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(54) Titre : INHIBITION DES LIAISONS CROISEES DU COLLAGENE EN EXCES AU COURS DU DIABETE PAR DES TETRACYCLINES, Y COMPRIS PAR DES TETRACYCLINES NON ANTIMICROBIENNES CHIMIQUEMENT MODIFIEES

(54) Title: TETRACYCLINES INCLUDING NON-ANTIMICROBIAL CHEMICALLY-MODIFIED TETRACYCLINES INHIBIT EXCESSIVE COLLAGEN CROSSLINKING DURING DIABETES

(57) Abrégé/Abstract:
A method for treating mammals suffering from excessive collagen crosslinking which is associated with diabetes, scleroderma and progeria by administering to the mammal an amount and/or type of a tetracycline that is not effectively antimicrobial but which effectively inhibits excessive collagen crosslinking.

ABSTRACT

A method for treating mammals suffering from excessive collagen crosslinking which is associated with diabetes, scleroderma and progeria by administering to the mammal an amount and/or type of a tetracycline that is not effectively antimicrobial but which effectively inhibits excessive collagen crosslinking.

TETRACYCLINES INCLUDING NON-ANTIMICROBIAL CHEMICALLY-MODIFIED TETRACYCLINES INHIBIT EXCESSIVE COLLAGEN CROSSLINKING DURING DIABETES

BACKGROUND OF THE INVENTION

This invention was made with Government support under R37 DE-03987, awarded by The National Institute of Dental Research. The Government has certain rights in the invention.

The present invention relates to a method of treating mammals suffering from conditions associated with an excessive amount of collagen crosslinking by administering to the mammal an amount and/or type of a tetracycline that is not effectively antimicrobial but which effectively inhibits excessive collagen crosslinking. Conditions associated with an excessive amount of collagen crosslinking include diabetes, scleroderma and progeria.

Diabetes mellitus (diabetes) is a complex disease that affects several hundred million people. Diabetes is characterized by an elevated level of glucose in the blood. Glucose cannot enter the body's cells to be utilized and therefore remains in the blood in high concentrations. When the blood glucose level exceeds the reabsorptive capacity of the renal tubules, glucose is excreted in the urine. Diabetes produces a number of debilitating and life-threatening complications.

A number of diabetes-induced abnormalities in collagen metabolism, such as pathologically-excessive collagenase activity in gingiva and skin, have been reported in the literature. Ramamurthy et al., J. Peridontal Res. 17: 455-462 (1983); Ramamurthy et al., Gerondontol. 2(1): 15-19 (1983). A complication of excessive collagenase activity is an unusually aggressive periodontal destruction which is frequently associated

with diabetes. Finestone et al., Diabetes 16: 336-340 (1967). This complication is particularly problematic when the disease is poorly controlled. Ainamo et al., J. Clin. Peridontol. 17: 22-28 (1990).

A "hallmark" abnormality in collagen metabolism in the connective tissues of the diabetic is a reduction in the extractability or solubility of collagen in either cold (0-4°C) neutral salt or dilute acid solutions. This reduction in collagen solubility reflects excessive inter- and intra- molecular covalent crosslinking of the collagen molecules. Diabetes-induced reduction in collagen solubility, due to excessive collagen crosslinking, has been seen in a variety of tissues including (but not limited to) skin, bone, tendon, gingiva and aorta. Ramamurthy et al., Gerondontology 2: 15 (1983); Brownlee et al., Science 232:1629 (1986); Buckingham et al., J. Clin. Invest. 86:1046 (1990); Dominiczak et al., Diabetes Care 13:468 (1990); Golub et al., Biochim. Biophys. Acta 534:73 (1978). Abnormally low solubility of collagen reflects the excessive crosslinking of collagen in the extracellular matrix which renders the collagen excessively polymerized and more resistant to degradation and turnover. (See Golub et al., Biochim. Biophys. Acta 534:73 (1978), for a review).

Hamlin et al., (Diabetes 24:902 (1975)) and others describe this reduced collagen solubility/increased collagen crosslinking, which characterizes the connective tissues of the diabetic, as an "aging-like" abnormality in collagen metabolism. This abnormality is increasingly viewed as a major cause of numerous complications of diabetes including (but not limited to) increased leatheriness of skin, limitation of joint movement, increased stiffness of arterial walls, impaired wound healing, decreased elasticity of lungs and nephropathy

including proteinuria.

Recent studies (Walton et al., Biochim. Biophys. Acta, 1138:172-183 (1992)) have demonstrated that cross-linking of basement membranes (type IV collagen) of the kidney glomerulus increases the permeability of the basement membrane to protein which, in turn, promotes proteinuria, a classic parameter of renal damage in the diabetic. As discussed earlier, diabetes mellitus results in increased crosslinking of proteins, including collagens. Cohen et al., (Biochem. Biophys. Res. Commun., 95:765-769 (1980)) have found that inducing diabetes in rats increases non-enzymatic glycosylation of glomerular basement membranes. Non-enzymatic glycosylation provides one mechanism for excessive collagen crosslinking. Reiser, Proc. Soc. Experiment. Biol. and Med., 196:17-29 (1991).

Collagen biosynthesis is a complex process characterized by extensive posttranslational modifications. Jackson, The Substrate Collagen, Chapter 1, in: "Collagenase In Normal And Pathological Connective Tissues," (eds: D.E. Woolley and J.M. Evanson), pp 1-10, John Wiley & Sons Ltd., N.Y., 1980. Collagen crosslinking results from two different biochemical pathways, enzymatic lysyl oxidase-dependent crosslinking and nonenzymatic glucose-derived collagen crosslinking. Buckingham et al., J. Clin. Invest. 86:1046 (1990).

Intracellular events include the hydroxylation of certain lysine and proline residues and enzymatically mediated glycosylation of hydroxylysine before the pro-collagen (collagen precursor) molecule is secreted from the cell. C-terminal and N-terminal extension peptides are subsequently cleaved. The collagen molecules become stabilized in their fibrillar arrays or networks by covalent cross-linking mediated initially by the enzyme,

lysyl oxidase. Nonenzymatic glycosylation of certain lysine and hydroxylysine residues also occurs in the extracellular matrix. This modification appears to have direct effects on collagen structure and function as well as indirect effects which arise as a result of further reactions of the glycosylated residues.

Increased collagen crosslinking during diabetes is mediated by both mechanisms. It is mediated enzymatically by the excessive activity of lysyl oxidase and non-enzymatically by the glucose-derived mechanism due to exposure to elevated blood and tissue fluid glucose concentrations. Makita et al., New Engl. J. Med. 325:836 (1991); Cerami et al., Diabetes Care 11 (Suppl.1:73) (1988).

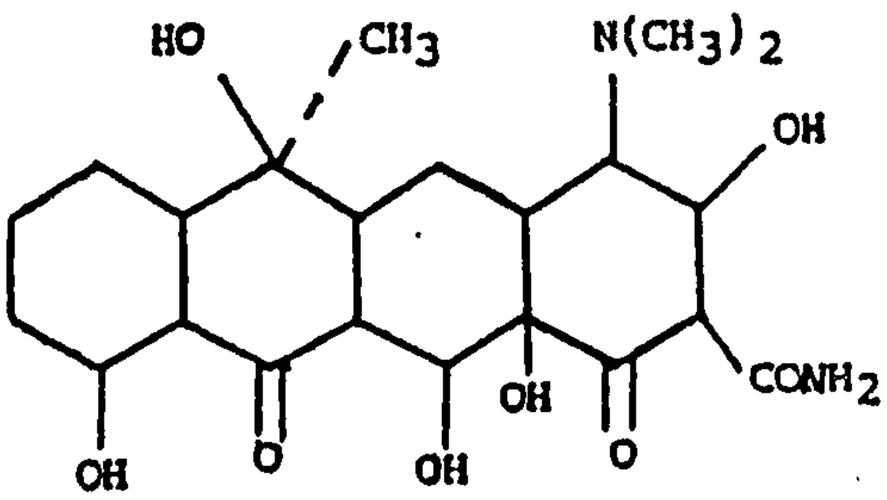
Enzymatic lysyl oxidase-dependent collagen crosslinking begins with lysyl oxidase-dependent oxidative deamination of certain lysine and hydroxylysine residues. The resultant aldehyde moieties may undergo further reactions with lysine, hydroxylysine, and histidine residues to form di-, tri- and tetrafunctional crosslinks. Robbins, Methods Biochem. Analysis 28: 330-379 (1982). In particular, lysyl oxidase converts ϵ-amino groups of certain lysyl and hydroxylysyl residues, in the non-helical regions of the collagen molecule, to aldehyde moieties which then form Schiff base crosslinks with adjacent molecules. Vader, et al., Biochem. J. 180: 639-645 (1979).

As previously mentioned, nonenzymatic glucose-derived crosslinking begins with the nonenzymatic glycosylation of lysine and hydroxylysine residues on the collagen molecules in the extracellular matrix. These early glycosylation products are believed to undergo a series of reactions to form complex fluorophores and chromophores collectively referred to as advanced

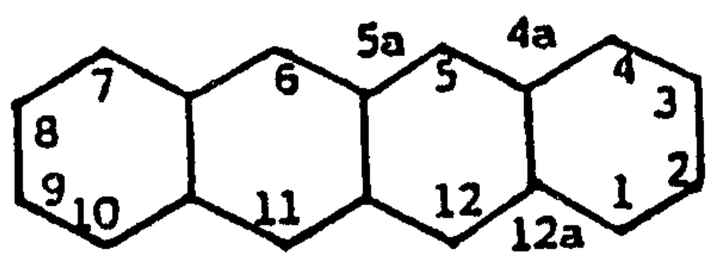
Maillard products or advanced glycosylation end-products ("AGES"). Brownlee et al., N. Engl. J. Med. 318: 1315-1322 (1988).

Brownlee et al., (Science 232: 1629-1632 (1986)) have reported that aminoguanidine is an effective inhibitor of nonenzymatic glucose-derived collagen crosslinking associated with diabetes in rats. However, aminoguanidine has not been approved for use in humans. Tetracycline, on the other hand, has been approved for use in humans.

The compound, tetracycline, exhibits the following general structure:



The numbering system of the ring nucleus is as follows:



Tetracycline as well as the 5-OH (Terramycin) and 7-Cl (Aureomycin) derivatives exist in nature, and are well known antibiotics. Natural tetracyclines may be modified without losing their antibiotic properties, although certain elements of the structure must be retained. The modifications that may and may not be made to the basic tetracycline structure have been reviewed by Mitscher in

The Chemistry of Tetracyclines, Chapter 6, Marcel Dekker, Publishers, N.Y. (1978). According to Mitscher, the substituents at positions 5-9 of the tetracycline ring system may be modified without the complete loss of antibiotic properties. Changes to the basic ring system or replacement of the substituents at positions 1-4 and 10-12, however, generally lead to synthetic tetracyclines with substantially less or effectively no antimicrobial activity. For example, 4-dedimethylaminotetracycline is commonly considered to be a non-antimicrobial tetracycline.

The use of tetracycline antibiotics, while effective, may lead to undesirable side effects. For example, the long term administration of antibiotic tetracyclines may reduce or eliminate healthy flora, such as intestinal flora, and may lead to the production of antibiotic resistant organisms or the overgrowth of opportunistic yeast and fungi.

In addition to their antibiotic properties, tetracyclines have been described for a number of uses. For example, tetracyclines are also known to inhibit the activity of collagen destructive enzymes such as mammalian collagenase, gelatinase, macrophage elastase and bacterial collagenase. Golub et al., J. Periodont. Res. 20: 12-23 (1985); Golub et al. Crit. Revs. Oral Biol. Med. 2: 297-322 (1991).

Tetracyclines, administered at both antimicrobial levels and non-antimicrobial levels, have been known to play a role in reducing collagenase and other collagenolytic enzyme activity as well as collagen breakdown, U.S. Patent Nos. 4,666,897; 4,704,383; 4,935,411; 4,935,412. In addition, tetracyclines have been known to inhibit wasting and protein degradation in mammalian skeletal muscle, U.S. Patent No. 5,045,538.

Furthermore, tetracyclines have been demonstrated to enhance bone formation in osteoporosis, U.S. Patent No. 4,925,833.

U.S. Patent No. 4,704,383 to McNamara et al. discloses that tetracyclines having substantially no effective antimicrobial activity inhibit collagenolytic enzyme activity in rats. McNamara et al. also report that non-antimicrobial tetracyclines reduce bone resorption in organ culture. Earlier, U.S. Patent No. 4,666,897 to Golub, et al. disclosed that tetracyclines in general, including commercially-available antimicrobial forms of the drug, inhibit excessive mammalian collagenolytic enzyme activity resulting in decreased connective tissue breakdown including that which occurs during bone resorption.

There have been a number of suggestions that tetracyclines, including non-antimicrobial tetracyclines, are effective in treating arthritis in rats. See, for example, Golub et al., "Tetracyclines (TCs) Inhibit Matrix Metalloproteinases (MMPs): In Vivo Effects in Arthritic and Diabetic Rats And New In Vitro Studies," Matrix, Suppl. No. 1:315-316 (1992); Greenwald et al. "CMT, A Matrix Metalloproteinase Inhibitor, Prevents Bone Resorption In Adjuvant Arthritis." Arthritis Rheum.: 34 (#9 suppl): S66 (abstract #A6), abstract presented at 55th Annual Meeting, Amer. College of Rheumatology, Boston MA, Nov. 18, 1991; Breedveld, "Suppression of Collagen And Adjuvant Arthritis By A Tetracycline," Northeastern Regional Meeting Of The Amer. Rheum. Assoc., Atlantic City, New Jersey, October 23, 1987. For a related commentary regarding the effect of non-antimicrobial tetracyclines on bone loss see Sipos et al., "The Effect of Collagenase Inhibitors On Alveolar Bone Loss Due To Periodontal Disease In Desalivated Rats," abstract presented at Matrix Metalloproteinase

Conference, Destin, Florida, September 11-15, 1989.

According to White, Lancet, April 29, p. 966 (1989) the tetracycline minocycline is effective in treating dystrophic epidermolysis bullosa, which is a life-threatening skin condition believed to be related to excess collagenase.

The effectiveness of tetracycline in skin disorders has also been studied by Elewski et al., Journal of the American Academy of Dermatology 8: 807-812 (1983). Elewski et al. disclosed that tetracycline antibiotics may have anti-inflammatory activity in skin and speculate that a portion of the therapeutic effect in skin diseases associated with bacteria, e.g., acne, may be due to inhibition of bacterially induced inflammation rather than a direct antimicrobial effect.

Similarly, Plewig et al., Journal of Investigative Dermatology 65: 352-532 (1975), disclose experiments designed to test the hypothesis that antimicrobials are effective in treating inflammatory dermatoses. The experiments of Plewig et al. establish that tetracyclines have anti-inflammatory properties in treating pustules induced by potassium iodide patches.

The use of tetracyclines in combination with non-steroidal anti-inflammatory agents has been studied in the treatment of inflammatory skin disorders caused by acne vulgaris. Wong et al., Journal of American Academy of Dermatology 11: 1076-1081 (1984), studied the combination of tetracycline and ibuprofen and found that tetracycline was an effective agent against acne vulgaris while ibuprofen was useful in reducing the resulting inflammation by inhibition of cyclooxygenase. Funt, Journal of the American Academy of Dermatology 13: 524-525 (1985), disclosed similar results by combining

antimicrobial doses of minocycline and ibuprofen.

Based on the foregoing, tetracyclines have been found to be effective in different treatments. However, there has been no suggestion whatsoever that tetracyclines can ameliorate the signs and symptoms of excessive collagen crosslinking. In addition, tetracyclines have been found to have no significant effect on reduction of the severity of hyperglycemia associated with diabetes. Golub et al., J. Periodontal Res., 18:516-526 (1983); Golub et al., Res.Commun. Chem. Path. Pharmacol., 68:27-40 (1990); Yu et al., J. Periodontal Res., submitted for publication (1992).

On the other hand, insulin, unlike tetracycline, is useful in the symptomatic treatment of diabetes, including reducing the severity of hyperglycemia. However, most insulin-treated diabetics still have some degree of hyperglycemia which ultimately leads to excessive collagen crosslinking. It is not clear which mechanism, enzymatic lysyl oxidase-dependent or nonenzymatic glucose-derived collagen crosslinking, is predominantly responsible for the excessive collagen crosslinking resulting from hyperglycemia.

The present invention is intended to provide a means for treating excessive collagen crosslinking. The present invention demonstrates that tetracyclines, including their chemically-modified analogs which have lost their antimicrobial efficacy, have a novel new use -- the ability to inhibit excessive collagen crosslinking in connective tissues of the diabetic. This non-antimicrobial property of tetracyclines reduces the severe complications of diabetes including the amelioration of proteinuria which is a sign of diabetes-induced nephropathy (a life-threatening complication of the disease).

SUMMARY OF THE INVENTION

The present invention provides a method for treating mammals suffering from excessive collagen crosslinking which is associated with diabetes, scleroderma and progeria. The method of the present invention includes administering to the mammal an amount and/or type of a tetracycline that is not effectively antimicrobial but which effectively inhibits excessive collagen crosslinking.

The invention also provides a commercial package comprising a pharmaceutically effective amount of a tetracycline sufficient to inhibit excessive amount of collagen crosslinking together with instructions for use thereof to treat a mammal suffering from conditions associated with an excessive amount of collagen crosslinking. Uses of such tetracyclines for such treatments as well as the preparation of medicaments for such treatments are also within the scope of the invention. The invention also provides compositions comprising the tetracyclines.

Chemically-modified tetracyclines, for example dedimethylaminotetracyclines, are useful in the present invention. Dedimethylaminotetracyclines include 4-dedimethylaminotetracycline, 4-dedimethylamino-5-oxytetracycline, 4-dedimethylamino-7-chlorotetracycline, 4-hydroxy-4-dedimethylaminotetracycline, 5a,6-anhydro-4-hydroxy-4-dedimethylaminotetracycline, 6α-deoxy-5-hydroxy-4-dedimethylaminotetracycline, 6-demethyl-6-deoxy-4-dedimethylaminotetracycline, 4-dedimethylamino-12a-deoxytetracycline and 4-dedimethylamino-11-hydroxy-12a-deoxytetracycline.

Further examples of chemically-modified tetracyclines useful in the present invention are 6a-benzylthiomethylenetetracycline, the 2-nitrilo analogs of tetracycline, the mono-N-alkylated amide of tetracycline, 6-fluoro-6-demethyltetracycline, 11a-chlorotetracycline and 12a-deoxytetracycline and its derivatives.

The non-antimicrobial tetracycline is administered in an amount of from about 0.5 mg/kg per day to about 50.0 mg/kg per day, preferably from about 1.0 mg/kg per day to about 15.0 mg/kg per day.

The method of the present invention ameliorates many of the complications associated with diabetes, for

example, increased leatheriness of skin, decreased lung elasticity, increased arterial wall stiffness, limitation of joint movement, impaired wound healing and nephropathy resulting in proteinuria.

For a better understanding of the present invention, reference is made to the following description, taken together with the accompanying drawings, and its scope will be pointed out in the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphic representation of the effect of different oral doses of CMT-1 on the blood glucose concentration (mg/dL) of streptozotocin-diabetic rats. Each value represents the mean ± S.E.M. for 4 rats per experimental group.

Figure 2 is a graphic representation of the effect of CMT-1 therapy on the acid-soluble fraction of collagen in skin of diabetic rats. Note: the solubility of collagen in dilute acid (4°C) was normalized to 100% for the non-diabetic control (NDC) rats and the data for the untreated diabetics (UD) and CMT-1 treated diabetics (D + mgs CMT-1) is expressed relative to the NDC values. * Indicates that value is significantly different from NDC ($p < 0.01$).

Figure 3 is a graphic representation of the effect of CMT-1 therapy on the salt-soluble fraction of collagen in skin of diabetic rats. Note: the solubility of collagen in neutral salt (4°C) was normalized to 100% for the non-diabetic control (NDC) rats and the data for the untreated diabetics (UD) and CMT-1 treated diabetics (D + mgs CMT-1) is expressed relative to the NDC values. * Indicates that value is significantly different from NDC ($p < 0.01$).

Figure 4 is a graphic representation of the effect of minocycline therapy on urine excretion and proteinuria in diabetic rats.

DETAILED DESCRIPTION OF THE INVENTION

It has been discovered that tetracyclines inhibit the excessive collagen crosslinking associated with diabetes, scleroderma and progeria. In particular, the inventors have discovered that the use of certain tetracyclines inhibits excessive collagen crosslinking. Various tetracyclines have been shown not to affect blood glucose levels. The tetracyclines of the present invention have clearly demonstrated an anti-collagen crosslinking effect. The tetracyclines of the present invention can be combined with insulin therapies, which are known to moderate blood glucose levels in the treatment of diabetes. While the tetracycline inhibits excessive collagen crosslinking, the insulin attempts to regulate hyperglycemia. As previously mentioned, the diabetic complications associated with excessive collagen crosslinking include increased leatheriness of skin, decreased lung elasticity, increased arterial wall stiffness, limitation of joint movement, impaired wound healing and nephropathy resulting in proteinuria.

The excessive collagen crosslinking inhibitory effect is associated with the unexpected ability of tetracyclines to reduce glycosylation of protein. While not wishing to be bound by any one theory, it is believed that this inhibitory effect on pathologically-excessive collagen crosslinking is associated with the unexpected ability of tetracyclines to reduce nonenzymatic glucose-derived collagen crosslinking and also to reduce enzymatic lysyl oxidase-dependent collagen crosslinking.

The conditions treated by the present invention

occur in mammals. Mammals include, for example, human beings and laboratory animals such as mice and rats.

The tetracyclines useful in the present invention may be any tetracycline administered to a mammal in a dose that is effectively non-antimicrobial in the mammal. Preferably, the tetracycline is modified so as to reduce its antimicrobial properties. Methods for reducing the antimicrobial properties of a tetracycline are disclosed in "The Chemistry of the Tetracyclines", Chapter 6, Mitscher, Marcel Dekker, Publishers, N.Y. (1978), at page 211. As pointed out by Mitscher, modifications at positions 1, 2, 3, 4, 10 and 12a lead to loss of bioactivity. Non-antimicrobial tetracyclines are preferred since they can be used at therapeutic levels which impart fewer side effects than antimicrobial tetracyclines at the same dosage level.

The preferred tetracyclines are those that lack the dimethylamino group at position 4. Such chemically modified tetracyclines include, for example, 4-dedimethylaminotetracycline, 4-dedimethylamino-5-oxytetracycline, 4-dedimethylamino-7-chlorotetracycline, 4-hydroxy-4-dedimethylaminotetracycline, 5a,6-anhydro-4-hydroxy-4-dedimethylaminotetracycline, 6-demethyl-6-deoxy-4-dedimethylaminotetracycline, 6α-deoxy-5-hydroxy-4-dedimethylaminotetracycline, 4-dedimethylamino-11-hydroxy-12a-deoxytetracycline and 4-dedimethylamino-12a-deoxytetracycline and its derivatives. Tetracyclines altered at the 2 carbon position to produce a nitrile, e.g., tetracyclinonitrile, may be useful as non-antimicrobial agents exhibiting anti-collagen crosslinking properties when administered via non-oral routes.

Further examples of tetracyclines modified for reduced antimicrobial activity include 6a-

benzylthiomethylenetetracycline, the mono-N-alkylated amide of tetracycline, 6-fluoro-6-demethyltetracycline, 11a-chlorotetracycline and 12a-deoxytetracycline and its derivatives.

The effective amount of tetracycline is that amount which effectively inhibits excessive collagen crosslinking while it is not effectively antimicrobial. For purposes of this invention, a tetracycline effectively inhibits excessive collagen crosslinking if it is present in an amount which significantly reduces excessive collagen crosslinking. Excessive collagen crosslinking is defined as collagen crosslinking which is greater than that found in connective tissues of a nondiabetic control of the same age. (See Hamlin et al., Diabetes 24:902 (1975)).

A tetracycline is considered effectively non-antimicrobial if it does not significantly prevent the growth of microbes. This of course may vary depending upon a number of factors, such as, type of tetracycline, disease state and type of microbe. The maximal useful dosage for humans is the highest dosage that does not cause adverse side effects. For example, for purposes of the present invention, side effects include clinically significant antimicrobial activity, as well as toxic effects. A dose in excess of about 50 mg/kg/day would produce side effects in most mammals, including humans. The non-antimicrobial tetracycline of the present invention may be administered in an amount of from about 0.5 mg/kg/day to about 50.0 mg/kg/day preferably from about 1.0 mg/kg/day to about 15.0 mg/kg/day.

The means of delivery of the tetracycline with a pharmaceutically acceptable carrier may be in a variety of forms including capsules, compressed tablets, pills, solutions or suspensions. It is contemplated that

carriers be included which are suitable for administration orally, topically, by injection and by other selected means.

EXAMPLES

The following examples serve to provide further appreciation of the invention but are not meant in any way to restrict the effective scope of the invention.

EXAMPLE I

Twenty-four adult male Sprague-Dawley rats were weighed (body weight between 350-375 g) and injected through the tail vein with either 0.9% saline (non-diabetic controls, NDC) or with the same solution containing streptozotocin (75 g streptozotocin/kg body weight) to induce diabetes. The diabetic rats were distributed into five experimental groups (n=4 rats per group) and daily administered by oral gavage either 0, 1, 2, 5 or 10 mg of CMT-1 (4-dedimethylaminotetracycline, a chemically-modified non-antimicrobial tetracycline) suspended in 2% carboxymethylcellulose (CMC) for 21 days. The non-diabetic control rats were gavaged with the carrier alone (2% CMC). Urine glucose levels were measured weekly with Tes-Tape (Eli Lily, Inc., Indianapolis, IN).

On the 21st day, the rats were weighed and anesthetized with halothane (Halocarbon Laboratories) before blood samples were collected for serum glucose (Sigma glucose oxidase kit, St. Louis, MO) and CMT-1 determinations. The rats were then killed by exsanguination and the skin from the entire torso was dissected, weighed, and minced as described by Schneir et al., Diabetes 31:426 (1982).

Skin samples were examined for collagen solubility using the techniques described previously (Golub et al., J. Periodont. Res. 12:402 (1977), Golub et al., Biochim. Biophys. Acta 534:73 (1978)) with some modifications. Except where indicated, all steps were carried out at 4°C.

Briefly, for the preparation of neutral salt-soluble collagen, minced rat skin was extracted with Tris-HCl buffer (pH 7.4) for 2 days followed by centrifugation at 11,000 xg for 20 min. The pellet was subjected to an additional extraction for 18 hr and centrifuged as described above. Both supernatants were then combined and dialyzed exhaustively against 3% acetic acid for 2 days.

For the preparation of acid-soluble collagen, the pellet was further extracted with 3% acetic acid as described for the neutral salt extraction. The neutral salt and dilute acid extracts were dried with a rotatory evaporator at 60°C under vacuum.

For the preparation of insoluble collagen, distilled water was added to the remaining pellet, which was heated at 121°C for 15 min. under pressure and then vacuum dried as previously described. Following acid hydrolysis, the dried samples from the two extractions were used for neutral salt- and acid-soluble collagen determinations, while the pellet was used to determine the amount of insoluble collagen.

The extraction and HPLC determination of the concentration of CMT-1 in rat serum was carried out as described by Yu et al., Biochem. Med. & Metabolic Biol. 47:10-20 (1992).

The data were subject to statistical analysis. The

standard error of the mean (S.E.M) was calculated from the standard deviation. The statistical significance between the groups were determined by analysis of variance while the significance of differences between groups was calculated by Tukey's test.

RESULTS

As seen in Figure 1, three weeks after inducing diabetes with streptozotocin, the rats were severely hyperglycemic; the non-diabetic controls (NDC group) and the untreated diabetics (UD group) exhibited blood glucose levels of 95 mg/dL ± 3 (S.E.M.) and 787 mg/dL ± 10, respectively ($p<0.01$). Treating the diabetic rats with the different oral doses of CMT-1, ranging from 1-10 mg/day, did not significantly alter the severity of hyperglycemia ($p>0.05$).

Serum CMT-1 concentration was assayed using the HPLC technique described by Yu et al., Biochem. Med. & Metabolic Biol. 47: 10-20 (1992). The results demonstrate that increasing the dose of the drug, orally administered to the diabetic rats, increased the serum concentration for individual rats from 0.6 (for the 1 mg oral dose) to 6.5 μg/ml (for the 10 mg oral dose) (data not shown). Table I shows that the serum CMT-1 concentration for the different experimental groups was increased from a mean of 0.75 (for 1 mg oral dose) to 5.8 μg/ml (for the 10 mg oral dose).

TABLE I

The Oral Administration of Increasing Doses of CMT-1 to Streptozotocin-Diabetic Rats: Resulting Serum CMT-1 Concentrationsθ

Experimental Groups	Serum CMT-1 Conc. (μg/ml)
NDC	0±0
UD	0±0
D+1mg CMT/day	0.75±0.04
D+2mg CMT/day	1.75±0.05
D+5mg CMT/day	3.90±0.16
D+10mg CMT/day	5.80±0.25

θ Each value represents the mean of 4 rats per group ± S.E.M.

The data presented in Figures 2 and 3 shows the effect of diabetes and increasing oral doses of CMT-1 on the solubility of skin collagen in cold (4°C) dilute acid and neutral salt solutions respectively. As expected, the bulk of the collagen in the tissue (92%-96%) was insoluble in these solutions for both the NDC and diabetic rats (data not shown). Inducing diabetes reduced the solubility of skin collagen in neutral salt and dilute acid solutions by 52% and 56%, respectively ($p<0.01$). Note that the collagen solubility data was "normalized" to 100% for the salt-soluble and acid-soluble fractions in the skin of the non-diabetic control rats. Increasing the oral doses of CMT-1 administered to the diabetics progressively increased the abnormally low collagen solubility in neutral salt solutions by 10% (for

the 1 mg CMT/day dose) to 91% (for the 10 mg/day dose), and increased the low collagen solubility in dilute acid solutions by 18% (1 mg/day dose) to 115% (10 mg/day dose). However, only the highest oral doses of CMT-1 administered to the diabetics, 5 and 10 mg/day, resulted in collagen solubility values that were similar to the normal values observed in the non-diabetic control rats (Figures 2 and 3).

CMT-1 therapy "normalized" the quality of collagen in the skin of diabetic animals by inhibiting the excess crosslinking which characterizes collagen in diabetic connective tissues.

As shown in Table II, a similar effect was seen when the diabetic rats were orally administered a commercially-available antimicrobial tetracycline, called minocycline (CMT-1, 4-dedimethylaminotetracycline is an analog of tetracycline which has lost its antimicrobial efficacy). As described above, the bulk of the collagen in the skin of the normal rats was insoluble in neutral salt and dilute acid solutions (4°C); only 5.8% and 10.7% of the collagen in the skin could be solubilized in 1 M NaCl and 3% acetic acid, respectively. When the rats were made diabetic, collagen solubility was reduced, particularly in the dilute acid solutions ($p<0.05$) and the relative amount of insoluble collagen in skin was increased from a normal level of 83.5% to an abnormally high level of 88.8% in the diabetics ($p<0.05$). However, when the diabetics were treated with minocycline, the collagen solubility was returned to normal levels.

TABLE II

The Solubility of Skin Collagen in Neutral Salt and Dilute Acid Solutions (4°C) in Diabetic Rats: Effect of Oral Administration of Minocycline (Mino)θ

Experimental Groups	Collagen Fractions (%)		
	Salt-Soluble	Acid-Soluble	Insoluble
NDC	5.8 ± 0.3	10.7 ± 1.9	83.5 ± 1.6
UD	4.5 ± 0.6	6.7 ± 0.2*	88.8 ± 0.5*
D + 20 mg Mino/day	5.8 ± 1.3	12.2 ± 1.9	82.0 ± 3.2

θ Each value represents the mean of 3 rats per group ± S.E.M.

* Significantly different from other 2 groups ($p<0.05$).

EXAMPLE II

Three groups of rats were established: a non-diabetic control group, an untreated streptozotocin-diabetic group and a streptozotocin-diabetic group treated orally with 2 mg doxycycline/rat/day over the 14-week time period. At the end of the 14 weeks, blood samples were collected from each rat and the serum was measured for glycosylated protein using an assay which is based on the ability of glucose to non-enzymatically bind to proteins by a ketoamine crosslink to form fructosamine. During the assay, the ketoamine bond reduces a tetrazolium dye under alkaline conditions producing a color change which is measured spectrophotometrically. Armbruster, Clin. Chem., 33:2153-2163 (1987). A fructosamine assay kit is available from Isolab Inc., Akron, Ohio 44321.

In particular, the serum samples were centrifuged to remove lipids. The clarified serum samples were treated with a reagent to remove ascorbic acid and other

interfering substances. The treated serum samples were then incubated with the fructosamine-bicarbonate reagent (Tetrazolium salt). The reaction was stopped by the addition of HCl and absorbance was measured at a wavelength of 500 nm in a spectrophotometer. The data was analyzed statistically.

RESULTS

As seen in Table III, chronic diabetes (14-weeks duration) and chronic hyperglycemia significantly ($p<0.01$) increased the fructosamine level in serum proteins. These results provide evidence of non-enzymatic glycosylation, a reaction which is widely believed to be a major cause of many medical complications of long-term diabetes including pathologically excessive collagen crosslinking. Table III further shows that when the diabetics were treated on a daily basis with doxycycline, the fructosamine levels in the serum proteins of the diabetics were significantly ($p<0.05$) reduced. These results indicate that administration of a tetracycline (e.g. doxycycline) inhibits non-enzymatic glycosylation of proteins during diabetes mellitus. However, this treatment does not reduce the severity of hyperglycemia in diabetic rats. Golub et al., Res. Commun. Chem. Pathol. Pharmacol., 68:27-40 (1990).

TABLE III

Experimental Groups of rats	Serum Fructosamine Concentration (mean ± standard error)
Non-diabetic Controls	1.33 ± 0.05
Untreated Diabetics	3.82 ± 0.17
Doxycycline-treated Diabetics	3.23 ± 0.20

EXAMPLE III

Example III was carried out to determine the effect of tetracycline therapy on proteinuria, a parameter of renal damage, in diabetic rats.

Streptozotocin-diabetic rats were treated by the daily oral administration of 20 mg minocycline/rat/day over a 4-week period. At the end of the protocol, 24-hour urines were collected to measure protein excretion.

RESULTS

As shown in Figure 4, the excretion of protein in the urine increased from 48 mg/24 hr in the normal (non-diabetic) rats to 280 mg/24 hr in the untreated diabetics, a 483% increase. Surprisingly, the daily oral administration of minocycline completely prevented the development of proteinuria in the diabetic rats. According to Walton et al., Biochim. Biophys. Acta, 1138:172-183 (1992), the prevention of proteinuria further demonstrates the ability of the tetracyclines of the present invention to inhibit excessive collagen crosslinking. Recent experiments using a chemically modified non-antimicrobial tetracycline (CMT-1; 4-dedimethylaminotetracycline) also found a reduction in proteinuria in diabetic rats (data not shown). Golub et al., Matrix, suppl 31: 315-316 (1992).

Figure 4 shows that the minocycline-treatment resulted in urine protein excretion of 52 mg/24 hr. Neither treatment with minocycline nor CMT-1 reduced the other signs of diabetes including hyperglycemia, glucosuria and polyuria.

These experimental results, using both antimicrobial and non-antimicrobial forms of tetracycline, demonstrate

a powerful new medical key for preventing apparent accelerated aging or excessive crosslinking of collagen, an abnormality widely believed to be responsible for many of the serious complications of diabetes.

Thus, while there has been described what are presently believed to be the preferred embodiments of the invention, those skilled in the art will understand that other and further modifications can be made without departing from the spirit of the invention. It is intended that the present invention includes all such modifications as come within the true scope of the invention as set forth in the claims.

CLAIMS:

1. Use of an effective amount of a tetracycline sufficient to inhibit an excessive amount of collagen crosslinking to treat a mammal suffering from conditions associated with an excessive amount of collagen crosslinking, wherein the tetracycline is not effectively antimicrobial.

2. The use according to claim 1, wherein the excessive amount of collagen crosslinking is associated with diabetes, scleroderma and progeria.

3. The use according to claim 1 or 2, wherein the tetracycline is a dedimethylaminotetracycline.

4. The use according to claim 3, wherein the dedimethylaminotetracycline is selected from the group consisting of 4-dedimethylaminotetracycline, 4-dedimethylamino-5-oxytetracycline, 4-dedimethylamino-7-chlorotetracycline, 4-hydroxy-4-dedimethylaminotetracycline, 5a,6-anhydro-4-hydroxy-4-dedimethylaminotetracycline, 6α-deoxy-5-hydroxy-4-dedimethylaminotetracycline, 6-demethyl-6-deoxy-4-dedimethylaminotetracycline, 4-dedimethylamino-12a-deoxytetracycline and 4-dedimethylamino-11-hydroxy-12a-deoxytetracycline.

5. The use according to claim 1 or 2, wherein the tetracycline is selected from the group consisting of 6a-benzylthiomethylenetetracycline, the 2-nitrilo analogs of tetracycline, the mono-N-alkylated amide of tetracycline, 6-fluoro-6-demethyltetracycline, 11a-chlorotetracycline and 12a-deoxytetracycline and their derivatives.

6. The use according to any one of claims 1 to 5, wherein the tetracycline is in a form administrable in an

amount of from about 0.5 mg/kg per day to about 50.0 mg/kg per day.

7. The use according to claim 6, wherein the tetracycline is in a form administrable in an amount of from about 1.0 mg/kg per day to about 15.0 mg/kg per day.

8. A commercial package comprising a pharmaceutically effective amount of tetracycline sufficient to inhibit excessive amount of collagen crosslinking, wherein the tetracycline is not effectively antimicrobial, together with instructions for use thereof to treat a mammal suffering from conditions associated with an excessive amount of collagen crosslinking.

9. A commercial package according to claim 8, wherein the tetracycline is a dedimethylaminotetracycline.

10. A commercial package according to claim 9, wherein the dedimethylaminotetracycline is selected from the group consisting of 4-dedimethylaminotetracycline, 4-dedimethylamino-5-oxytetracycline, 4-dedimethylamino-7-chlorotetracycline, 4-hydroxy-4-dedimethylaminotetracycline, 5a,6-anhydro-4-hydroxy-4-dedimethylaminotetracycline, 6α-deoxy-5-hydroxy-4-dedimethylaminotetracycline, 6-demethyl-6-deoxy-4-dedimethylaminotetracycline, 4-dedimethylamino-12a-deoxytetracycline and 4-dedimethylamino-11-hydroxy-12a-deoxytetracycline.

11. A commercial package according to claim 8, wherein the tetracycline is selected from the group consisting of 6a-benzyl-thiomethylenetetracycline, the 2-nitrilo analogs of tetracycline, the mono-N-alkylated amide of tetracycline, 6-fluoro-6-demethyltetracycline, 11a-chlorotetracycline and 12a-deoxytetracycline and their derivatives.

12. A commercial package according to any one of claims 8 to 11, wherein the tetracycline is in a form administrable in an amount of from about 0.5 mg/kg per day to about 50.0 mg/kg per day.

13. A commercial package according to claim 12, wherein the tetracycline is in a form administrable in an amount of from 1.0 mg/kg per day to about 15.0 mg/kg per day.

14. A composition for treating a mammal suffering from conditions associated with an excessive amount of collagen crosslinking, said composition comprising an effective amount of a tetracycline sufficient to inhibit an excessive amount of collagen crosslinking, wherein the tetracycline is not effectively antimicrobial, and a pharmaceutically acceptable carrier.

15. The composition according to claim 14, wherein the excessive amount of collagen crosslinking is associated with diabetes, scleroderma and progeria.

16. The composition according to claim 14 or 15, wherein the tetracycline is a dedimethylaminotetracycline.

17. The composition according to claim 16, wherein the dedimethylaminotetracycline is selected from the group consisting of 4-dedimethylaminotetracycline, 4-dedimethylamino-5-oxytetracycline, 4-dedimethylamino-7-chlorotetracycline, 4-hydroxy-4-dedimethylaminotetracycline, 5a,6-anhydro-4-hydroxy-4-dedimethylaminotetracycline, 6α-deoxy-5-hydroxy-4-dedimethylaminotetracycline, 6-demethyl-6-deoxy-4-dedimethylaminotetracycline, 4-dedimethylamino-12a-deoxytetracycline and 4-dedimethylamino-11-hydroxy-12a-deoxytetracycline.

18. The composition according to claim 14 or 15, wherein the tetracycline is selected from the group consisting of 6a-benzylthiomethylenetetracycline, the 2-nitrilo analogs of tetracycline, the mono-N-alkylated amide of tetracycline, 6 fluoro-6-demethyltetracycline, 11a-chlorotetracycline and 12a-deoxytetracycline and their derivatives.

19. The composition according to any one of claims 14 to 18, wherein the tetracycline is in a form administrable in an amount of from about 0.5 mg/kg per day to about 50.0 mg/kg per day.

20. The composition according to claim 19, wherein the tetracycline is in a form administrable in an amount of from about 1.0 mg/kg per day to about 15.0 mg/kg per day.

21. The composition according to any one of claims 14 to 20 in the form of a capsule, compressed tablet, pill, solution or suspension.

22. Use of the composition according to any one of claims 14 to 21 for treating a mammal suffering from conditions associated with an excessive amount of collagen crosslinking.

23. Use of the composition according to any one of claims 14 to 20 for preparing a medicament for treating a mammal suffering from conditions associated with an excessive amount of collagen crosslinking.

24. A commercial package comprising a pharmaceutically effective amount of the composition according to any one of claims 14 to 21 together with instructions for use thereof to treat a mammal suffering from conditions associated with an excessive amount of collagen crosslinking.

25. Use of an effective amount of a tetracycline sufficient to inhibit an excessive amount of collagen crosslinking, wherein the tetracycline is not effectively antimicrobial, for preparing a medicament to treat a mammal suffering from conditions associated with an excessive amount of collagen crosslinking.

26. The use according to claim 25, wherein the excessive amount of collagen crosslinking is associated with diabetes, scleroderma and progeria.

27. The use according to claim 25 or 26, wherein the tetracycline is a dedimethylaminotetracycline.

28. The use according to claim 27, wherein the dedimethylaminotetracycline is selected from the group consisting of 4-dedimethylaminotetracycline, 4-dedimethylamino-5-oxytetracycline, 4-dedimethylamino-7-chlorotetracycline, 4-hydroxy-4-dedimethylaminotetracycline, 5a,6-anhydro-4-hydroxy-4-dedimethylaminotetracycline, 6α-deoxy-5-hydroxy-4-dedimethylaminotetracycline, 6-demethyl-6-deoxy-4-dedimethylaminotetracycline, 4-dedimethylamino-12a-deoxytetracycline and 4-dedimethylamino-11-hydroxy-12a-deoxytetracycline.

29. The use according to claim 25 or 26, wherein the tetracycline is selected from the group consisting of 6a-benzylthiomethylenetetracycline, the 2-nitrilo analogs of tetracycline, the mono-N-alkylated amide of tetracycline, 6-fluoro-6-demethyltetracycline, 11a-chlorotetracycline and 12a-deoxytetracycline and their derivatives.

30. The use according to any one of claims 25 to 29, wherein the tetracycline is in a form administrable in an amount of from about 0.5 mg/kg per day to about 50.0 mg/kg per day.

31. The use according to claim 30, wherein the tetracycline is in a form administrable in an amount of from about 1.0 mg/kg per day to about 15.0 mg/kg per day.

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Figure 1

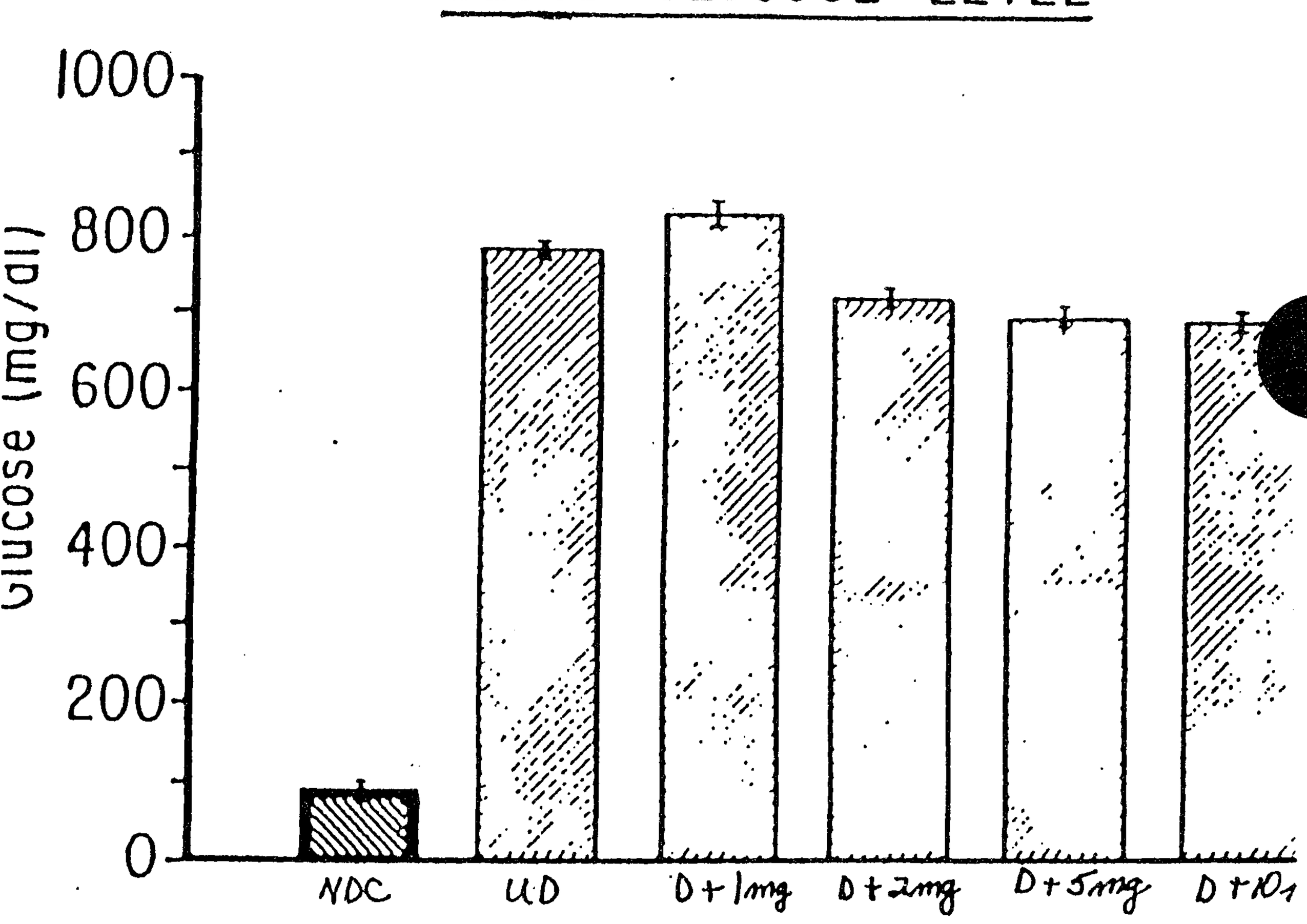

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Figure 2

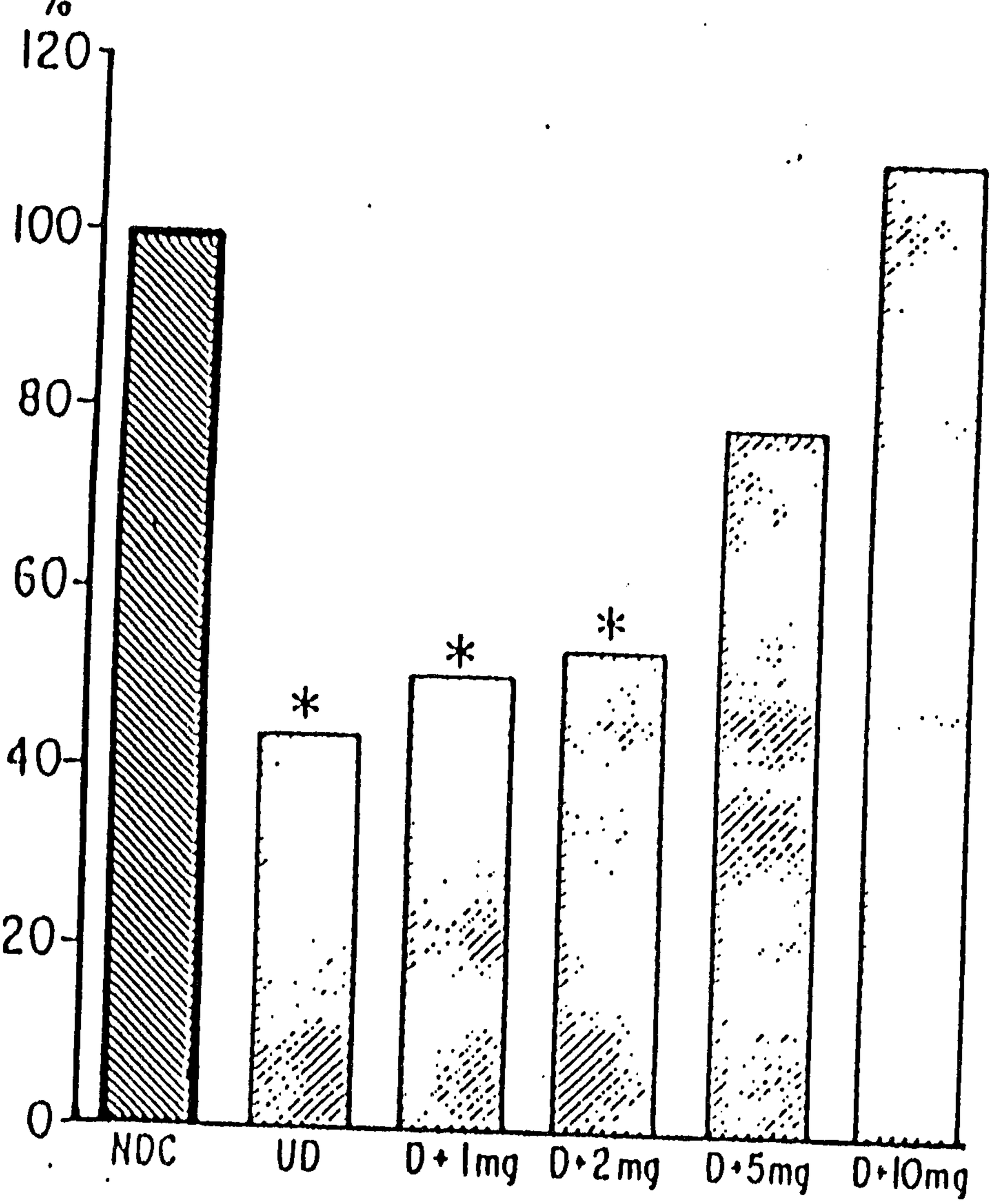

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Figure 3

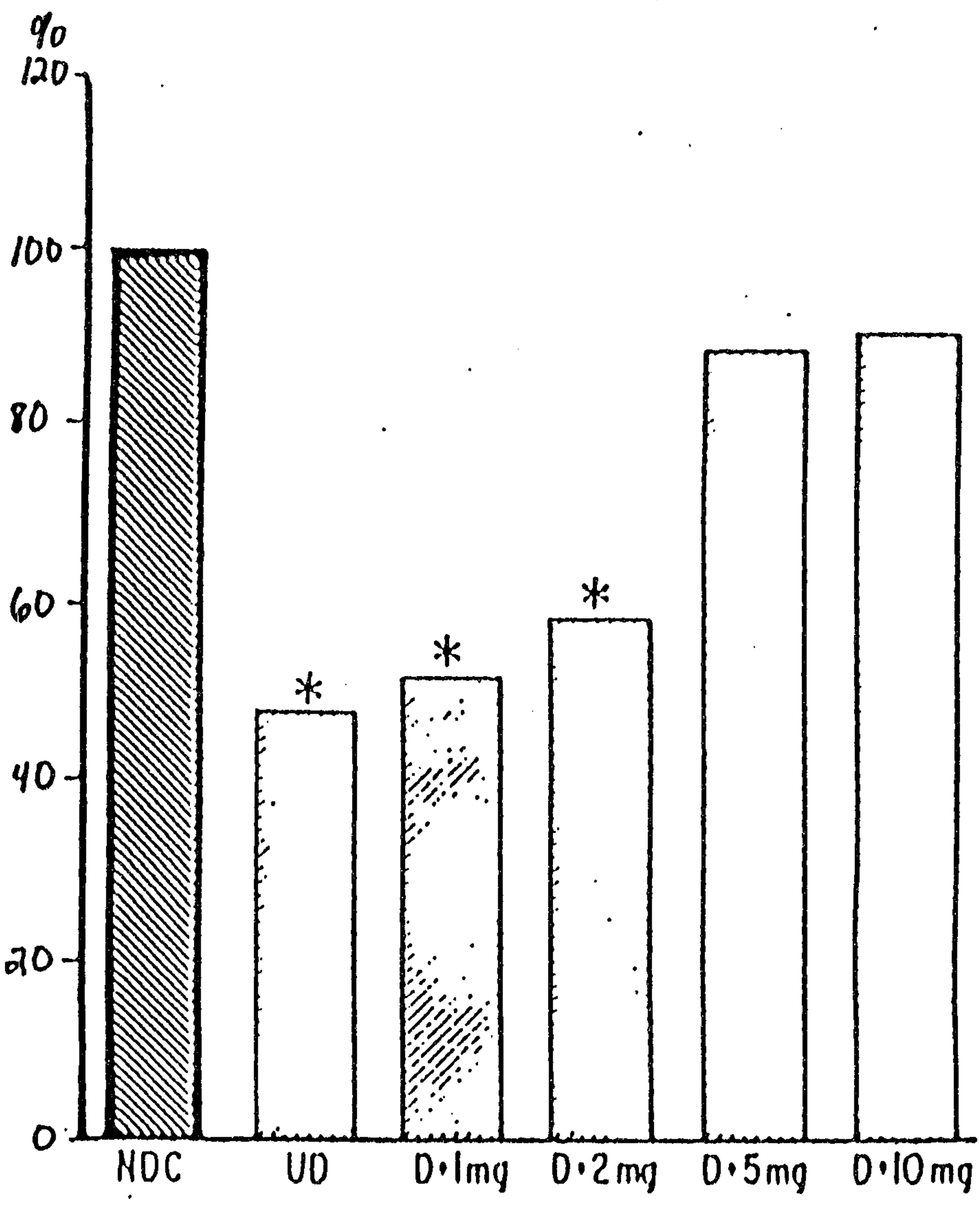

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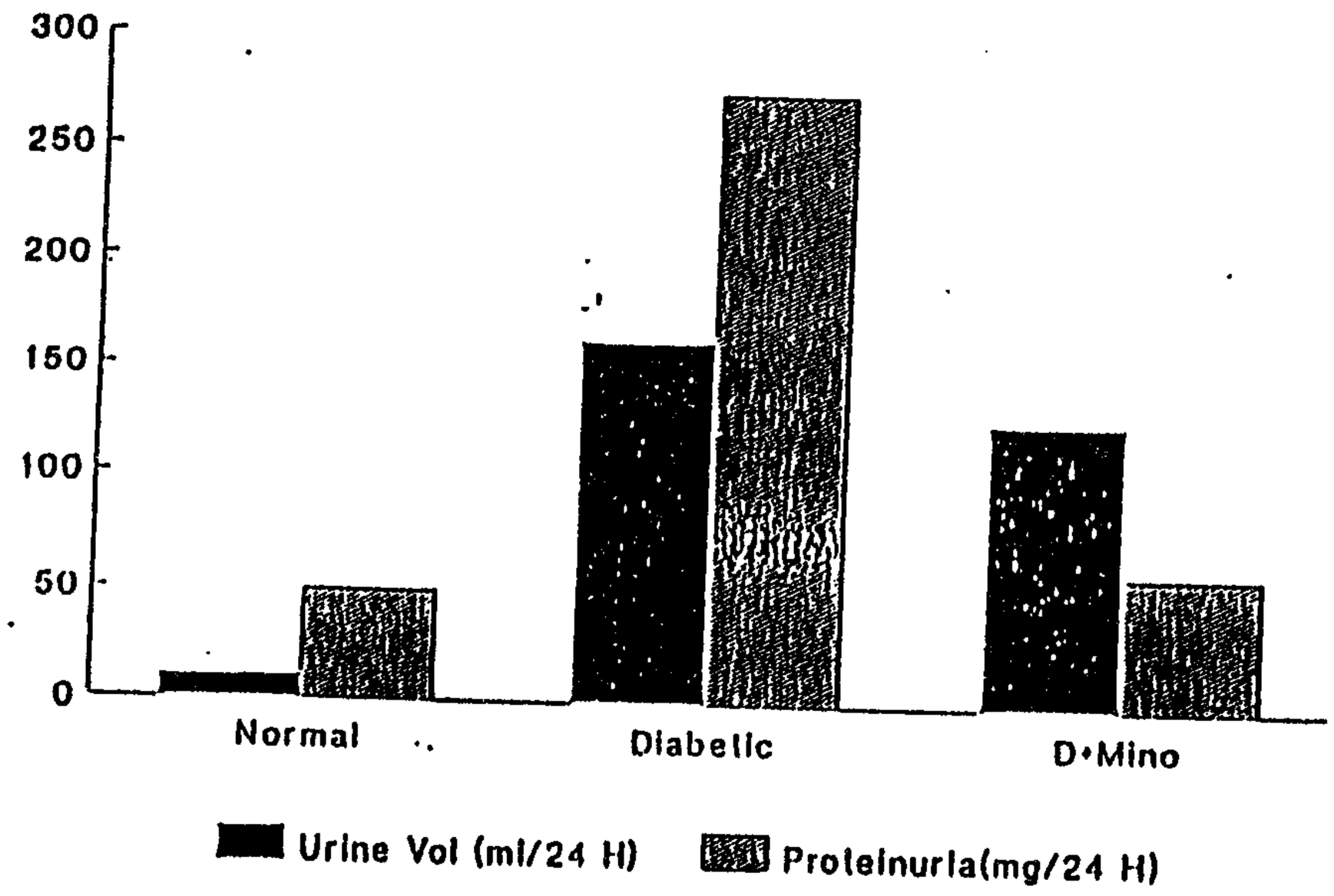
Figure 4



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