Biochemical Pharmacology, Vol. 17, pp. 1479–1484. Pergamon Press. 1968. Printed in Great Britain

EFFECT OF TETRACYCLINE ON SYNTHESIS OF COLLAGEN AND INCORPORATION OF ^{45}CALCIUM INTO BONE IN FOETAL AND PREGNANT RATS

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(*Received* 30 *January* 1968; *accepted* 13 *March* 1968)

Abstract—The effect of tetracycline on calcification and on the synthesis of collagen in both foetal and maternal bone was studied. ^{45}Ca and ^{14}C-proline were injected into pregnant rats and, after administration of tetracycline, the specific and total activities of ^{45}Ca and ^{14}C-hydroxyproline were determined. In the foetal calvaria, tetracycline reduced the amount of calcium and hydroxyproline, and the total activities of ^{45}Ca and ^{14}C-hydroxyproline were also significantly lower in the tetracycline-treated group than in the controls. Between the corresponding values for maternal femora there was no significant difference.

The effect of tetracycline on the synthesis of soluble collagen in foetal skin was studied by determining the specific and total activities of ^{14}C-hydroxyproline after injection of ^{14}C-proline. Both the amount of hydroxyproline and the sp. act. of ^{14}C-hydroxyproline in the soluble collagen of foetal skin were lower in the tetracycline-treated animals than in the controls, the total activity of ^{14}C-hydroxyproline in the treated animals being only about one-third of that in the controls. The total incorporation of ^{14}C-proline into the soluble protein fraction in the same foetal skin was not affected by tetracycline.

The results of the present study suggest that tetracycline, when injected into pregnant rats in therapeutic doses, inhibits the calcification of foetal bones and the biosynthesis of collagen in foetal bone and skin but does not significantly affect the maternal tissues. In addition, the inhibition of collagen biosynthesis by tetracycline seems to be selective.

TETRACYCLINES have an unusual affinity for the skeletal system, especially for the areas of bone growth.[1, 2] After administration of tetracycline to animals it can be detected as yellow fluorescence in the growing portion of bone.[1, 2] An interaction occurs between tetracycline and calcium,[1, 3] but it is not clear whether it is an absorption of the antibiotic on the precipitate of calciumorthophosphate or a more complex reaction at the nucleation sites, seeded with hydroxyapatite, on the collagen fibres.[4]

Other authors have shown that tetracycline, when administered to the premature infant, produces a marked depression of normal skeletal growth as measured by inhibition of fibula growth.[5] Tetracycline seems also to cause reduction of foetal size at term when administered on the 10–15th days of gestation in the rat,[5] although some workers have failed to demonstrate such inhibition of growth or incorporation of ^{45}Ca into embryos *in vivo*.[6, 7] At all events, a considerable body of evidence has accumulated showing that tetracyclines do inhibit ^{45}Ca incorporation into foetal bone rudiments *in vitro*[8–10] at concentrations comparable with those produced *in vivo* by therapeutic doses.[11, 12] Moreover new evidence indicates that in a medium containing

tetracycline at least at higher concentrations, incorporation of labelled thymidine or proline into cultured bones is also inhibited.[13]

Quite recently evidence has been presented that a close correlation exists between collagen and calcium metabolism in bones and that changes in calcium and collagen metabolism are usually parallel.[14] Collagen is the major protein of connective tissue, accounting for about 95 per cent of the organic matrix of bone, and its composition is unique, because up to 14 per cent of its amino acid content is hydroxyproline, an amino acid found exclusively in collagen (for review see ref. 15). The object of the present experiments was to discover whether tetracycline given to pregnant rats in therapeutic doses would have any effect on the synthesis of collagen or on bone calcification in the foetuses. This question was studied by injecting ^{14}C-proline into pregnant rats and analysing the specific and total activities of ^{14}C-hydroxyproline in foetal and maternal bones and skin. Similarly the incorporation of ^{45}Ca into foetal and maternal bones was studied after injection of ^{45}Ca into the rats.

MATERIAL AND METHODS

The experimental animals were female albino Wistar rats weighing 210–230 g. They were fed on a hydroxyproline- and calcium-deficient diet *ad libitum* and allowed free access to water. One hundred female rats were divided in groups of four rats and male rats were mated with these for 20 hr. Eight of these female rats became pregnant and they were divided in two groups, four rats in each. Tetracycline hydrochloride (Orion Oy, Finland) was injected s.c. in a dose of 40 mg/kg body wt. during 16–20th days of pregnancy to rats of one group. ^{14}C-proline (uniformly labelled 12·6 μC/μM, The Radiochemical Centre, Amersham) was injected s.c. in 1·0 ml of 0·9% sodium chloride solution, 15 μC to each animal. ^{45}Ca (2–5 C/g Ca, The Radiochemical Centre, Amersham) 25 μC per rat was injected simultaneously.

The animals were killed by decapitation 8 hr after isotope injection and the embryos dissected free from the surrounding membranes and placed on ice-cooled trays. The mean number of embryos per litter was 9 in both groups. The skins of the embryos of one litter were pooled, weighed and homogenized in cold 0·45 M sodium chloride (4 ml/g of skin) with an Ultra-Turrax Homogenizer and fractionated for soluble collagen as follows:[16] The homogenates were extracted at + 4° for 24 hr with occasional stirring, after which they were centrifuged at 60,000 *g* for 60 min. The supernatants obtained were precipitated with 4 vol. of cold ethanol and after centrifugation extracted twice with 80% ethanol, twice with absolute ethanol and twice with warm ethanol-ether (1:2).

The residues were gelatinized with distilled water at 124° for 3 hr and after filtration a sample of the gelatin solution was hydrolysed with an equal volume of 12 N HCl for 6 hr at 138° and used for determination of the quantity[17] and sp. act. of the soluble collagen hydroxyproline.[18] Two specimens of maternal skin were taken from each animal and fractionated and analysed in the same way as foetal skin.

To analyse the foetal calvaria for collagen and calcium radioactivity, the calvaria were dissected, weighed and hydrolysed with concentrated HCl. Samples of the hydrolysate were used for analyses of the radioactivity due to ^{45}Ca and ^{14}C-hydroxyproline.[18] The maternal femora were dissected out, fractured transversely, washed free of marrow with ice-cold distilled water, placed in acetone for 1 week with one change of acetone, hydrolysed and analysed as above. Calcium was titrated with EDTA,

using calcein[19] as indicator. The radioactivity of calcium, precipitated with ammonium oxalate, was measured with a gas-flow counter. Corrections were made for self-absorption and decay.

To study the total radioactivity of ^{14}C-proline in the crude 0·45 M sodium chloride-soluble protein fraction in foetal skin,[20] aliquots of the 60,000 *g* supernatant were dialysed 3 times against 0·45 M sodium chloride at 4° with carrier proline, hydrolysed with equal volume of 12 N HCl for 6 hr, evaporated to dryness, and dissolved in a small volume of distilled water. An aliquot of this solution was used for the determination of the total radioactivity of the crude 0·45 M sodium chloride-soluble protein fraction with the counting system described by Prockop and Ebert.[21]

RESULTS

After preliminary tests an experiment was carried out comprising four rats in each of two groups. Administration of tetracycline was begun on the 16th day of gestation and on the 20th day the animals were given ^{45}Ca and ^{14}C-proline. Tetracycline administration did not affect foetal weight in our experiments. The effect of tetracycline on the incorporation of ^{45}Ca into maternal and foetal bones is shown in Table 1. The content of calcium, calculated as μg Ca/mg foetal calvaria, was lower in the tetracycline-treated rats than in the controls, and the total activity of ^{45}Ca in the foetal calvaria was only 56 per cent of that in the controls. In the maternal femora no significant effect could be seen.

TABLE 1. EFFECT OF TETRACYCLINE ON THE UPTAKE OF ^{45}Ca IN MATERNAL AND FOETAL BONES AFTER ADMINISTRATION OF ^{45}Ca

Source	Group	Calcium content μg/mg bone	Sp. act. of ^{45}Ca cpm/μg	Total activity of ^{45}Ca cpm/mg bone
Foetal calvaria	Controls	1·21 (1·04–1·48)	148 (140–159)	178 (158–209)
	Tetracycline	0·78 (0·67–0·85)	126 (108–147)	99 (82–124)
Maternal femora	Controls	211 (174–261)	4·23 (2·74–5·44)	890 (712–1110)
	Tetracycline	201 (139–259)	4·47 (2·72–6·00)	898 (674–1215)

^{45}Ca was injected 25 μc to each animal 8 hr before they were killed. Four rats in each group.

The incorporation of ^{14}C-proline into ^{14}C-hydroxyproline in the unfractionated collagen of foetal and maternal bone is shown in Table 2. The content of collagen hydroxyproline per mg of foetal calvaria was considerably lower in the tetracycline-treated animals than in the controls. The sp. act. of ^{14}C-hydroxyproline was also lower than in the controls and thus the total activity in the embryos of the tetracycline-treated animals was only 46 per cent of that in the controls. In the corresponding values of the maternal bones there was no significant difference between the tetracycline-treated animals and the controls.

In order to study the effect of tetracycline on the synthesis of soluble collagen in the skin, the incorporation of ^{14}C-proline into ^{14}C-hydroxyproline in the 0·45 M sodium chloride-soluble collagen fraction of maternal and foetal skin was determined. The results are shown in Table 3. In the tetracycline-treated animals the content of soluble collagen hydroxyproline per g of foetal skin was about 62 per cent of that in the

controls. The sp. act. of ^{14}C-hydroxyproline was significantly lower and the total activity was only about 37 per cent of that in the controls. The corresponding values in the soluble collagen of maternal skin in the rats receiving tetracycline were somewhat lower than in the controls, but the difference is not significant.

TABLE 2. EFFECT OF TETRACYCLINE ON THE CONTENT OF HYDROXYPROLINE AND ON THE SPECIFIC AND TOTAL ACTIVITIES OF ^{14}C-HYDROXYPROLINE IN THE NON-FRACTIONATED COLLAGEN OF THE EMBRYONIC AND MATERNAL BONES 8 HR AFTER INJECTION OF ^{14}C-PROLINE

Source	Group	Hydroxyproline μg/mg bone	Sp. act. of ^{14}C-hydroxyproline dpm/μg	Total activity dpm/mg bone
Foetal calvaria	Controls	0·835 (0·735–0·935)	1·77 (1·51–2·01)	1·49 (1·11–1·74)
	Tetracycline	0·665 (0·429–0·857)	1·03 (0·87–1·21)	0·681 (0·519–0·780)
Maternal femora	Controls	43·2 (40·4–45·1)	0·0149 (0·0112–0·0184)	0·655 (0·448–0·780)
	Tetracycline	42·5 (34·4–48·4)	0·0167 (0·0121–0·0192)	0·708 (0·523–0·840)

The same animals as in Table 1. ^{14}C-proline was injected 15 μC to each animal.

TABLE 3. EFFECT OF TETRACYCLINE ON THE CONTENT OF HYDROXYPROLINE AND ON THE SPECIFIC AND TOTAL ACTIVITY OF ^{14}C-HYDROXYPROLINE IN THE SOLUBLE COLLAGEN OF FOETAL AND MATERNAL SKIN AFTER INJECTION OF ^{14}C-PROLINE

Source	Group	Hydroxyproline μg/g of skin	Sp. act. of ^{14}C-hydroxyproline dpm/μg	Total activity dpm/g of skin
Foetuses	Controls	119 (106–141)	19·2 (17·7–21·6)	2330 (2020–2690)
	Tetracycline	73 (63– 84)	12·1 (8·8–14·1)	865 (740–1020)
Mothers	Controls	437 (389–503)	5·23 (4·23–6·27)	2280 (1640–3024)
	Tetracycline	391 (341–467)	4·79 (3·96–5·41)	1870 (1370–2200)

Four rats in each group. ^{14}C-proline was injected 15 μC to each animal.

To study whether tetracycline had an effect on the synthesis of other proteins of foetal skin, the total incorporation of ^{14}C-proline into the soluble proteins of the skin was determined. Table 4 shows the total radioactivity of the crude 0·45 M sodium chloride-soluble fraction of foetal skin compared with the total activity of soluble collagen ^{14}C-hydroxyproline. The total activity of the crude 0·45 M sodium chloride-soluble fraction of the tetracycline-treated animals was about the same as in the controls and therefore the ratio of the total ^{14}C-hydroxyproline activity to total radioactivity was lower in the tetracycline-treated animals than in the controls, suggesting that the synthesis of collagen was inhibited compared with the synthesis of other proteins of foetal skin.

DISCUSSION

The results of the present study indicate that tetracycline given to the mother in therapeutic doses can inhibit the incorporation of radiocalcium into foetal bones

while having no significant effect on its incorporation into maternal bones. Our study confirms the results of *in vitro* studies,[8–10] which have shown the inhibition of incorporation of ^{45}Ca into embryonic bone rudiments, but adds the new finding that the same dose has no effect on the maternal bone.

TABLE 4. EFFECT OF TETRACYCLINE ON THE TOTAL RADIOACTIVITY OF CRUDE 0·45 M SODIUM CHLORIDE-SOLUBLE PROTEIN FRACTION IN FOETAL SKIN 8 HR AFTER ADMINISTRATION OF ^{14}C-PROLINE

Group	0·45 M NaCl-soluble protein fraction	
	Total activity of ^{14}C-proline dpm/g of skin	Ratio ^{14}C-hydroxyproline activity:* total activity × 100
Controls	59,000 (54,000–64,000)	3·95 (3·34–4·90)
Tetracycline	60,000 (56,000–69,000)	1·42 (1·32–1·51)

Same rats as were used for the isolation of soluble collagen.
* For ^{14}C-hydroxyproline values, see Table 3.

Since hydroxyproline is an amino acid highly specific for collagen, and since proline, and not free hydroxyproline, is the source of collagen-linked hydroxyproline,[15] the biosynthesis of collagen can readily be studied by determining the specific and total activities of collagen ^{14}C-hydroxyproline after administration of ^{14}C-proline.[15] In the present study the specific and total activities of ^{14}C-hydroxyproline in the non-fractionated collagen of the foetal calvaria were significantly lower in foetuses of rats receiving tetracycline than in controls. This indicates that tetracycline inhibits the over-all synthesis of collagen in foetal bone tissue. In the maternal femora no similar effect could be demonstrated.

Because the distribution of tetracyclines after injection is not limited to bones but has also been found in other tissues, for example epithelial tissue in cases of malignancy,[22] and traumatized and proliferating tissue,[23] we studied the effect of tetracycline on the synthesis of soluble collagen in foetal and maternal skin. In the 0·45 M sodium chloride-soluble collagen of foetal skin administration of tetracycline to the mother led to specific and total activities significantly lower than those of the controls. This fraction consists of recently synthesized collagen[24] and this finding thus indicates that relatively early stages of collagen biosynthesis can be affected by tetracycline. Moreover, it seems possible that this inhibition is a selective one, because tetracycline did not inhibit the incorporation of ^{14}C-proline into the crude soluble protein fraction of foetal skin.

Our study indicates that tetracycline in therapeutic doses inhibits calcification and collagen biosynthesis in foetal tissues but does not significantly affect maternal tissues. We do not yet know whether this difference between foetal and maternal tissues depends on the different concentrations of tetracycline in the tissues or solely on the greater metabolic turnover in foetal tissue. The nature of the inhibition of collagen biosynthesis and bone calcification and the question which of these processes is primarily affected by tetracycline remain unsettled. But it seems clear that the inhibitory effect on collagen biosynthesis is not limited to bone. This universal effect could be

taken as evidence that the primary action is on collagen biosynthesis, which is immediately followed by arrest of calcification of bone.

From the clinical point of view our study confirms the earlier conclusions that long-term tetracycline treatment during pregnancy is contraindicated.

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