxylase in the presence of ferrous iron, α-ketoglutarate, molecular oxygen and ascorbate (*Hutton et al.*, 1967). Since iron plays an important role in the synthesis of collagen (*Prockop*, 1971) iron chelating agents exert a specific effect on collagen synthesis which is more apt to be inhibited than the synthesis of other proteins by these agents (*Chvapil et al.*, 1967).

In the present experiments tetracyclines exerted an inhibitory effect on the hydroxylation of [^{14}C]proline by pulpal protocollagen proline hydroxylase. The inhibition was related to the concentration of ferrous iron in the incubation solution. It thus seems that tetracyclines may suppress collagen biosynthesis through chelation of ferrous iron, an important cofactor of protocollagen proline hydroxylase. This hypothesis is supported by the in vivo experiments. Demethylchlortetracycline injected to rats inhibited formation of collagen hydroxyproline and diminished the activity of protocollagen proline hydroxylase in incisor pulps. The inhibitory concentration for collagen synthesis in vitro was found to be 10^{-5} M, which corresponds to the serum concentration in adult humans given therapeutic doses of tetracycline. In the pulp of the developing teeth of premature children the concentration during tetracycline therapy is likely to be large enough to depress the formation of dentinal organic matrix. The inhibition of collagen synthesis in the pulp at pharmacological concentrations seems to be specific since total protein synthesis was

not inhibited by tetracyclines until at concentrations over 10^{-4} M. At these concentrations the inhibition of protein synthesis is shown to parallel the inhibition of DNA synthesis (*Bennet et al.*, 1967). No clear differences were found in the extent to which oxytetracycline and demethylchlortetracycline affected collagen synthesis in the pulp in vitro. In vivo, however, only demethylchlortetracycline inhibited collagen synthesis and protocollagen proline hydroxylase activity. Thus, the stronger deleterious side effects exerted by demethylchlortetracycline may, at least partly, be explained by the pharmacokinetic properties of different tetracyclines.

Some compounds of tetracyclines are designed to the topical treatment of the dental pulp. According to the present results tetracyclines exert a direct suppressive effect on the collagen synthesis in the pulp. Since the mineralization in calcifying tissues that follows the formation of the organic matrix is even more apt to be inhibited by tetracyclines (*Kaitila*, 1971) the formation of reparative dentine by the injured pulp may be suppressed in the tooth under tetracycline medication.

The results presented here are in accordance with those of *Halme et al.* (1969) obtained from studies with cultured bones and they point to the conclusion that mineralization disturbances exerted by tetracyclines in developing bone and teeth may be partly due to an interaction of these antibiotics with ferrous iron and to the subsequent inhibition of the conversion of proline into collagen hydroxyproline of the organic matrix.

Acknowledgements. This study has been supported by grants from the Finnish Dental Society and the National Research Council for Medical Sciences, Finland.

REFERENCES

André, T., 1956. Studies on the distribution of tritium-labelled dihydrostreptomycin and tetracycline in the body. Acta Radiol., Suppl. 142

Bennet, I. C., Proffit, W. R. & Norton, L. A. 1967. Determination of growth inhibitory concentrations of tetracycline for bone in organ culture. Nature 216, 176—177

Bevelander, G., Rolle, G. K. & Cohlan, S. Q. 1961. The effect of the administration of tetracycline on the development of teeth. J. Dent. Res. 40, 1020—1024

Bevelander, G. 1964. The effect of tetracycline on mineralization and growth. Adv. Oral. Biol. 1, 205—223

Bridges, J. B., Owens, P. D. S. & Stewart, D. J. 1969. Tetracyclines and teeth. An experimental investigation into five types in the rat. Brit. Dent. J. 126, 306—311

Caswell, A. H. & Hutchinson, J. D. 1971. Selectivity of cation chelation to tetracyclines: Evidence for special conformation of calcium chelate. Biochem. Biophys. Res. Commun. 43, 625—630

Chvapil, M., Hurych, J., Ehrlichová, E. & Tichy, M. 1967. Mechanism of the action of chelating agents on proline hydroxylation and its incorporation into collagenous and non-collagenous proteins. European J. Biochem. 2, 229—235

Grant, M. E. & Prockop, D. J. 1972. The biosynthesis of collagen. New Engl. J. Med., 286, 194—199, 242—247, 291—300

Hakala, P. E. & Mäkelä, P., 1963. Tetracycline and prematures. Suom. Hammaslääk. Toim. 59, 284—293

Hamp, S-E. 1967. The tetracyclines and their effect on teeth. Odont. T. 75, 33—48

Halme, J., Kivirikko, K. I., Kaitila, I. & Saxén, L. 1969. Effect of tetracycline on collagen biosynthesis in cultured embryonic bones. Biochem. Pharmacol. 18, 827—836

Hutton, J., Tappel, A. L. & Udenfriend, S. 1967. Cofactor and substrate requirements of collagen proline hydroxylase. Arch. Biochem. Biophys. 118, 231—240

Ibsen, K. R., Urist, M. R. & Sognnaes, R. 1965. Differences among tetracyclines with respect to the staining of teeth. J. Pediat. 67, 459—462

Juva, K. & Prockop, D. J. 1966. Modified procedure for the assay of H^3 or C^{14} labelled hydroxyproline. Anal. Biochem. 15, 77—83

Kaitila, I., Wartiovaara, J., Laitinen, O. & Saxén, L. 1970. The inhibitory effect of tetracycline on osteogenesis in organ culture. J. Embryol. Exp. Morph. 23, 185—211

Kaitila, I. 1971. The mechanism by which tetracycline hydrocloride inhibits mineralization in vitro. Biochim. Biophys. Acta 244, 584—594

Kivirikko, K. I. & Prockop, D. J. 1967. Enzymatic hydroxylation of proline and lysine in protocollagen. Proc. Nat. Acad. Sci. U.S. 57, 782—789

Prockop, D. J. & Ebert, P. S. 1963. A simple method for differential assay of tritium and carbon-14 in water-soluble biological materials. Anal. Biochem. 6, 263—271

Prockop, D. J. 1971. Role of iron in the synthesis of collagen in connective tissue. Fed. Proceedings 30, 984—990

Saxén, L. 1966a. Effect of tetracycline on osteogenesis in vitro J. Exp. Zool. 162, 269—294

Saxén, L. 1966 b. Drug-induced teratogenesis in vitro: Inhibition of calcification by different tetracyclines. Science 153, 1384—1386

Uitto, J. & Prockop, D. J. 1974. Intracellular hydroxylation of non-helical protocollagen to form triple-helical procollagen and subsequent secretion of the molecule. Eur. J. Biochem. 43, 221—230

Uitto, V-J. & Antila, R. 1971. Characterization of collagen biosynthesis in rabbit dental pulp in vitro. Acta Odont. Scand. 29, 609—617

Wallman, I. S. & Hilton, H. B. 1962. Teeth pigmented by tetracycline. Lancet 1, 827—829

Witkop, C. J. & Wolf, R. O. 1963. Hypoplasia and intrinsic staining of enamel following tetracycline therapy. J.A.M.A. 185, 1008—1011

Yen, P. K.-J. & Shaw, J. H. 1972. Preliminary study of inhibitory effects of tetracyclines on membranous bone growth in rhesus monkeys. J. Dent. Res. 51, 1651—1657