DOI: 10.7860/JCDR/2017/31235.10803

Original Article

Biochemistry Section

Effect of Oxytetracycline on In vitro Mineralization and Demineralization Reactions in the Absence and Presence of Collagen

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ABSTRACT

Introduction: Oxytetracycline and its derivatives are routinely used to treat various ailments have also been shown to inhibit embryonic bone formation, mineralization in pregnant female rats and parathyroid hormone induced demineralization of bones. Oxytetracycline has also been routinely used as bone fluorochrome to study bone metabolism. However, despite the above observations, its mechanism of action is not clearly understood. Some studies tend to suggest that it acts by inhibiting collagen biosynthesis while others indicate that it acts without influencing collagen metabolism.

Aim: To study the mechanism by which oxytetracycline influences the mineralization and demineralization reactions.

Materials and Methods: Homogeneous and heterogeneous systems of in vitro mineralization under physiological conditions of temperature, pH and ionic strength were used to investigate the effect of oxytetracycline not only on initial mineral phase formation but also on its subsequent growth or demineralization. In the Homogenous system, supersaturated conditions with respect to calcium and phosphate ions were employed to study their precipitation as mineral phase resembling hydroxyapatite in nature. However, in the heterogeneous system, collagen isolated from sheep tendons was used to induce identical mineral phases under saturated conditions with respect to calcium and phosphate ions prevailing in the body fluids.

Results: The study demonstrated that in the homogeneous reaction system (mineralization in the absence of collagen) oxytetracycline inhibited both the initial mineral phase formation and its subsequent growth without influencing its demineralization. However, in the heterogeneous system, oxytetracycline was found to inhibit not only the initial mineralization but also its subsequent growth or demineralization.

Conclusion: Oxytetracycline acted like crystal poisons to inhibit the mineralization and demineralization reactions by tightly associating with the mineral phase.

Keywords: Bone metabolism, Inhibition, Mineral phase

INTRODUCTION

Mineralization of bone and teeth is known to occur under physiological conditions, whereas mineralization of aorta, urinary or salivary system takes place under specific pathological conditions [1,2]. However, in both the above conditions, mineral phase has always been found to be associated with an organic matrix. Although the role of the matrix in physiological mineralization of bones and teeth is clearly understood, the controversy still exists regarding its role during pathological mineralization [3-5]. The extent of mineralization at any given time is determined by the balance between initial mineral phase formation and its subsequent growth or demineralization [6].

Oxytetracycline and its derivatives have not only been routinely used to treat various ailments but have also been experimentally used as a fluorochrome to study various aspects of bone metabolism e.g., embryonic bone formation, mineralization in pregnant female rats, parathyroid hormone induced demineralization of bones, osteogenic and osteoclastic activities osteointegration of implants, etc. [7-9]. However, despite all the above observations, the mechanism of action of oxytetracycline and its derivatives is not clearly understood. Many in vivo and in vitro studies tend to suggest that it acts by influencing cartilaginous matrix biosynthesis and/or osteogenic and osteoclastic activities, while others indicate that it acts without influencing the above activities [10-12].

During the present study, both homogeneous and heterogeneous systems of in vitro mineralization, were developed and standardised as done by earlier studies, [13,14] which have been employed to study initial mineral phase formation and its subsequent growth or demineralization in the absence and presence of collagen respectively.

Various concentrations of oxytetracycline were employed to study its effect on the above reactions and the mechanism by which oxytetracycline acts to influence these reactions.

MATERIALS AND METHODS

This experimental study (not involving human subjects) was conducted in the Department of Biochemistry, Himalayan Institute of Medical Sciences, Dehradun, Uttarakhand, India. in collaboration with Department of Biochemistry, Panjab University Chandigarh, India and had the clearance from the Ethical Committee of the two institutes. The study was carried out over a period of 22 months (March 2015-December 2016).

Homogeneous System of Mineralization

The reaction system consisted of 5 mM Calcium chloride ($CaCl_2$), 5 mM Monopotassium phosphate (KH_2PO_4), 87.5 mM Tris (hydroxyl methyl) aminomethane (Tris–HCl) buffer (pH 7.4) and 105 mM Sodium chloride (NaCl), in a final volume of 5.0 ml. The pH of the solutions added to the reaction was pre-adjusted to 7.4. To obtain precipitates, the reaction tubes were incubated for 10-12 minutes at 37°C. The supernatants obtained were discarded and the precipitates thus formed were dissolved in 5.0 mL of 0.1 N Hydrochloric acid (HCl). To study the growth of the preformed mineral phase, the precipitates of calcium phosphate formed by

the above method were resuspended in the reaction system given above. In order to conduct demineralization studies, the preformed mineral phase was resuspended in the above mentioned reaction system but without the presence of calcium and phosphate ions.

Heterogeneous System of Mineralization

Collagen was used to induce matrix bound initial mineral phase formation and its subsequent growth or demineralization. Collagen was isolated from sheep tendon by the method of Thomas and Tomita as modified by Jethi RK and Wadkins CL [13]. About 100 mg of collagen fibres so obtained were incubated at 37°C in the assay system consisting of 87.5 mM Tris-HCl Buffer (pH 7.4), 105 mM NaCl, 1.3 mM $CaCl_2$ and 1.2 mM KH_2PO_4 in a final volume of 25 mL. The final volume was made by using glass distilled water. Aliquots removed at different time intervals were filtered to determine the concentration of calcium and phosphate ions.

To study the subsequent growth of the mineral phase bound to collagen, the premineralised collagen preparations were obtained by incubating the known amounts of collagen fibres in the reaction system mentioned above for 6-24 hours at 37°C. The mineralised matrices thus prepared were washed twice with Tris-HCl buffer before resuspending in the fresh incubation media. For demineralization studies, the premineralised matrices were resuspended in various reaction systems without calcium and phosphate ions.

The concentration of calcium and phosphate in the sample obtained from homogenous and heterogeneous systems of mineralization and demineralization reactions were determined by methods of Baginski ES et al., and Amador E and Urban J respectively [15,16].

To study the effect of oxytetracycline (Pfizer) on mineralization and demineralization reactions, its various amounts were dissolved in glass distilled water to obtain its specific concentrations between 0.1-50 mg% as specifically mentioned. The pH of the oxytetracycline samples was adjusted to 7.4 before addition to the various reaction systems.

STATISTICAL ANALYSIS

Data was analysed using Statistical Package for Social Sciences (SPSS) version 23.0 (IBM Chicago, USA). Simple proportion, mean, standard deviation, as well as level of significance were calculated for the data. A p-value less than 0.05 were considered to be significant.

RESULTS

Both homogenous and heterogeneous systems of in vitro mineralization were used to study not only the effect of various amounts of oxytetracycline on initial mineral phase formation and its subsequent growth or demineralization but also the mechanism of action of oxytetracycline to influence the above three reactions.

Effect of Oxytetracycline on Mineralization (Both Initial Mineral Phase Formation and its Subsequent Growth) in the Absence and Presence of Collagen

Initial mineralization: The results presented in [Table/Fig-1] demonstrate that oxytetracycline inhibited the initial mineral phase formation (mineralization) both in the absence and presence of collagen in homogeneous and heterogeneous systems respectively. Addition of oxytetracycline at 0.2 mg% concentrations resulted in approximately 25% inhibition of initial mineral phase formation in the presence of collagen without having any effect on mineral phase formation in the absence of collagen. At all other higher concentrations oxytetracycline was also found to be a better inhibitor of initial mineralization (Ca^{2+} and HPO_4^{2-} precipitation) in the heterogeneous system as compared to the homogeneous system. Approximately 50% inhibition of initial mineralization in the absence and presence of collagen was obtained with 2.0 mg% and 0.5 mg% concentrations of oxytetracycline respectively.

Growth of preformed mineral phase: The present study [Table/Fig-2] further revealed that similar to the studies on initial mineralization, oxytetracycline was also found to inhibit the growth of the preformed mineral phase present as such (homogeneous system) and when

Exp.	Concentration of Oxytetracycline used (mg%)	Percentage inhibition of initial mineral phase formation (disappearance of ions from media).			
		Homogeneous System		Heterogeneous System	
		Ca^{2+}	HPO_4^{2-}	Ca^{2+}	HPO_4^{2-}
1	0.10	0	0	15±4*	12±3*
2	0.20	0	0	29±4†	24±3†
3	0.50	15±6	14±4	55±3‡	50±3‡
4	1.0	30±5	25±3	75±3‡	70±2‡
5	2.0	50±4	45±3	92±2‡	88±2‡
6	4.0	75±3	70±2	100†	100†
7	8.0	85±3	80±3	100*	100*
8	16.0	95±2	90±2	100§	100§

[Table/Fig-1]: Effect of oxytetracycline on in vitro initial mineral phase formation in the absence (homogeneous system) and the presence of collagen (heterogeneous system).
Note: 100% inhibition of initial mineral phase formation was obtained in both the systems at oxytetracycline concentration ≥ 16 mg%.
All values are mean ± S.D of seven replicates.
*p<0.05, †p<0.01, ‡p<0.001, §Not Significant as compared to the homogenous system at identical concentrations of oxytetracycline.

Exp.	Concentration of oxytetracycline used (mg%)	Percentage inhibition of ions uptake by the pre-formed mineral phase (i.e., its growth).			
		Homogeneous System		Heterogeneous System	
		Ca^{2+}	HPO_4^{2-}	Ca^{2+}	HPO_4^{2-}
1	0.20	0	0	9±6§	8±5§
2	0.50	0	0	22±4†	20±4†
3	1.0	0	0	40±3‡	36±3‡
4	2.0	7±4	5±3	66±3‡	50±2‡
5	4.0	15±4	12±4	95±2‡	90±3‡
6	8.0	28±4	25±3	100‡	100‡
7	16.0	53±3	50±3	100‡	100‡
8	32.0	78±3	72±2	100*	100*
9	50.0	95±2	90±3	100§	100§

[Table/Fig-2]: Effect of oxytetracycline on the growth of pre-formed mineral phase present as such (homogeneous system) and when bound to collagen (heterogeneous system).
Note: No inhibition of ions uptake by pre-formed mineral phase was observed in both the systems at oxytetracycline concentration ≤ 0.2 mg%.
All values are mean ± SD of seven replicates
*p<0.05, †p<0.01, ‡p<0.001, §Not Significant as compared to the homogeneous system at identical concentrations of oxytetracycline.

Exp.	Concentration of oxytetracycline used (mg%)	Percentage inhibition of release of ions (from the mineral phase present as such & bound to collagen) into the media.			
		Homogeneous System		Heterogeneous System	
		Ca^{2+}	HPO_4^{2-}	Ca^{2+}	HPO_4^{2-}
1	1.0	0	0	38±8†	44±7†
2	2.0	0	0	42±9†	40±5†
3	4.0	0	0	50±4†	48±4†
4	8.0	0	0	56±4§	50±3§
5	16.0	0	0	62±3§	56±5§
6	32.0	0	0	65±4§	60±3§
7	50.0	0	0	68±4§	64±4§

[Table/Fig-3]: Effect of oxytetracycline on in vitro demineralization of the pre-formed mineral phase present as such (homogeneous system) and when bound to collagen (heterogeneous system).
Note: All values are mean ± S.D of seven replicates
§Not Significant as compared to 4 mg% concentration of oxytetracycline (Exp-3).
*p<0.05, †p<0.01, ‡p<0.001 as compared to the control system in the absence of oxytetracycline.

Exp.	Concentration of reacting ions (μmoles) in the reaction system		Percentage inhibition of mineralization by oxytetracycline in the absence of collagen (Homogeneous system)			
			Initial Mineral phase formation‖		Growth of the preformed mineral phase**	
	Ca^{2+}	HPO_4^{2-}	Ca^{2+}	HPO_4^{2-}	Ca^{2+}	HPO_4^{2-}
1	15	25	55±3	47±2	54±3	48±2
2	25	25	50±4††	45±3††	52±3††	50±3††
3	50	25	48±5††	46±3††	58±2††	54±3††
4	25	15	52±3	47±2	55±3	48±2
5	25	25	50±4‡‡	45±3‡‡	56±3‡‡	50±3‡‡
6	25	50	55±3‡‡	48±3‡‡	54±3‡‡	52±3‡‡

[Table/Fig-4]: Effect of changing the concentration of calcium and phosphate ions in the reaction system on the ability of a fixed amount of oxytetracycline to inhibit in vitro initial mineral phase formation and its sequent growth in the absence of collagen.
Note: All values are mean ± S.D of seven replicates
‖ Concentration of oxytetracycline used was 2.0 mg%.
** Concentration of oxytetracycline used was 16.0 mg%.
†† Not Significant as compared to exp,1.
‡‡Not Significant as compared to exp.4.

Exp.	Concentration of reacting ions(μmoles) in the reaction system		Percentage inhibition of collagen induced mineralization by oxytetracycline (Heterogeneous system).			
			Initial Mineral phase Formation‖		Growth of the preformed mineral phase **	
	Ca^{2+}	HPO_4^{2-}	Ca^{2+}	HPO_4^{2-}	Ca^{2+}	HPO_4^{2-}
1	25.5	40	54±3	48±2	65±2	62±2
2	51.0	40	55±3††	50±3††	66±3††	60±2††
3	68.0	40	52±2††	49±2††	62±2††	58±4††
4	51.0	24	58±2	55±3	60±3	57±2
5	51.0	40	55±3‡‡	50±3‡‡	66±3‡‡	60±2‡‡
6	51.0	56	52±3‡‡	50±33‡‡	58±2‡‡	55±3‡‡

[Table/Fig-5]: Effect of changing the concentration of calcium and phosphate ions in the reaction system on the ability of a fixed amount of oxytetracycline to inhibit in vitro initial mineral phase formation and its sequent growth in the presence of collagen.
Note: All values are mean ± S.D of seven replicates
‖ Concentration of oxytetracycline used was 0.5 mg%.
** Concentration of oxytetracycline used was 2.0 mg%.
†† Not Significant as compared to exp.1.
‡‡ Not Significant as compared to exp.4.

Exp.	Amount of Collagen used to induce mineralization (mg)	Percentage inhibition of collagen induced mineralization by Oxytetracycline (Heterogeneous system).			
		Initial mineral phase formation‖		#Growth of the preformed mineral phase**	
		Ca^{2+}	HPO_4^{2-}	Ca^{2+}	HPO_4^{2-}
1	100	55±3	50±2	66±3	60±3
2	200	35±4*	30±3*	45±3*	40±2*
3	300	20±3†	15±4†	28±4†	24±3†

[Table/Fig-6]: Effect of changing the amount of collagen in the reaction system on the ability of a fixed amount of oxytetracycline to inhibit in vitro initial mineral phase formation and its sequent growth in the presence of collagen.
Note: All values are mean ± S.D of seven replicates
#For the growth studies, different amounts of collagen had approximately identical amounts of calcium and phosphate ions present in the mineral phase.
‖Concentration of oxytetracycline used was 0.5 mg%.
¨Concentration of oxytetracycline used was 2.0 mg%.
*p<0.05, †p<0.01 as compared to exp.1

bound to collagen (heterogeneous system). Comparison of the studies presented in [Table/Fig-1] with those of [Table/Fig-2] further revealed that at identical concentrations, oxytetracycline acted as a better inhibitor of initial mineral phase formation as compared to its subsequent growth. As compared to initial mineral phase formation, approximately 3 and 2 times higher concentrations of oxytetracycline were required to inhibit the growth of the preformed mineral phase present as such and when bound to collagen respectively.

Effect of oxytetracycline on demineralization of preformed mineral phase present as such and when bound to collagen: Results presented in [Table/Fig-3], demonstrate that up to a concentration of 50 mg% oxytetracycline was found to have no effect on demineralization of preformed mineral phase present as such. However, when preformed mineral phase bound to collagen was employed, oxytetracycline was found to significantly ($p<0.01$) inhibit its demineralization. Interestingly, although 50% demineralization inhibition of the collagen bound mineral phase was attained by an oxytetracycline concentration of 4 mg%, increasing its concentration further even up to 50 mg% was found to have no significant additional inhibitory effect on the demineralization of collagen bound mineral phase.

Mechanism of action of oxytetracycline to influence mineralization and demineralization reactions: Studies further revealed that changing the soluble concentration of either calcium or phosphate ions was found to have no significant effect on the ability of oxytetracycline to inhibit both the initial mineral phase formation and its subsequent growth either in the absence [Table/Fig-4] or the presence of collagen [Table/Fig-5].

Results presented in [Table/Fig-6], however, revealed that when different amounts of collagen were used in the assay systems, while keeping the concentrations of both of calcium and phosphate ions constant, to study the effect of a fixed amount of oxytetracycline (0.5 mg% and 2.0 mg% in case of initial mineral formation and its subsequent growth respectively), increasing the amounts of collagen was found to inversely effect the ability of oxytetracycline to inhibit both the collagen induced initial mineral phase formation and its subsequent growth.

DISCUSSION

Homogeneous and Heterogeneous systems of in vitro mineralization have routinely been employed not only to study the mechanism of initial mineral phase formation and its subsequent growth or demineralization but also the effect of various molecules/samples on these reactions [11-14]. These studies have clearly shown that collagen induces mineralization in a step-wise mechanism to convert calcium and phosphate ions present in the soluble phase to the matrix bound mineral phase. These studies have further shown that mineral phase formed gets tightly associated with the collagen and this matrix bound mineral phase can either undergo further growth or get demineralised (dissolution) to form free ions depending upon the concentration of the reacting ions in the soluble phase. Interestingly demineraliztion was found to occur not by the reversal of various steps involved in mineralization [13,14].

Effect of oxytetracycline on mineralization and demineralization reactions in the absence and presence of collagen: Results presented in [Table/Fig-1,2], clearly demonstrate that oxytetracycline acted as a better inhibitor of both the initial mineral phase formation and its subsequent growth in the presence of collagen (heterogeneous system) as compared to when these reactions were studied in the absence of collagen in the homogeneous system. In comparison to the homogeneous system, in the heterogeneous system, much lower concentrations of oxytetracycline were required to get identical inhibitions of both the initial mineral phase formation and its subsequent growth. However, whereas in the heterogeneous system, oxytetracycline was found to significantly ($p<0.01$) inhibit the demineralization of collagen bound mineral phase, it was found to have no effect on the demineralization of free mineral phase [Table/Fig-3].

Mechanism of action of oxytetracycline to influence mineralization and demineralization reactions: In vitro precipitation of calcium and phosphate ions as mineral phase (mineralization) and its subsequent growth or demineralization (release of ions from the solid mineral phase into the aqueous soluble phase) can be influenced by oxytetracycline either by

binding with the reacting ions present in the soluble phase thus decreasing their effective concentrations or by binding with the mineral phase similar to the action of crystal poisons [13]. To differentiate between the above two mechanisms, the inhibition of initial mineral phase formation and its subsequent growth, caused by a fixed concentration of oxytetracycline (0.5 mg% and 2.0 mg% in case of initial mineral phase formation and its subsequent growth respectively) was studied by changing either the concentration of reacting calcium and phosphate ions in the soluble phase or having different amounts of collagen in the assay systems during initial mineral phase formation and its subsequent growth. Having approximately the same amounts of calcium and phosphate ions present in the mineral phase bound to increasing amounts of collagen would lead to a proportional increase in the surface area available for binding of inhibitors which act like crystal poisons.

Results presented in [Table/Fig-4,5] demonstrated that increasing the soluble phase concentrations of either calcium or phosphate ions was found to have no significant effect on the inhibition of either the initial mineral phase formation or its subsequent growth caused by a fixed concentration of oxytetracycline. However, results presented in [Table/Fig-6], clearly demonstrated that when different amounts of collagen were employed in the studies, the percentage inhibition of both the initial mineral phase formation and its subsequent growth caused by a fixed concentration of oxytetracycline was found to be inversely related to the amounts of collagen used. The above observation along with the one made earlier that at all the concentrations oxytetracycline was found to be a better inhibitor of initial mineral phase formation as compared to its subsequent growth, strongly suggests that like crystal poisons, oxytetracycline influenced both the mineralization and demineralization reactions by associating with the mineral phase.

Better inhibition of mineralization by oxytetracycline in the presence of collagen and differential effect of oxytetracycline on demineralization in the absence and presence of collagen clearly suggests that oxytetracycline may also be acting by influencing the activities of various sites of collagen involved in converting free calcium and phosphate ions present in the soluble phase to the collagen bound mineral phase and the subsequent growth or demineralization of this matrix bound mineral phase.

LIMITATION

As seen under in vivo conditions, various biochemical transformations/processes are regulated not only by the concentration of reactants and products but also by the interaction of vitamins, hormones and other bioregulatory biomolecules circulating in body fluids, questions can always be raised regarding the physiological significance of the results obtained during the in vitro studies. To overcome the above limitation, it is recommended that to fully appreciate the significance/clinical implications of the results obtained during the present studies, further in vivo studies in animals should be conducted to study the effect of administered oxytetracycline on mineralization and demineralization reactions using available model systems.

CONCLUSION

The results obtained in the present in vitro study clearly demonstrate that under physiological conditions of temperature, pH and concentration of reactants, oxytetracycline can not only inhibit initial mineral phase formation and its subsequent growth but also the demineralization of the mineral phase bound to an organic matrix like collagen.

The above observations may have important implications in the prescription of oxytetracycline to patients of various age groups at different stages of skeletal development.

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FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: **Jun 29, 2017**
Date of Peer Review: **Jul 24, 2017**
Date of Acceptance: **Sep 25, 2017**
Date of Publishing: **Nov 01, 2017**