Histopathological Studies in Long-term Tolbutamide-administered Alloxan Diabetes Rabbits

Setsuya TAKEUCHI* Tadao OKAMURA* Kazuhiro SHICHINOHE*
and Kazumasa NAKAMA*

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* Department of Pharmacology, Nippon Medical School, Tokyo

Effect of long term administration of tolbutamide was histopathologically studied in 36 male alloxan diabetes rabbits. After the continuous tolbutamide administration for 3~6 months, the heart and kidney were light- and electron-microscopically examined. Severe focal degenerative lesions, consisting of damages in small blood vessel and cardiac muscle, were found in the heart from the alloxan rabbits, and not a few exudative lesions and sclerotic changes of nodular or diffuse forms were seen in their renal glomeruli. From the tolbutamide-administered alloxan rabbits, qualitatively similar findings to the above were obtained, but there were many severe cases in this group. Especially, the incidence of severe cardiac lesions significantly increased in the rabbits which received tolbutamide for 6 months.

It was suggested that tolbutamide might indirectly act on the diabetic process in the alloxan rabbits.

Key Words : Alloxan diabetes, Tolbutamide

Since the publications of University Group Diabetes Program (UGDP) in 1970 and 1975 (Karam et al.), the results of chronic toxicity tests for tolbutamide in the experimental diabetic animals have been reported by Nakama (1977) and Takeuchi et al. (1978). They have suggested in their reports the presence of an unknown mechanism of toxic action of tolbutamide, which has never been found in the experiments with the normal experimental animals. Nakama, who studied in mongolian gerbils, took note of three possibilities that tolbu-

tamide (a) might accelerate essentially diabetic process, (b) might indirectly act as an unfavorable substance to diabetes, or (c) might stimulate the microangiopathic process, which had previously been started by their genes. On the other hand, Takeuchi et al., who studied alloxan rabbits, reported that long term administration of tolbutamide produced significant increase in the incidence of abnormal ECG patterns. The present communication is concerned with the histopathological examination on kidney and heart of the alloxan diabetes rabbits,

which was performed for the object of elucidating the mode of tolbutamide action and the essential nature of pathological process in the alloxan diabetes.

Materials and Methods

Thirty six male alloxan rabbits used in the present experiment were same as those which had previously been experimented by Takeuchi et al. (1978), and the details had been described in their reports. They were divided into three groups: control, and two tolbutamide groups, given 100 mg/kg and 200 mg/kg, respectively. After the feeding for 3~6 months, the animals were sacrificed by exsanguination, and the heart and kidney were removed for the light- and electron-microscopic examinations.

For the electron microscopic examination the isolated tissues were prefixed in 2% glutar-aldehyde and postfixed in 1% osmium tetroxide, buffered with phosphate (pH 7.4). Furthermore, they were dehydrated in a graded series of ethanol and embedded in epon. Thin sections were cut with the Porter Blum (MT-1) ultramicrotome and stained with uranyl acetate and lead hydroxide. Photographs were taken with an electron microscope (HITACHI 12-A).

For the light microscopic examination the tissues were fixed in Helly's solution or 20% formalin, embedded in paraffin and stained with Hematoxylin and Eosin (HE), Periodic-Acid Methenamine Silver (PAM), Periodic-Acid-Schiff (PAS) or Azan.

Results

Findings in heart: Main pathological findings in light-microscopic examination could be arranged into two series; one comprised of vascular changes and the other, the myocardial alterations. As shown in a representative case

Photo. 1 Endothelial cell swelling, medial fibrosis and perivascular fibrosis in a small artery of cardiac muscle, which were graded to "moderate change". No. 14, Azan stain $\times 280$

Photo. 2 Obliterations in small arteries of heart, which were graded to "severe change". No. 146, Azan stain $\times 560$

in Photo. 1, there were found some typical initial changes in and/or on the relatively small blood vessels. And they consisted of endothelial cell swelling, medial edema and fibrosis in the walls, which eventually resulted in luminal narrowing and obliteration. At the same time, the alterations developed in perivascular space, that is, perivascular edema and fibrosis, a part of which gradually invaded intermuscular space. Photo. 2, shows a complete obliteration in small arteries, the consequence of which would

Photo. 3 Electron microphotograph of myocardium from the alloxan rabbit (No. 14). Increases of pinocytotic vesicles (V) in endothelial cell of a capillary (Cp), and collagen fibrils (Cf) in perivascular edema (Ed). There are also observable disorderly intercalated-disc (Id) and mitochondria edema. Uranyl acetate and lead hydroxide stain

Photo. 4 Electron microphotograph of precapillary arterioles (CP) in myocardium (No. 14). Endothelial cell (EN) swelling, increase in the population of pericytes, perivascular edema and a lytic process in myofibrils (arrow). MC : medial cell. Uranyl acetate and lead hydroxide stain

be the focal degeneration in cardiac muscle.

The photographs obtained with electron microscope supported the above observation. As shown clearly in Photo. 3, swelling of endothelial cells associated with increase of pinocy-

Photo. 5 Electron microphotograph of myocardium (No. 14). Stasis in capillary, fibrotic process in perivascular space, a part of which penetrates into interstitium of myocytes (arrows). Uranyl acetate and lead hydroxide stain

totic vesicles in capillary wall and perivascular edema were found as the preceding processes, and even when some collagen fibrils began to increase, the microstructure in cardiac muscle cells still displayed only slight alterations such as disorderly intercalated-discs and edema in mitochondria. Photo. 4, illustrates the changes of capillary wall in cardiac muscle, that is, endothelial cell swelling, increase in the population of pericytes and perivascular edema. And a lytic process was also observable in peripheral area, where cardiac muscle cell bordered on the perivascular space. Other representative changes are given in Photo. 5. There are seen the stasis in capillary, the fibrotic process in perivascular space which have penetrated into interstitium of myofibrils and tremendous increase of mitochondria. Consequently, the detectable changes in cardiac muscle from the alloxan rabbits—focal myopathy consisting of lysis or degeneration of myofibril, disappearance of Z-band and waxy degeneration—were considered to be due to the angiopathy above mentioned.

Photo. 6 Hyalin substance depositions at glomerular axis and endothelial cell swelling in afferent vessel (Fig. B.). "moderate change" Fig. A : No. 38, Fig. B : No. 146. PAM stain $\times 560$

Photo. 7 Axial fibrosis, thickening of basement membrane in Bowman's capsule and endothelial cell swelling in afferent vessel of an another glomerulus (Fig. B). "severe change" Fig. A : No. 38, Fig. B : No. 157, PAM stain $\times 560$

Findings in kidney : Swelling of basement membrane of Bowman's capsule, interstitial edema, fibrosis, round-cell infiltration and glomerular sclerosis and luminal narrowing in blood vessel, and perivascular fibrosis were commonly found in the renal preparations from the alloxan rabbits. The pathological findings in glomeruli were very characteristic, and the representative cases are shown in Photo. 6 and 7. There were deposition of hyalin substance at the glomerular axis and endothelial cell swelling in the afferent vessel accompanied with swelling of basement membrane in Bowman's capsule. The case shown in Photo. 7-A displays

the so-called diffuse sclerosis, and the case in Photo. 7-B was glomerular fibrosis. Photo. 8 illustrates the ultramicrostructure of glomeruli from alloxan diabetes rabbit's kidney, showing endothelial cell swelling, epithelial cell degeneration, cytoplasmic fatty degeneration and disarrangement of the endothelial pores. The photograph clearly supports the fact that the changes belong to typical diffuse sclerosis. As shown in Photo. 9, ultramicrostructure of the hyalin substance depositions, where can be seen considerable swelling of the endothelial cells of the capillary loop in glomerulus and the increase of the mesangial matrix as well as ir-

Photo. 8 Electron microphotograph of the renal glomerulus (No. 44). Endothelial cell swelling, epithelial cell degeneration (arrows ↓) in capillaries, increase of fatty droplets in mesangial cell (MC) and disarrangement of the endothelial pores (arrows ↑). Uranyl acetate and lead hydroxide stain

Photo. 9 Electron microphotograph of an area of mesangial matrix for the hyalin substance deposition showing accumulation of microfibrils (mf, arrows). No. 146 Uranyl acetate and lead hydroxide stain

regular thickening of the basement membrane owing to accumulation of microfibrils, indicate that the glomerular change can be named nodular-exudative sclerotic lesions. These findings in the alloxan rabbit's kidney were often accompanied by inflammation in the interstitial tissue, that is, round-cell infiltration or inter-

stitial edema. They might be closely related with the pyelonephritis, which were occasionally observed as the complication in this experiment.

Table 1 summarizes the histopathological findings arranged in the order of incidences in each individuals. Symbol + indicates "moderate change" and ++ "severe change (showing large number or area of alterations and severe pathological process, for example, blood vessel obliteration, myofibrosis or waxy degeneration in the heart, and nodular or diffuse glomerulosclerosis, or glomerulofibrosis)". As shown in Table 2, the result from chi-square test for the distribution probability of the case of severe change revealed a significant increase of the incidence of the positive cardiac findings in the tolbutamide-administered alloxan rabbits (6 months), while the differences in the probabilities were within the insignificant range in their renal findings. And, furthermore, the variances due to dose-levels or durations of administration were found to be insignificant.

Discussion

Since it was reported by Dunn et al. in 1943 that alloxan injected into rabbits produced the pancreatic β -cell necrosis and diabetes-like syndromes such as glycosuria and hyperglycemia, the alloxan diabetes has been recommended as one of the models for human diabetes (DM). It has, however, been well known that the experimental diabetes manifests not a few different characters from those of DM; the former's insulin-requirement for keeping normoglycemia is larger (Thorogood et al., 1945), sulfonyleurea-resistance is higher (Gordon et al., 1957), development of diabetic complication is slower (Janes, 1959) and so on. Consequently, one must be subtly cautious in deciding whether

Table 1 Summary of the Histopathological Findings on Heart and Kidney of Alloxan Rabbits

Group	Months	No. of animals	Pathological findings **															Clinical findings						
			Heart							Kidney								Blood glucose ***	Urinary sugar	Cataract	Abnormal ECG findings			
			Myocardium				Vasculature			Glomerulus			Interstitial edema	Interstitial fibrosis	Tubular degeneration	Vasculature								
			Edema	Waxy degene.	Loss of striation	Fibrosis	Endo. cell swelling	Medial fibrosis	Luminal narrowing	B.M.thick.	Diffuse sclerosis	Nodular sclerosis				Thickening	Edema					Fibrosis		
Alloxan diabetes(A.D.)	3	44	+		+					+			+	+	+	+	+	+	+	+	+	+	+	+
		51	+				+		+				+	+	+	+	+	+	+	+	+	+	+	+
		57	+								+			+	+	+	+	+	+	+	+	+	+	+
		67	+	+							+			+				+	+	+	+	+	+	+
	6	7	+	+			+	+		+			+	+	+	+	+	+	+	+	+	+	+	+
		34								+	+		+	+	+	+	+	+	+	+	+	+	+	+
		38								+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		122	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		134	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		151	+	+	+		+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
152		+	+			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
156			+			+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
159				+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
*96		+							+			+			+	+	+	+	+	+	+	+	+	
A.D. + Tolb. 100mg	3	72	+	+	+		+	+		+			+	+	+	+	+	+	+	+	+	+	+	
		82	+	+	+		+	+	+	+			+	+	+	+	+	+	+	+	+	+	+	
		84	+	+	+	+	+	+	+	+			+	+	+	+	+	+	+	+	+	+	+	
	6	11	+	+			+	+		+		+	+	+	+	+	+	+	+	+	+	+	+	+
		90		+			+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		108	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		126	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		145	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A.D. + Tolb. 200mg	3	49	+	+	+			+	+	+	+		+		+	+	+	+	+	+	+	+	+	
		53	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		58	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		62	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		66	+			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
	6	14	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		110	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		125	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		133	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		153	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

degene. : degeneration, Endo. : endothelial, B.M. : basement membrane, thick. : thickening of Bowman's capsule

Tolb. : tolbutamide, * 12months, ** + : moderate change # : severe change

*** + : normoglycemia (150mg % >) # : mild hyperglycemia (151mg % ~ 250mg %) # : hyperglycemia (251mg % <)

Table 2 Chi-square Table for the Distribution Probabilities of the Cases Showing Severe Pathologic Change in Heart and Kidney (Shown in Table 1)

Organ	Morphologic change	Alloxan diabetes (A.D.)		A.D. + Tolbutamide	
		3 mon.	6 mon.	3 mon.	6 mon.
Heart	severe	0	0	4	7
	no or moderate	4	10	4 n.s. $\chi^2 = 1.172$	7* $\chi^2 = 4.846$
Kidney	severe	1	1	3	5
	no or moderate	3	9	5 n.s. $\chi^2 = 0.047$	9 n.s. $\chi^2 = 0.914$

* $P < 0.05$, n.s : non significant, mon. : months

the finding detected in the chronic toxicity test on alloxan diabetes animals is related to the essential process of diabetes or not. Some of the histopathological findings as described in the present communication, for example, the inflammation of renal interstitial tissue, can be regarded, strictly speaking, as non-diabetic. Although it is clear that there is no influence on the genetical factor, the double exertion of the complicated effects must be assumed to be found in the rabbits after alloxan administration; one is the manifestation of direct toxic effect of alloxan, and the other the development of alloxan diabetic process. The former is generally expected to be severe immediately after the injection and, thereafter, to cure gradually. The latter, by contrast, is highly known to manifest 3 months later and to be progressive. The cardiac findings, which were suspected to be produced by the blood vessel damage in the present experiment, do not seem to be the result of the direct toxic effect of alloxan, since they are always progressive. It goes, however, without saying that they can always be considered to belong to the diabetic angiopathy, since they may likewise occur in pathological conditions other than diabetic. On the other hand, the findings in renal glomeruli pro-

vided an evidence that the essential diabetic process, which was easily supported by the nodular-exudative sclerosis in glomerulus, continuously developed during 6 months in the present alloxan rabbits. The diffuse glomerulosclerosis possessing nearly the same detection probability with that of nodular sclerosis in the present observation, was recently reported by Sakaguchi et al. (1965) to be in a pre-step of nodular glomerulo-sclerosis. Almost all of the present renal findings can be said to agree well with the results by Mann et al. (1951), Ricketts et al. (1959), Greenberg (1962) and Bloodworth (1965).

The significant effect of the long term administration of tolbutamide was found only in the cardiac findings. Two explanations can be considered from these results; tolbutamide may act upon (a) the heart, or (b) the diabetic metabolism. The lack of qualitative distinction in histo-pathological findings between the tolbutamide-treated and untreated groups of alloxan-rabbits allows us to deny the assumption that the drug may have a specific cardio-tropic action. Levey et al. (1971), Lasseter et al. (1972), and Pogatsa et al. (1977) reported on the positive inotropic-like action of tolbutamide. But their experiments can hardly be said to always

reveal the present histological findings, since the blood vessel damage has been established in our work to precede the cardiac muscle lesions. If the pharmacological data related to our histological findings are to be sought, the report by Wales et al. (1971) will be preferred over the above communications. The latter has suggested in their report that tolbutamide caused a slight increase in blood pressure in dogs. Naturally, the relationship between the Wales' hypothesis and ours should not conclusively be taken up in the present state of studies. And, furthermore, the afore mentioned possibility (b) has not been warranted by the renal glomerular findings, since there has only been insignificant difference in the incidences of the lesion between the control and the tolbutamide-given group. The present result, indicating that tolbutamide has little affected on the metabolic disturbance in alloxan diabetes leads us to the last explanation that tolbutamide may exert some indirect influences on metabolic phase in alloxan rabbits. The experiment by Nakama (1977) on mongolian gerbils seems to reveal data compatible with our present results from the alloxan rabbits. Since mongolian gerbils show the normo- or hyperinsulinemic hyperglycemia while alloxan rabbits exhibits oligoinsulinemic hyperglycemia, some influences by the long term administration of tolbutamide can be considered to be indifferent to insulin activity in animals. It is for the moment expected that the mode for such actions may be elucidated by the investigation of tolbutamide on cardiac microcirculation.

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