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Biochemical role of oxygen free radicals in progression of diabetic nephropathy

[1]Dr. Shivaji Mane, Professor and Head Department of Biochemistry H B T Medical College and Dr R N Cooper Municipal General hospital Juhu Mumbai-56

[2]Shilpa Sanjay Ninawe, Laboratory Technician, EMS Department, LTMMC & LTMG Hospital Mumbai -400022

Corresponding Author: Dr. Shivaji Mane, Professor and Head Department of Biochemistry H B T Medical College and Dr R N Cooper Municipal General hospital Juhu Mumbai- 400056, India.

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Abstract

Background: Diabetic nephropathy (DN) is a serious complication of diabetes mellitus (DM), which is a major public health problem in the world. Data on oxidative stress in diabetic patients with DN is scant. The present study was undertaken to determine the role of free radical and changes in natural antioxidant defense in diabetic patients with DN.

Method: Total 64 in-door and out-door patients with DN and 44 normal healthy controls were included in the study. Selection criteria for DN was that patient must have fasting blood glucose >120 mg/dL and urinalysis show microalbuminuria and glycosuria as confirmed by Ames reagent strip at least two consistent positive confirmatory tests. DN patients were subdivided into four groups on the basis of rate of urinary albumin excretion.

Results: The patients with DN have higher levels of plasma lipid peroxides (LPO) (17.80±2.83nmol/ml) than normal controls (9.53±1.93nmol/ml). The activities of SOD, CAT and GPx were significantly lowered in patients with progressive DN. Transferrin was increased while ceruloplasmin and albumin concentration was significantly decreased in patients with progressive DN. When compared to healthy controls plasma ascorbic acid was reduced whereas bilirubin and uric acid levels were increased in patients with progressive DN.

Conclusion: All the above factors discussed in result increases the free radical generation and lipid peroxidation enhancing the oxidative stress in diabetes mellitus leading to progression of DN complications.

Keywords: Diabetic nephropathy; Oxidative stress; Free radical; Antioxidant; Microalbuminuria; Glycosuria; Ames reagent

Introduction

Diabetic nephropathy (DN) is one of the major complications of diabetes mellitus (DM) and is now the leading cause of endstage renal disease (ESRD) that affects about 30% of diabetic subjects, with a higher rate in persons with type 1 DM (30% to 40 %) than in those with type 2 DM (10% to 20%) [1]. In both forms of diabetes the initial changes includes glomerular hyper-filtration and hyper-perfusion functional changes which is associated with subtle morphological changes including thickening of glomerular basement membrane, glomerular hypertrophy, meningeal expansion and modest expansion of the tubulointerstitium [2]. This

phase is followed by a phase known as microalbuminuria or incipient DN, defined as a urinary albumin excretion rate of 20-200 ug/min [3]. After the phase of microalbuminuria there is a continued increase in urinary protein excretion with declining glomerular filtration rate. This results in the development of albustix positive proteinuria and known as overt nephropathy or macroproteinuria [4].

Moreover, DM is now believed to be associated with increased free radical activity. Lipid peroxidation and oxidative stress leads to severe diabetic complications such as retinopathy, nephropathy, atherosclerosis and cardiovascular diseases. In diabetes, chronic hyperglycemia dependent processes are involved in DN. Increased production of free radicals have been strongly implicated in the pathophysiology of DN [5]. Microalbuminuria is the early indicator of diabetic nephropathy. Progression of microalbuminuria to overt proteinuria results in the decline renal function. There are increasing reports in literature that human and animal models of microalbuminuria in DN are associated with hyperglycemia, hypertension and oxidative stress [6].

Oxidative stress is greatly increased in diabetes because of prolonged exposure to hyperglycemia. Glucose combines with plasma proteins and lipoproteins in non-enzymatic glycation reaction [7]. There may auto-oxidize in situ, generating free radicals, causing local oxidative damage. Relevant metabolic factors include glucose dependent pathways such as advanced glycation, increased formation polyols and activation of the enzyme proteinkinase C. These have been implicated in the development of diabetic complications [8]. In the present study we have studied plasma lipid peroxides (LPO) a marker of free radical activity and free radical detoxifying defense potential in patients with progressive DN. The plasma LPO was measured as thiobarbituric acid reactive substances (TBARS) and erythrocyte antioxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were measured. Non enzymatic plasma antioxidants such as transferrin, ceruloplasmin, albumin, uric acid, bilirubin, and ascorbic acid were also measured in plasma.

Materials and Methods

A total of 64 in-door and out-door patients with DN, age ranged from 35-80 years were included in the study, of them 43 were males and 21 were females. Total 44 age and sex matched normal healthy controls (aged 20 to 60 years) including students and staff from Krishna Institute of Medical Sciences Karad, who free from diseases including, diabetes mellitus, infection, hypertension, coronary artery disease, atherosclerosis and any renal dysfunction and no history of smoking were selected. The diagnosis of DN was confirmed by clinical examination and appropriate laboratory investigations. The selection criteria for DN was that patient must have fasting blood glucose >120 mg/dL and urinalysis should show microalbuminuria and glycosuria as confirmed by Ames reagent strip at least two consistent positive confirmatory tests. Ames multiple reagent strips for urinanalysis were firm plastic strips on which were affixed several separate reagent areas. These strips provide test for glucose and proteins in urine.

Plasma glucose concentration was determined by enzymatic method [9] i. e. based on a double sequential enzyme reaction, one enzyme, glucose oxidase, catalyzes the formation of gluconic acid and hydrogen peroxide from the oxidation of glucose. A second enzyme peroxidase catalyzes the reaction of hydrogen peroxide with a potassium iodide to oxidize the chromogen to colours ranging from green to brown. The colour so

developed was measured at 505 nm, which was directly proportional to glucose concentration.

Method: In three different test tubes, 10 µl plasma in test tube marked 'test' and 10 µl standard solution in tube marked standard and 1000 µl each of working glucose reagent was added. Blank tubes containing 1000 µl reagent Solution in each tube was mixed well and incubated at 37°c for 15 minutes. Program in autoanalyser after sequentially aspiration of blank, standard and test.

The protein test was based on the protein error of indicator principle. At a constant pH the development of any green colour was due to the presence of protein. Colour range from yellow for Negative through yellow-green and green to green-blue for Positive reaction [10].

Method: Fresh urine sample was collected from suspected patients in a clean and dry container. Sample was mixed well before testing. Urine specimen tested by immersing reagent area of the strip in urine sample. Comparison of colour between reagent areas and corresponding colour chart on the bottle label gives approximate concentration of glucose and protein in urine. Small amount of glucose is normally excreted by kidney. This amount is usually below the sensitivity of this test. Result at the first positive level may be significantly abnormal if found consistently. Normally proteins are not excreted in detectable amounts in urine. A colour matching with any block greater than trace indicates significant proteinuria. Diabetic Nephropathy patients were subdivided into four groups on the basis of rate of urinary albumin excretion. These groups are as follows-

Patients Group	Urinary Protein in mg %	Ames strip test
I	15-30	+
II	30-100	++
III	100-300	+++
IV	>300	++++

Results

The mean values of plasma lipid peroxides (LPO) and important antioxidants (SOD, CAT, GPx, ascorbic acid, bilirubin, uric acid, transferrin (TFR), ceruloplasmin (CER) and albumin) were significantly changed in diabetic nephropathy patients when compared with normal control group which is depicted in table 1.

Table 1: The mean values of plasma lipid peroxides (LPO) and important antioxidants (SOD, CAT, GPx, ascorbic acid, bilirubin, uric acid, TFR, CER and albumin) in patients with progressive diabetic nephropathy and healthy normal controls

Parameters	Normal controls (n=44)		Patients with progressive diabetic nephropathy (n=64)	
	Mean ± SD	Range	Mean ± SD	Range
LPO nmols / ml	9.53±1.93	6.86-14.06	17.80±2.83*	13.27-25.02
SOD unit/mg Hb	22.53±3.06	16.66-26.78	8.72±2.53*	5.72-15.20
CAT#	543.82±69.67	465.76-823.92	324.35±51.18*	210.85-419.81
GPx Unit/L	576.04±100.65	460.24-685.80	368.17±105.07*	280.11-580.18
TFR mg/dL	204.81±38.69	142.00-321.18	569.48±61.12*	485.20-692.11
CER mg/dL	38.47±6.86	26.65-52.90	22.35±5.61*	15.20-33.45
Albumin gm/dL	3.94±0.49	3.00-4.50	3.32±0.35**	2.80 - 3.86
Ascorbic acid mg/dL	0.57±0.17	0.28-0.96	0.22 ±0.083*	0.09-0.43
Bilirubin mg/dL	1.08±0.25	0.80-1.80	1.22±0.42**	0.8 - 2.6
Uric Acid mg/dL	3.16±0.64	1.60-4.62	6.02±1.02*	3:92-8.40

- The activity of catalase expressed as mm of H_20_2 decomposed /mgHb/min

**p<0.001 and *p<0.05 Vs normal control group

The values of all parameter except diastolic BP (dBP) in various groups of diabetic nephropathy patient were significantly different from the normal control group. There was significant increase in systolic BP ($_sBP$) with increasing microalbuminuria. Whereas dBP was insignificantly changed in the progression of diabetic nephropathy when compared to normal controls as shown in table 2.

Table 2: Age, $_sBP$, $_dBP$, plasma glucose (PG), and plasma lipid peroxide (LPO) levels in various groups of progressive diabetic nephropathy patients and normal control group

Parameters	Controls (N=44)	Group I (N=12)	Group II (N=19)	Group III (N=16)	Group IV (N=15)
Age in years	43.95±8.91	50.26±15.63*	51.37±9.34*	56.27±13.45**	57.34±11.64*
SBP mmHg	116.28±4.65	131.05±14.49*	133.75±17.07*	137.27±19.54**	143.24±15.18*
DBP mmHg	76.77±4.23	81.57±11.18#	81.25±9.57#	80.90±7.00#	82.60±8.24#
PG mg/dL	94.90±15.00	195.73±61.73*	206.37±70.44*	211.73±71.66**	218.78±68.76*
P. LPO nmol/ml	9.53±1.93	14.05±2.24*	16.65±2.92*	18.68±1.80**	20.66±2.12*

** P < 0.001- Highly significant; * P<0.05- Significant; # - Non-significant

From the figure 1 it was observed that there was increased in plasma glucose (PG) level from group I to group IV as compared to control, this increases the plasma lipid peroxide (LPO) levels and higher levels of plasma LPO increasing the degree of microalbuminuria in DN.

Figure 1: Comparison of plasma glucose (PG), and plasma lipid peroxide (LPO) levels

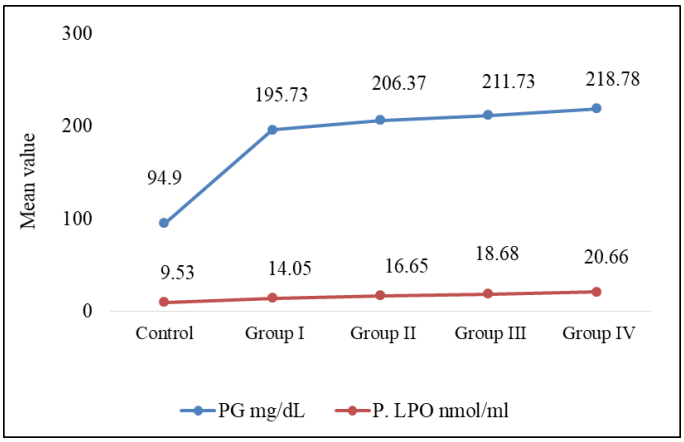

Table 3 illustrate effect of progressive DN on erythrocyte enzymatic antioxidant defense, preventive antioxidants and chain breaking antioxidant. SOD, catalase and GPx activity steadily lowered with progression of diabetic nephropathy. It was decreased from group I to Group IV. The levels of TFR, CER and albumin were significantly (P<0.001) changed with progression of DN, there was significant increase in transferrin shown by all group of patients (P<0.001).

Scavenging antioxidants like ascorbic acid, bilirubin and uric acid levels were significantly changed in patients with progressive DN. The ascorbic acid level was gradually decreased with progression of DN while bilirubin and uric acid levels were increased in patients with progressive DN as compared to the normal controls. The increase in uric acid level from control to group I was very slow and fast in group II, group III and group IV respectively indicating a close relationship between renal injury and uric acid level.

Table 3: Effect of progressive diabetic nephropathy- on enzymatic antioxidant; on preventive antioxidants of plasma and on chain breaking antioxidants

Antioxidants		Group I (N=14)	Group II (N=19)	Group III (N=16)	Group IV (N=17)
Enzymatic	SOD unit/	14.08±3.66*	10.80±2.65*	8.12±3.26*	7.02±2.14*

antioxidants	mg Hb				
	CAT#	355.76±48.19*	318.60±35.95*	320.44±70.50*	288.90±60.16*
	GPx unit/L	324.48±79.82*	317.99±68.52**	312.62±59.73*	309.73±66.63*
Preventive antioxidants in plasma	TFR mg/dL	490.82±48.89*	433.55±100.03*	590.70±60.12*	612.43±83.14*
	CER mg/dL	33.14±4.92*	28.13±4.78*	21.55±5.02*	18.76±4.12*
	Albumin gm/dL	3.60±1.02**	3.05±0.78*	3.00±0.93*	2.86±0.72
Chain breaking antioxidant in plasma	Ascor. acid mg/dL	0.28±0.090*	0.22±0.078*	0.19±0.090*	0.18±0.082*
	Bilirubin mg/dL	1.26±0.90*	1.20±0.72*	1.24±0.48*	1.15±0.082*
	Uric acid gm/dL	4.90±1.03**	5.38±1.12*	5.90±1.72*	6.40±1.40*

#the unit of enzyme activity expressed as mM of H_20_2 decomposed / mgHb /min

*P< 0.05- significant with progression of diabetic nephropathy. **P<0.001 highly significant

Discussion

The incidence of DN disease is increased in patients with diabetes. Increased oxidative stress in diabetes is believed to play an important role in the pathogenesis of glomerularosclerosis in diabetes. However, data on oxidative stress in DN are scanty. The present study clearly indicates that diabetic patients' increases the plasma glucose (PG) level which increases the plasma lipid peroxide (LPO) levels and this higher levels of plasma LPO increased with increasing degree of microalbuminuria in DN. The increased plasma LPO clearly indicates increased oxidative stress in DN. A report by Uzel et al [11] showed susceptibility of erythrocyte to OFR in diabetic individuals and suggested that increased plasma lipid peroxides levels might originate from the per-oxidative destruction of membrane lipid [11]. Increased LPO in plasma may have effect on glomerular basement membrane (GBM) of nephron through oxidized LDL and lipid peroxide products in the plasma. Active oxygen species are known to be involved in the mechanism of proteinuria, lipid peroxide products and hydroxy radicals attack are also incriminated for the development of proteinuria in nephritis and amino-nucleoside nephritis [12]. The results in this study favor these hypothesis plasma LPO were significantly increased with progression of DN and that there was steady raise in plasma LPO with increasing urinary albumin concentration. The relationship between plasma LPO and albuminuria were closely related with each other as disease progresses. This strong close relationship confirms a role of OFR in progression of microalbuminuria to overt proteinuria in the diabetic end stage renal disease (ESRD).

In the present study the activities of SOD, CAT and GPx were significantly lowered in patients with progressive DN indicating decreased enzymatic antioxidant defense. The activity of SOD rapidly decreased with increase in urinary protein excretion. It is evident from these results, that free radical production was higher in DN which was in excess of beyond the capacity of SOD to neutralize all O_2 Radicals. The SOD activity thus is found to be rapidly decreased with progression of diabetic renal disease. Similarly GPx and CAT also decreased in chronic patients of DN. In diabetes chronic hyperglycemia leads to enhanced generation of free radical such as O_2, OH and H_2O_2. Hydrogen peroxide formed in erythrocytes during prolong diabetes exceed the capacity of GPx and

catalase. Both of these enzymes detoxify H_2O_2 in to water and O_2. The third antioxidant enzyme catalase (CAT) in etythrocytes was also significantly lowered in DN patients than normal healthy controls. The decrease in the activity of CAT during the progression of DN showed slow decline whereas in overt DN there was fast decrease in CAT activity. This is because of effective decomposition of H_2O_2 at the stage of initiation of microalbuminuria and there may be lowering of activity of GPx in prolong diabetes. The present result of in this decreased erythrocyte antioxidant enzymes such SOD, GPx and CAT in DN might have occurred due to protease action i.e. self-destruction the possible causes were discussed here, exact mechanism is unclear. Moreover, the depletion of enzymatic defense in DM results in increased oxidative stress. The increased free radical activity and decreased antioxidant defense potential contributes to progression of microalbuminuria to overt proteinuria in progressive DN.

Plasma proteins such as transferrin, ceruloplasmin and albumin are also important antioxidant components of body defense. These proteins have ability to chelate metal ions such as copper and iron. These metal ions are capable of generating free radicals by Fenton and Haber-Weiss reaction which consequently induces tissue damage. In current study transferrin measured on the basis of plasma iron levels was increased in patients with progressive DN. This may result due to ischemic reperfusion renal injury and cell death, which release ferritin iron into plasma. Whereas ceruloplasmin another protein with ferroxidase activity, known to enhance the rate of binding Fe^{+2} to apotransferrin. In our study ceruloplasmin concentration was significantly decreased. (The consequence and Haber-Weiss reaction resulting in free radical production in DN). Increased oxidative stress damages the glomerular membrane, it causes hyper-filtration, microalbuminuria and finally proteinuria. Therefore the results that the albumin concentration is decreased in patients, with DN and shown a decreasing trend in different groups of nephropathy were well explained.

There was statistically significant increase in sBP but not dBP in progressive DN patients. Increase in sBP accelerates arterial blood pressure. Hypertension could be due to abnormal hemodynamic adoption of renal circulation resulted due to renal injury and microalbuminuria. This is supported by the report that increased prevalence of hypertension with increasing albuminuria [13]. Hyperglycemia is a major determinant of progression of DN. In current study plasma glucose levels were significantly higher in patients than controls, ($P< 0.001$) and which showed increasing trend with increased albuminuria. There are increasing evidences which suggests that chronic hyperglycemia mid hypertension are the risk factors in the development of DN [14]. In diabetic patients oxidative stress is greatly increased because of overload of glucose due chronic hyperglycemia and increased oxidative stress has been strongly implicated in the pathophysiology of DN [14].

Plasma concentration of antioxidant ascorbic acid was significantly decreased in patients with DN. The decline in plasma vitamin C was very fast with increasing albuminuria. The low level of ascorbic acid found in DN probably resulted from greater consumption in antioxidant due to ongoing oxidant (OFR) load in diabetes [15]. In the present study bilirubin levels were slightly higher in DN patients than normal controls. This increase was not uniform with progression of renal disease. However, the increase in plasma bilirubin is most likely due to increased heamolysis as a result of oxidative stress rather than other causes. Uric acid was significantly increased than normal controls. Plasma uric

acid concentration steadily increased with progression of DN. There was fast increase in plasma uric acid various groups of progressive DN. Very high levels were found in patients with severe DN than mild DN. These increased plasma uric acid levels are most probably due to mitochondrial dysfunction, which leads to depressed phophorylation. Thus increased catabolism of AMP to uric acid by xanthine oxidase system must have resulted into increased levels of uric acid. Xanthine oxidase not only generates uric acid but also superoxide radicals are simultaneously generated. Uric acid is a weak antioxidant which react with hydroxyl radical, singlet oxygen and superoxide radicals. However the rate of generation of free radicals is very high, which causes oxidative stress and in diabetes mellitus increased blood glucose and hypertension promotes free radical activity.

Ceruloplasmin levels were significantly decreased (P (P<0.001) in patients with DN when compared to the normal controls. Ceruloplasmin levels were directly related with progression of DN. There was a slow decrease in patients with mild microalbuminuria and fast decrease in chronic patients of DN with massive protein loss. Group III and Group IV patients showed very low levels of ceruloplasmin. The decreased ceruloplasmin level may be due to loss in heavy proteinuria in chronic DN. The decrease in plasma ceruloplasmin was closely related to the concentration of urinary protein. Because of decrease in plasma ceruloplasmin concentration, total peroxidase activity in the plasma declines consequently the conversion of Fe++ to Fe+++ is decreased. Thus elevated iron level in plasma causes enhanced rate of chain reaction of lipid peroxidation by Haber-Weiss reaction. Very high levels of transferrin were observed in progressive DN in this study. According to the hypothesis proposed, the rate of erythrocyte destruction was increased in DN due to increased oxidative stress. These results also showed high bilirubin levels and free iron levels. The over load of iron due to hemolysis increases the transferrin saturation. The antioxidant activity shows a storage inverse correlation with percent saturation of transferrin and correlates positively with the ceruloplasmin /differed transferrin ratio in human serum. Under such condition, serum ceruloplasmin or apotransferrin concentrations were decreased or transferrin percent saturation by iron is increased. In other words, a deficit in serum antioxidant capacity is observed [16].

In this study transferrin was not estimated as true transferrin. Transferrin level was estimated from plasma iron levels as calculated transferrin. From this derived value higher level of transferrin was observed in progression of DN. Another weak copper chelator protein, albumin levels showed significant decrease in DN than in normal healthy controls (P< 0.001) Albumin undoubtedly cleared by kidney in the form microalbuminuria. Urinary protein increases with progression of renal disease and similar results are obtained in this study. There was a fast decrease in albumin level with progression of DN. In chronic cases albuminuria increased showing decreasing trend as disease progresses. Therefore albumin may loss as an albuminuria. And thus in diabetic nephropathy there was steady loss of albumin, through urine may causes low levels of albumin.

Conclusion

The present study revealed that the increased in plasma glucose (PG) level in diabetic patients increases the plasma lipid peroxide (LPO) levels and this higher levels of plasma LPO increasing the degree of microalbuminuria in diabetic nephropathy. However, in diabetic nephropathy there was increased free radical generation occurs by uncontrolled levels of high blood

glucose and hypertension; increased transferrin level (free iron in plasma) increases metal catalyzed generation of free radicals; increased uric acid by enhanced xanthine oxidase system; decreased ascorbic acid, ceruloplasmin, albumin and erythrocyte enzymatic antioxidants defense such as SOD, CAT and GPx. These are the possible causes of progression of diabetic nephropathy. All of above factors increase the free radical generation and lipid peroxidation enhancing the oxidative stress in diabetes mellitus leading to progression of diabetic nephropathy complications.

References

1. Macisaac RJ, Ekinci EI, Jerums G. Markers of and risk factors for the development and progression of diabetic kidney disease. Am J Kidney Dis. 2014;63:S39–62.
2. Zeni L, Norden, AGW, Cancarini G & Unwin, RJ. A more tubulocentric view of diabetic kidney disease. Journal of Nephrology 2017; 30(6):701–717.
3. Al-Maskari F, El-Sadig M & Obineche E. Prevalence and determinants of microalbuminuria among diabetic patients in the United Arab Emirates. BMC Nephrol 2008;9(1): 1-8.
4. Vora J, Ibrahim H & Bakris G. Responding to the challenge of diabetic nephropathy: the historic evolution of detection, prevention and management. Journal of Human Hypertension 2000; 14(10-11):667–685.
5. Matough FA, Budin SB, Hamid ZA, Alwahaibi N, Mohamed J. The role of oxidative stress and antioxidants in diabetic complications. Sultan Qaboos Univ Med J. 2012;12(1):5-18.
6. Lim AKh. Diabetic nephropathy - complications and treatment. Int J Nephrol Renovasc Dis. 2014;7:361-381.
7. Asmat U Abad K & Ismail K. Diabetes mellitus and oxidative stress—A concise review. Saudi Pharmaceutical Journal 2016; 24(5):547–553.
8. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circ Res. 2010;107(9):1058-1070.
9. Blood Glucose GOD / POD Method By Autospan Reagent Span Diagnostics (Surat) India Ltd.
10. Ames™ Multiple Reagent Strips -Bayer Diagnostics India Ltd. 274 / AEPIV 6 / 95.
11. Uzel N, Sivas A, Uysol M and OZ H. Horm Met Res. 1987;19:89-90.
12. Hara T, Miyai H, Idia T, fotenma A, Nakamura S, Kato ,K. 1990 Proc XI,b Int ! , Congr. NephrolJPN P 442 A.
13. Porush JG. AMJ Kidney DIS. 1998 31: 177 -184
14. Sharma BK, Shanna N, Jain S. Journal of the Indian Medical Association 1999; 97(3): 91-95.
15. Johnes AF, Winkles JW, Jennings PE. et al Diabetes Res. 1988 7; 89-92.
16. Carle A, Hubei Copy right (c) 1996 by Mosby Yearbook Inc: Vol. 175 AM J Obstet Gynecol.