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Studies on the Effect of Antidiabetic Drugs on Collagen in Rats

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Abstract: To assess the effects of antidiabetic drugs on collagen, the study has been carried out. However, at present, no conclusive data available on the *In vivo* effect of antidiabetic drugs in collagen. It was therefore thought worthwhile to undertake a systemic study on the metabolic turnover of connective tissue proteins, particularly collagen which will help to understand the various changes taking place in conventional antidiabetic drug treated rats. The hydroxyproline content was determined by the method of Neumann and Logan. There was no significant difference in mean body weight of animals in treated groups when compared to control animals on 50th day. In drug treated group G5, urinary excretion of hydroxyproline was slightly increased in contrast to that of the rats in other groups and showed a significant difference ($p<0.001$) between control and G5 group. The proportion of soluble collagen in treated group was somewhat higher than in the control but while taking the significance value there are no statistically significant changes. Liver showed the highest concentration of 0.98 g collagen/100 g tissue, whereas kidney and heart showed 0.57 and 0.72 g collagen/100 g tissue. Observations of the present study collectively indicate that the anabolic rate of collagen metabolism is not altered significantly by the administration of antidiabetic drugs in normal rats.

Key words: Collagen, hydroxyproline, antidiabetic drugs, urinary excretion, skin

INTRODUCTION

The cost of treating diabetes involve not just the therapies used to lower blood glucose concentrations but also measures for preventing and/or treating diabetes related complications. Along with the improved glycemic control there is a need for reducing the risk of long term complications. Both frequencies of dosing and number of tablets consumed by each patient during their life time affect adherence to diabetic regimens (Paes *et al*., 1997). Collagen is one of the important proteins which can be nonenzymatically glycosylated under the conditions presented by diabetes. Two metabolic pathways are thought to underlie the pathogenesis. The first is known as the Maillard reaction in which sugars react with protein amino groups and form Schiff base mediated adducts that rearrange into stable glycoconjugates. The subsequent transformation is known as the Amadori rearrangement and leads to stable keto- amine-linked-1-deoxyhexose. Salicylates, a commonly used drug have also been found to inhibit glycosylation (Yue *et al*., 1984). Investigations of the effect of insulin on collagen metabolism have been mostly focused on clinical and experimental diabetes mellitus (Bonadonna *et al*., 1965). Urinary excretion of hydroxyproline was found to be normal in most patients with diabetes mellitus (Benoit *et al*., 1963; Kivirikko, 1970). Increased values were found in insulin dependent diabetes (Mani and Mani, 1986). On the other hand, a decrease in the urinary excretion of hydroxylysylglycosides was found in patients with diabetes (Sato *et al*., 1980). Microangiopathy in diabetic patients is due to elevated serum level of an aminopropeptide of type III procollagen and is a good noninvasive marker for measuring vascular changes in patients with diabetes mellitus (Okazaki *et al*., 1988). In diabetes patients, an increase in the serum level of 7S collagen, the cross linking domain of type IV collagen was reported. In such cases, the amount of collagen per square unit of the skin is significantly enhanced, while the concentration of collagen, expressed as amount per unit of weight of dry skin, is unaltered (Perejda, 1987). The diameter of the collagen fibres in the skin remains unaltered. An increase in hexose bound nonenzymatically to collagen was found in diabetic scelrosis when compared with normal control. A major matrix alteration in diabetes mellitus is thickening of the basement membrane and is associated with cardiovascular, renal, ocular, skeletal and neuropathic complications. Thickening of the basement membrane is known to progress with the duration of diabetes and correlates with proteinuria and a

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reduced glomerular filtration rate and is mainly due to type IV collagen accumulation. The mechanism of this phenomenon is not fully understood. Streptozotocin induced diabetes treated with insulin was associated with a new expression of type III collagen (Abrass *et al*., 1988). The effect of antidiabetic drugs has so far been studied only from clinical, biochemical and haematological points of view. It was therefore thought worthwhile to undertake a systemic study on the metabolic turnover of connective tissue proteins, particularly collagen which will help to understand the various changes taking place in antidiabetic drug treated rats. In the present study, an investigation on the effect of commercial antidiabetic drugs such as Repaglinide, Pioglitazone, Glipizide and Metformin on collagen has been carried out using experimental animal.

MATERIALS AND METHODS

Wistar female albino rats (120-150 g) used for this study were procured from King Institute, Guindy, Chennai, India and housed in the Institutional animal house under standard environmental conditions (23±1°C, 55±5% humidity and 12 hours/12 hours light/dark cycle) and maintained with free access to standard diet (Hindustan Lever Ltd, Bangalore, India) and water *ad libitum*. The protocol of the animal study was approved by Institutional Animal Ethics Committee (IAEC 03/003/08). After acclimatization, animals were divided into five groups, each group containing 6 animals. In this study, wistar albino rats (four groups) were exposed to repeated oral dose administration of drugs like Repaglinide, Pioglitazone, Glipizide and Metformin for 50 days following Organization for Economic Cooperation and Development (OECD) test guideline 425, by applying Good Laboratory Practice. One group served as control. Each of the four drugs was tested at the highest dose. Body weights were taken every week. At the end of each week, urine samples were collected in flask containing toluene for 24 h following gastric loading with 3 mL of saline/100 g b.wt. of rat, on keeping the animals in individual metabolic cages. The food and water were analyzed and are considered not to contain any contaminants that could reasonably be expected to affect the purpose or integrity of the study. On the 50th day of drug administration all the animals were sacrificed by euthanasia method and all the organs were collected, observed for gross pathology and immediately stored at -80°C. Skin and tail from all the animals were collected and stored appropriately.

Body weight: The mean body weight of all the animals was taken from the start of the study, weekly and on the final day of sacrifice. Observations of animals were made after dosing during the first day and daily thereafter for 50 days. The time onset, intensity and duration of symptoms, if any, were recorded. Appearance of skin, fur and eyes were observed during these days.

Hydrolysis for urine, tissues: One milliliter urine was hydrolyzed with concentrated HCl in a sealed tube for 24 h and the standard procedure of Neumann and Logan was followed. Aliquots of the tissues were cut into small pieces, dehydrated in acetone, defatted with light petroleum ether and air dried. Defatted tissue samples were hydrolyzed in a sealed tube with 6N HCl for 24 h at 110°C. The hydroxyproline content was determined by the method of Neuman and Logan (1950).

Analysis of skin and tail collagen: Skin samples were hydrolyzed in a sealed tube with equal quantity of 12 N HCl for 24 h at 110°C and analyzed for total hydroxyproline content.

Soak the tail in 70% ethanol to remove debris. Tendons were separated using forceps and collected in Phosphate Buffer Saline (PBS) and washed thrice. Then the tendons were dissolved by adding 0.2% acetic acid and stirred for 48 h at 4°C. The state of collagen solution was checked periodically, if too viscous more amount of 0.2% acetic acid solution is added. The resulting viscous solution was centrifuged at 4°C, resulting supernatant is the collagen stock solution and dialyzed against 0.02 M Na_2HPO_4, followed by exhaustive dialysis against 0.05 M acetic acid and then freeze dried. The total collagen content was then calculated in each case by multiplying the hydroxyproline content by a factor 7.46 (Neuman and Logan, 1950).

Fractionation of collagen

Total collagen: The total collagen content was determined by estimating the amount of hydroxyproline which is a characteristic aminoacid of collagen.

Neutral salt soluble collagen: The neutral salt soluble collagen was extracted as described by Levene and Gross (1959). The tissues were cut into small fragment and homogenized and were extracted thrice with 0.45 M NaCl (pH 7.4) at 4°C for 24 h with one drop of octan-2-ol added as a preservative. The combined extract was centrifuged for 1 h at 20,000×g in a refrigerated centrifuge. An aliquot of the supernatant was hydrolyzed with an equal volume of 6N HCl and the collagen content and hydroxyproline were determined.

Insoluble collagen: The residue left after 0.45 M NaCl extraction, consisting of insoluble collagen was analyzed for collagen content.

RESULTS

Body weight and observation of animals: No morbidity/mortality was observed in experimental rats. None of the animals exhibited any clinical signs of toxicity. No adverse effects were seen on the body weight. However, mean body weight of animals showed significant difference on 50th day when compared with the first day of treatment. There was no significant difference in mean body weight of animals in treated groups when compared with the mean body weight of animals in G1 on 50th day (Fig. 1). No changes were observed in feed and water consumption. Behavioral and all other observational parameters remained normal in all experimental rats.

Urinary hydroxyproline: Rats subjected to treatment with antidiabetic drugs for 50 days showed no extreme marked changes in the urinary hydroxyproline excretion when compared with control group except G5. The data in the Fig. 2 represents the quantitative changes in the urinary excretion of hydroxyproline levels in both normal and treated rats. At the beginning of the experiment, the mean excretion of urinary hydroxyproline was approximately about 250 μg 24 h in all the groups. In the drug treated group G5, urinary excretion of hydroxyproline was slightly increased in contrast to that of the rats in other groups and showed a significant difference ($p<0.001$)between control and G5 group.

Skin collagen: The values for the control group are the mean of the values obtained from the analyses of six individual skins (Fig. 3). The weight of the skin was that of shaved and scraped skin. As would be expected with animals, the drug treated group showed no significant difference in the collagen content as compared with the control group. The proportion of soluble collagen in treated group was somewhat higher than in the control but while taking the significance value there are no statistically significant changes. Total collagen concentration in the control and treated groups are shown in the Fig. 3. The result of neutral salt soluble collagen and insoluble collagen are shown in the Fig. 4 and 5.

Tissue hydroxyproline: Figure 6 shows the mean concentration of collagen in various tissues. In control group as well as in treated group, liver had the highest concentration of hydroxyproline. But the variation between the concentration of hydroxyproline in liver and other tissues were not significant. Liver showed the highest concentration of 0.98 g collagen/100 g tissue, whereas kidney and heart showed 0.57 and 0.72 g collagen/100 g tissue.

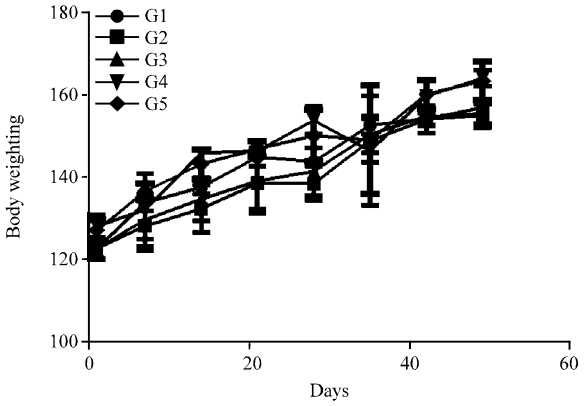

Fig. 1: Changes in the body weight gain of all the drug treated groups compared with control group. Values are expressed in Mean±SD. G1-Control, G2-Repaglinide, G3-Pioglitazone, G4-Glipizide, G5-Metformin treated groups. No significant differences between control and drug treated groups on day 50

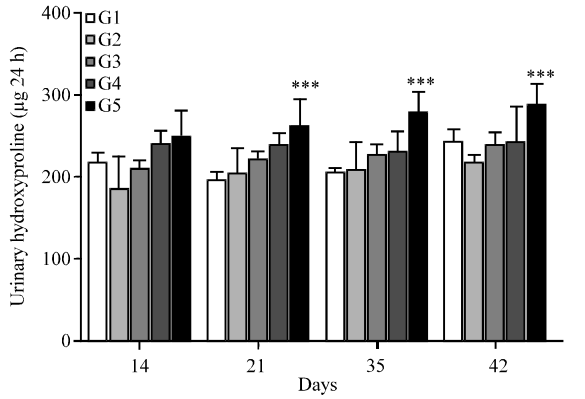

Fig. 2: Changes in the urinary hydroxyproline excretion of all the drug treated groups compared with control. Values are expressed in Mean±SD. G1-Control, G2-Repaglinide, G3-Pioglitazone, G4-Glipizide, G5-Metformin treated groups. No significant differences between control and drug treated groups. Dunnet comparison test between G1 and G5 showed significantly different ($p<0.001$) on 42nd day

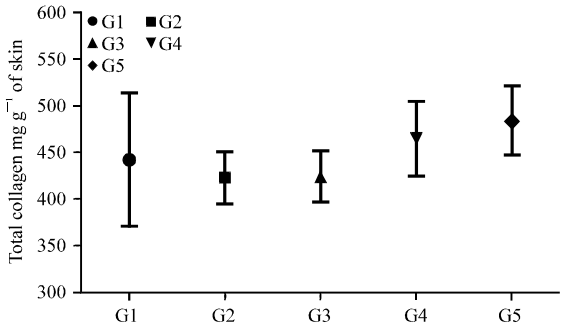

Fig. 3: Total collagen concentration of all the drug treated groups compared with control. Values are expressed in Mean±SD. G1-Control, G2-Repaglinide, G3-Pioglitazone, G4-Glipizide, G5-Metformin treated groups. No significant differences between control and drug treated groups

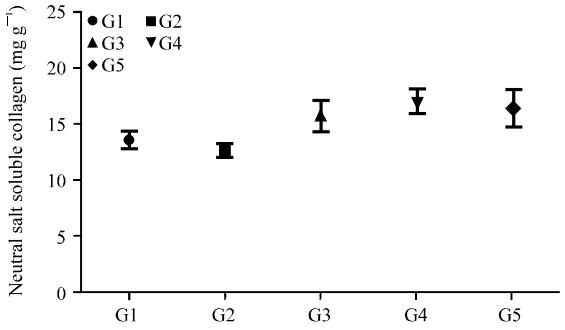

Fig. 4: Neutral salt soluble collagen concentration of all the drug treated groups compared with control. Values are expressed in Mean±SD. No. significant differences between control and drug treated groups

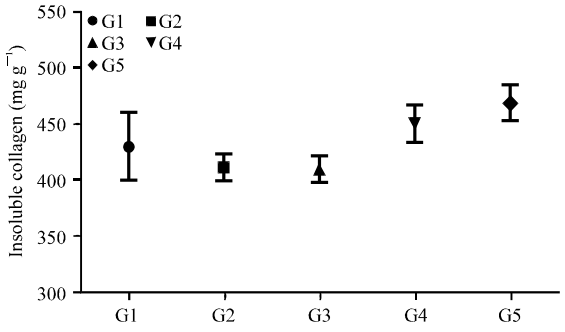

Fig. 5: Insoluble collagen concentrations of all the drug treated groups compared with control. Values are expressed in Mean±SD. No. significant differences between control and drug treated groups

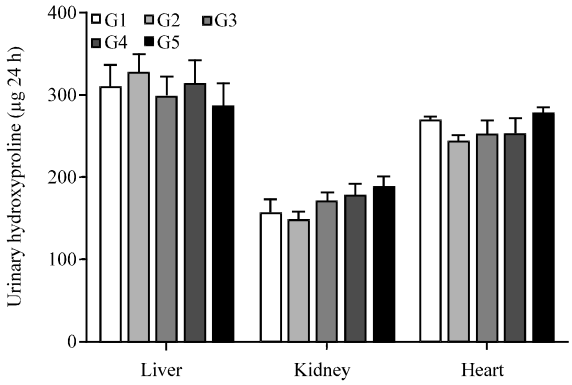

Fig. 6: Changes in the hydroxyproline content of all drug treated groups compared with control. Values are expressed in Mean±SD. G1-Control, G2-Repaglinide, G3-Pioglitazone, G4- Glipizide, G5-Metformin treated groups. No significant differences between control and drug treated groups

DISCUSSION

In the present study, the animals treated with Metformin 2000 mg kg^{-1} b.wt. did not show any prominent changes in body weight. But reports showed that the patients receiving sulfonylurea therapy for diabetes and those who receive Metformin generally maintain or lose body weight, with loss of adipose tissue reason for most of the weight loss. Clinical studies reported that Metformin, stabilizes or reduces body weight over the short term and after years of follow up Moreover an increase in body weight with sulfonylurea treatment may be lessened or avoided by the addition of Metformin. A 5% reduction in bodyweight can be achieved by those patients who are all under metformin therapy. The present results showed no changes in body weight of rats. Feed and water consumption also remained normal in all experimental rats and there is no significant changes observed. No combination therapy has been carried out in this study and hence the result may show difference when compared with other reports. Other researchers reported a loss in bodyweight with Metformin monotherapy whereas Repaglinide plus Metformin and Repaglinide monotherapy leads to respective weight gains (Julio *et al*., 2004). In the present study, body weight did not differ significantly between groups during the treatment of sulphonylurea. The results obtained in the present study support the previous claim and reveals that these drugs at maximum concentrations did not appear to retard growth or affect food consumption and all experimental animals remained healthy throughout the

study period. The feed consumption of the different groups followed a similar pattern indicating normal metabolism. This finding indicates that the feed intake and utilization of proteins and other nutrients were not affected by the intake of drugs. But the claim of reduction in body weight produced by Metformin due to anorectic effect is doubtful because there was no reduction in food consumption observed during the present study. reduction in body weight is a simple and responsible indicator of toxicity after exposure to toxic substances (Raza *et al*., 2002).

The therapeutic effect of antidiabetic drugs, in experimental animals and humans involving administration of larger amounts, life long, has been well documented and reviewed. However, the effect of such high dose for lifelong therapy on the biochemical process, particularly on skin, tendons and lysosomal enzyme activities has received scant attention. Earlier some authors had claimed significant and no significant changes in enzyme level after administration of some drugs and it's not proved (Hanefeld *et al*., 2001). There is a critical opinion among researchers that many of the vascular consequences of diabetes may arise even under drug therapy (Haller, 1997; Lefebvre and Scheen, 1998). So, it is useful to find out whether the intake of antidiabetic drugs for management of diabetes, plays any role in diabetic complications or not.

Therefore, the effect of antidiabetic drugs on skin collagen and other tissues which constitute the absorption site and action site comparable to other action has been exposed. Hence, it was felt worthwhile to investigate whether the conclusion derived from other studies also applies to our protocol and to find out the extent as well as the nature of changes in the collagen of wistar albino rats-due to 50 days administration of antidiabetic drugs.

Collagen constitutes 60-80% of the dry weight of fat free skin, the major dermal constituent (Hamlin *et al*., 1975; Schnider and Kohn, 1981). Some changes on collagen appear to be accelerated in diabetes. Age related change of collagen cross linking was increased in diabetes (Haller, 1997; Gerstein, 1998).

Tetracycline, a drug which produces effect on bones is well documented, but incidentally it was found that the drug showed some changes on mechanical properties on skin. This finding focused attention on collagen and then healthy rats were treated with oxytetracycline for 14 days. At the end of the study, the soluble fraction of collagen was elevated in skin and bones (Engesaeter and Skar, 1978). In the present study, after 50 days of drug administration, no significant differences in collagen solubility between drug treated and control group rats were found. The amount of collagen in skin was not affected by treatment. In rats receiving drugs, increased serum levels of hydroxyproline were reported. The possible role of this in the therapeutic properties of the drug remains unknown. Collagen abnormalities are shown to accompany pathophysiological changes of almost all organs of the body. The indices are classified for practical purposes into the following groups: (1) Measured in body fluids (2) Determined in tissue samples (3) Measured in *In vitro* cultured cells or tissues. Serum and urine are the biological materials most commonly used in clinical practice. The activity of serum enzymes as related to collagen degradation and their inhibitors has been shown to alter in patients with abnormalities in collagen metabolism. Liver function is suggested to affect the level of enzymes and inhibitors. It is significant that almost all serum and urine markers of collagen metabolism have been found to be related to the age of the individual. Thus, it is difficult to elaborate creditable normal values for the healthy population.

Hydroxyproline(Hyp), is an amino acid found in the tissues almost exclusively in collagen is synthesized by hydroxylation of proline a precursor of collagen viz., protocollagen (Prockop and Kivirikko, 1967). An increase in the urinary excretion of hydroxyproline has been reported in growing children and in many pathological conditions (Gerstein, 1998). Other condition that may be accompanied with altered hydroxyproline values include certain skin diseases, thermal burns, some condition associated with changes in hormonal levels and administration of certain drugs. In the present study, no significant difference was found between the study groups indicating that the end products of collagen metabolism are the same in both. Small local changes in connective tissue usually do not cause significant changes in hydroxyproline excretion. Since the hydroxyproline levels in urine indicate the index of collagen degradation *in vivo*, the amount of hydroxyproline specimens of normal and treated rat's urine sample indicates the toxicity of drug to collagen- In the present study, hydroxyproline values of the urine, skin, tail and tissue, indicate the amount of total collagen present in these tissues without any significant alterations. The result gives valuable information about the effect of antidiabetic drugs on the metabolic rate of collagen synthesis and degradation *in vivo* and it clearly demonstrate that the amount of hydroxyproline in skin, tail, urine and organs was not affected and hence the antidiabetic drugs have no effect on collagen and they are safe to use for longer terms. Overall, the observations collectively indicate that the anabolic rate of collagen metabolism is not altered.

While investigating the effect of antidiabetic drugs on collagen and its metabolism, the study concludes that the effect of antidiabetic drugs in normal rats not producing much changes in the collagen but further studies in future has to be carried out in diabetic rats, because pathological condition may change the effect of drug and its action.

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