Role of Fas/Fas Ligand Pathway in a Sample of Iraqi Diabetic Foot Patients

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Abstract

Background: Diabetic foot (DF) is one of diabetes mellitus (DM) complication, it is delayed wound healing and may end with amputation of limbs. Involvement of Fas/Fas ligand (FasL) pathway in the pathogenesis of diabetic complications has been proposed. Apoptosis via Fas/FasL interactions has been proposed to be a major T-cell-mediated effector mechanism in autoimmune diabetes.

Objectives: To investigate Fas/Fas Ligand, in patients with type 2 diabetes who have diabetic foot ulcerations.

Methods: sFas, sFasL and high sensetive-CRP were measured with the ELISA method in thirty(30) normal controls (group I), twenty five patients with type 2 DM with no diabetic foot (group II) and twenty five diabetic foot patients with type 2 DM (group III). Besides, serum glucose (Fasting), lipid profile (total cholesterol, triacylglycerol, HDL, LDL and VLDL), body mass index, Waist circumstance and age were determined.The study was carried out in National center of diabetes, Al-Mustansirya University, Baghdad, Iraq.

Results: The patients with diabetic foot lesions were found to be poorly controlled and had significantly higher levels of fasting blood glucose (FBG) ($p<0.05$, $p<0.001$) compared to patients without diabetic foot lesions and healthy control groups.Also the patients with diabetic foot lesions were found to be significantly have higher levels of sFas and FasL compared to patients without diabetic foot lesions and healthy control groups ($p<0.001$, $p<0.05$) respectively. Serum levels of total cholesterol, triacylglycerol, LDL and vLDL were significantly higher ($p<0.05$) in diabetic patients (with and without foot ulceration) in comparison with healthy normal control. While HDL-c level was lower in both groups than in the control group, it was reach statistical significance.There are no significant different in lipid profile between diabetic foot patients and without diabetic foot patients. sFas serum levels were significantly increased in both diabetic groups compared with normal controls (12.2±2.18 ,10.55±2.57 ng/ml Vs 5.91±3.1 ng/ml, $p<0.001$), but the levels were significantly higher in patients with diabetic foot when compared with those without diabetic foot patients ($p<0.05$). sFasL serum levels were also increase in both diabetic patients as compared with normal controls(2.55±1.06, 1.75±0.62 ng/ml Vs 1.59±0.32 ng/ml,$p<0.05$), but the levels were higher in diabetic foot patients than those without diabetic foot patients. The duration of DM of patients were with >10 yrs, age > 58 yrs, and BMI >25 Kg/m^2.

A significant positive correlation was observed between sFas and (sFasLand hs-CRP) in diabetic foot patients. Also the same with sFas and (FBG, and lipids).

Conclusion: We conclude that the apoptotic pathway in the development of diabetic foot increases by means of the Fas/FasL,and the development of new treatment against apoptosis may play an important role in the management of diabetic foot lesions.

Key words: Diabetic foot,Fas/Fas ligand,apoptosis,type2 diabetes .

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Introduction

Diabetes mellitus is a carbohydrate metabolic disorder disease companied with increasing of blood glucose "hyperglycemia", and at the top 5 of diseases all over the world [1, 2]. DM is a chronic disease cannot be cured, and is combined with serious complications especially after 10-20 years. The high levels of glucose lead to macro and micro vascular diseases [2].

Macrovascular diseases cause cardiovascular disease such as atheroseclerosis and heart ischemic [2, 3]. The microvascular complications "damage the small blood vessels when coated with sugar" lead to retinopathy with affects blood vessels of retina of eyes and also can lead to nephropathy (damage of kidney). Diabetic neuropathy is the impact of diabetes on the nervous system, which causing numbness, tingling, and pain in the feet and also increasing the risk of skin damage due to altered sensation [2, 3, 4]. Neuropathy with vascular disease, in the legs contributes the risk of diabetes-related foot problems, such as diabetic foot ulcers and infection that can be difficult to be treated and occasionally require amputation [5, 6, 7].

Diabetes mellitus is characterized by increased production of Reactive Oxygen Species (ROS), sharp reduction in antioxidant defense and altered cellular redox status.

Hyperglycemia, is a key clinical manifestation of diabetes mellitus, not only generates more reactive oxygen species, but also attenuates antioxidative mechanisms by scavenging enzymes and substances [8]. Oxidative stress, a potentially harmful imbalance between the level of pro-oxidants and anti-oxidants. It can cause cellular injury and tissue damage by promoting several reactions e.g., lipid perxidation, DNA damage, protein glycation [9, 10]. Lipid peroxides may increase the participation of advanced glycation end-products in the development of chronic vascular complications [11]. The chemical modifications of proteins and lipids by ROS is contribute to the pathogenesis of diabetic complications [12, 13]. ROS also causes nitrogen base modifications and strand breaks in DNA [14]. It increases the conversion of Deoxyguanosine (dG) to 8-hydroxydeoxyguanosine (8-OHdG) in DNA which is linked to increased mitochondrial DNA deletions [15]. Indeed, when defense mechanisms cannot prevent the accumulation of ROS, there is an increase in cellular damage proteins, lipids and nucleic acids. Accumulation of such injury ultimately leads to cell death through necrotic or apoptotic mechanisms [16]. Oxidative stress is a critical part of the apoptoting agent. If the DNA is severely damaged, the cell will undergo apoptosis [17]. Proteins secreted by these cells including soluble Fas (sFas) and soluble Fas ligand (sFasL) circulate in small, but detectable amounts. Fas is generated by alternative messenger RNA splicing capable of encoding a soluble Fas molecule lacking the transmembrane domain[18], whereas sFasL is released in serum from membrane-bound FasL processed by a metalloproteinase[19] and its ligand are typicall members of The Tumor Necrosis

Factor (TNF) receptor super family.Many studies have demonstrated that importance of Fas-mediated apoptosis in tumorigenesis[20]. Fas (Apo-1 or CD95) is a cell-surface receptor that transduces apoptotic signals from Fas ligand (FasL).

The Fas/Fas ligand system is a key regulating system responsible for activation of apoptosis in various cell types including cellular constituents of the vessel wall [21,22].

Methods

This cross sectional study population included 80 subjects. All subjects were divided into 3 groups:

- Group I; Control group: comprising 30 apparently healthy subjects who matched with age and sex the diabetic patients. They had no recognizable diseases or previous history of endocrine disturbances. They were clinically free from any abnormality. They were not receiving and medications.
- Group II; Diabetic group: including 25 patients with type 2 diabetes (diagnosis according to criteria of the ADA, 2006) [23]. Diabetes duration ranged from 1 to 32 years. The age ranged from 46 to 68 years.
- Group III; Diabetic foot group: including 25 diagnosed type 2 diabetic patients with diabetic foot lesions. Diabetes duration ranged from 2 to 31 years. The age ranged from 36 to 77 years.

All patients and participants gave their informed consent for the study in National center of diabetes, Al-Mustansirya University, Baghdad, Iraq. The following variables were recorded: age, BMI, gender. BMI was calculated as weight divided by height squared (kg m^{-2}) the cutoff point of abnormal BMI was 25 kg m^{-2} [24]. Waist Circumference (WC) was measured, with the subject standing, at the level midway between the lower rib margin and the iliac crest [25]. In all cases blood samples were taken after 12 h overnight fasting. Venous blood was collected in vacutainers without additive, allowed to clot for 30 min at room temperature and centrifuged at 3000 rpm for 10 min to get serum for immediate measurement of glucose (fasting) and lipid profile. Hemolysed samples were excluded. The remaining serum of the control subjects, diabetic patients were separated from their whole blood, divided into aliquots and were stored at-80°C until the measurement of sFas and FasL hs-CRP.

Glucose, total cholesterol, triacylglycerol and HDL were determinated using the methods

By Barham and Trinder (1972) [26]; Allain et al (1974) [27]; Fossati and Prencipe (1982) [28] and Finley *et al*. (1978) [29] respectively. LDL-c was calculated by the Friedewald *et al*. (1972) [30] formula.VLDL concentration is calculated as one – fifth of the serum TG.

sFas concentration was assessed using the sFas Enzymelinked Immunosorbent Assay (ELISA) kit (Cusabio biotech co.,LTD,China), with a sensitivity less than 0.3 ng mL−1 in serum. sFasL concentration was assayed using the sFasL ELISA kit (Demeditecdiagnostics Gmbh**.**Lise-Meitner-StraBe2, Germany), with a sensitivity of 0.07 ng mL_1 in serum. All procedures were performed according to the manufacturer's instructions.

Chemicals and Instruments

Kits,chemicals and Instruments employed in the present study are given in table 1:

Table (1): Chemicals, Instruments and their suppliers**.**

Items (Chemicals or Instruments)	Supplier
Soluble "Human" Factor-related Apootosis (sFas) ELISA Kit	CUSABIO BIOTECH Co. LTD. China
Soluble "Human" Factor-related Apootosis Ligand (sFasL) ELISA Kit	Demeditec Co., Germany
hs-CRP ELISA Kit	Demeditec Co., Germany
HDL-Cholesterol Kit	Biomaghreb, Sa, France
Glucose Kit	Spinreact, Spain
Total Cholesterol Kit	Biomaghreb, Sa, France
Triglyceride Kit	Biomaghreb, Sa, France
ELISA	Micro ELISA system (washer and reader) (Thermo, Germany)
Centrifuge	Hettich Universal-Germany
Balance	Saturius Lab-Germany
Incubator	BDH
Spectrophotometer	Cecil CE 72000, France
Refrigerator	Arcelik, Turkey
Timer with alarm	Junghans, Germany
Length scale micropipettes and Multichannel	Salter, England

Statistical Analysis

All results were expressed as the mean ± SD. Statistical analysis was performed with Statistical Package for the Social Science for Windows (SPSS). The data were analyzed by one-way Analysis Of Variance (ANOVA). To compare the difference among the groups, post hoc testing was performed by the Bonferroni test. The p value less than 0.05 were considered statistically significant [31].

Results

The baseline characteristics of all the groups included in the study were summarized in Table 2. The diabetic studied groups and control group were comparable to each other.The patients and controls were age matched ($p>0.05$). A significant difference was detected between diabetic group and control group in WC ($p<0.05$). It was seen that, no significant difference detected between diabetic groups and control group in BMI ($p>0.05$). FPG was significantly higher in the studied groups compared to control group as represented by $p<0.05$.It was also seen signifigant difference between diabetic foot patients and without diabetic foot patients ($p<0.05$). Table 3 demonstrates the changes of lipid profile of diabetic patient's type 2. Total Cholesterol (TC), triacylglycerol (TG), LDL-c and vLDL manifested significant elevations ($p<0.05$) in diabetics as when compared to normal control subjects, while HDL-c level showed no significant difference ($p<0.05$) among the study groups. TC, TG and LDL-c represented pronounced increases in diabetic patients with long duration compared to diabetic patients of short duration.It was seen no significant difference in lipid profile

between diabetic foot patients and without diabetic foot patients ($p>0.05$). Table 4 showed the levels of, sFas, and sFasL in the diabetic groups and controls.sFas and FasL were higher in patients of diabetes mellitus (both groups) as compared to controls. The level of sFas represented significant elevation ($p<0.05$) in both groups in comparison to normal subjects but diabetic foot patients showed pronounced increase compared to without diabetic foot patients.Also FasL showed higher levels in diabetic patients than control($p<0.05$), but the levels were higher in diabetic foot patients than whom without diabetic foot patients.The hs-CRP was measured in three groups and showed signifigantly higher levels indiabetic foot patients than diabetic patients and control as showen in table (4).

Table (2): Baseline characteristics of the subjects studied.

Parameters	Group 1	Group II	Group III	P value
Subjects (n)	30.0	25.0	25.0	
Age (years)	53.13±8.61	59.60±9.27	58.12±9.29	0.072
Duration of DM (years)		14.24±11.07	13.28±7.73	0.268
BMI (kg/ m^2)	29.44±5.11	31.79±5.84	28.84±3.88	0.563
WC (Cm)	104.9±12.07	110.0±12.10	106.1±11.56	0.009*
F.B.G (mg/dl)	96.23±9.03	193.68±62.53	229.84±92.38	0.0001*
-The values expressed as mean ± S.D *Significant using ANOVA test at 0.05 level				

BMI: Body mass index; DM: Diabetes Mellitus;WC: Waist circumstance F.B.Glucose: fasting blood glucose .

Table (3): Serum lipid profile in different studied groups.

Parameter		Group III	Group II	Group 1	p value
Cholesterol (mg/dl)	**Mean ± SD**	181.44±63.47	188.60±43.28	144.13±12.42	0.0001*
	Range	(100.0-307.0)	(100.0-250.0)	(125.0-170.0)	
Triglycerides (mg/dl)	**Mean ± SD**	157.88±104.55	158.68±78.98	113.47±9.37	0.035*
	Range	(88.0-600.0)	(90.00-400.0)	(101.0-140.0)	
HDL (mg/dl)	**Mean ± SD**	43.20±7.97	43.88±7.52	43.00±1.97	0.983
	Range	(30.0-60.0)	(30.0-60.0)	(40.0-47.0)	
LDL (mg/dl)	**Mean ± SD**	120.12±74.39	120.24±42.98	86.30±5.55	0.012*
	Range	(28.0-297.0)	(37.0-190.0)	(75.0-96.0)	
VLDL (mg/dl)	**Mean ± SD**	25.88±7.97	29.04±11.08	21.97±1.81	0.004*
	Range	(17.0-43.0)	(18.0-56.0)	(19.0-26.0)	
-Data were presented as Mean ± SD (Range). -*Significant using ANOVA test at 0.05 level					

Table (4): sFas and sFasL in different studied groups.

Parameter		Group			p value
		Diabetic foot	Diabetic	Control	
Fas(ng/ml)	Mean±SD	12.20±2.18	10.55±2.57	5.91±3.10	0.0001*
	Range	4.79-16.57	4.94-15.53	0.82-12.93	
FasL (ng/ml)	Mean±SD	2.55±1.06	1.75±0.62	1.59±0.32	0.028*
	Range	1.17-2.20	0.63-4.04	0.67-2.22	
hs-CRP (mg/l)	Mean±SD	13.96±12.62	4.83±4.02	3.89±4.21	0.0001*
	Range	0.87-35.37	0.54-12.34	0.27-13.03	
-Data were presented as Mean ± SD (Range). -*Significant using ANOVA test at 0.05 level					

Serum Human Soluble Factor-related Apoptosis (Fas):

Table (5) below showed that mean Fas was found to be significantly elevated ($p<0.05$) in diabetic foot patients and without diabetic foot patients when compared with healthy control. In the same table, mean Fas concentrations in patients with diabetic foot was found to be significantly elevated ($p<0.05$) when compared with patients whom without diabetic foot ulcers.

Table (5): mean ± SD and range values of serum Fas in patients and control.

Groups	Serum FAS (ng/ml)		P value in comparison to	
	Mean ± SD	Range	Diabetic	Control
Diabetic foot	12.28±3.07	4.79-16.57	0.019*	0.0001*
Diabetic	10.55± 2.57	4.94-15.53		0.0001*
Control	5.91± 3.10	0.82-12.93		
*significant using student-t- test two independent means at 0.05 level of significant.				

Serum Human Soluble Factor-related Apoptosis Ligand (FasL):

Table (6) below showed that mean FasL was found to be significantly elevated ($p < 0.05$) in diabetic patients when compared with healthy subject. And the mean also FasL of diabetic foot patients was found to be significantly ($p < 0.05$) when compared with diabetic patients whom without diabetic foot ulcers.

Table (6): mean ± SD and range values of serum FasL in patients and control.

Groups	Serum FasL (ng/ml)		P value in comparison to	
	Mean ± SD	Range	Diabetic	Control
Diabetic foot	2.55±1.06	1.17-440	0.036*	0.014*
Diabetic	1.75±0.62	0.63-404		0.244
Control	1.59±0.32	0.63-2.22		
* Significant using student-t- test two independent means at 0.05 level of significant.				

Correlations

The correlations between sFas and other parameters in all groups included in the study were summarized in table 4. There was negative significant correlation between sFas and age (r =-0.494, p<0.05) in diabetic foot patients.There was also negative correlation between sFas and BMI and waist (r=-0.23,p>0.05), (r=-0.3347,p>0.05) respectively in diabetic foot patients. There were positive correlation between sFas and duration and FBS(r=0.033,p>0.05), (r=0171,p>0.05) respectively in diabetic foot patients. Also there were positive between sFas and cholesterol, triglyceride, LDL and vLDL (r=0.129,p>0.05),)(r=0292,p>0.05), (r=0.185,p>0.05) and (r=0,208,p>0.05) respectively but there was negative correlation with HDL(r=-0.125,p>0.05) in diabetic foot patients.There were positive significant correlation between sFas and FasL and hs-CRP. Also the correlations between sFas and this parameters in diabetic group and control group showed in table 7.

The correlation between FasL and other parameters in all studied groups were summarized in table 5.There were negative significant correlation between FasL and age (r=-0.453,p<0.05), and there were negative correlation between FasL and BMI and waist (r=-0.157,p>0.05),(r=-0.145,p>0.05) respectivrly in diabetic foot patients.There were positive correlation between FasL and duration and FBS(r=0.062,p>0.05),(r=0.297,p>0.153) respectively indibetic foot patients. Also there were positive correlation with cholesterol(r=0.059,p>0.05), triglyceride (r=0.364,p>0.05), LDL, (r=0.031,p>0.05), and vLDL (r=0.191,p>0.05).But there was negative correlation with HDL(r=-0.139,p>0.05).There was positive significant correlation between FasL and hs-CRP. The correlation between FasL and this parameters in diabetic patients whom without diabetic foot lesions and control also showed in table 8.

Table (7): The correlation of sFas with other parameters in three studied groups.

Parameter		Fas (ng/ml)		
		Diabetic foot	**Diabetic**	**Control**
Age (years)	r	-0.494*	-0.084	-0.016
	P	0.012	0.689	0.935
BMI (Kg/m^2)	r	-0.230	-0.077	-0.198
	P	0.269	0.715	0.298
Waist (cm)	r	-0.347	-0.138	0.018
	P	0.089	0.511	0.924
Duration (years)	r	0.033	0.045	-
	P	0.876	0.83	-
FBG (mg/dl)	r	0.171	0.034	0.218
	P	0.413	0.871	0.248
Cholesterol(mg/dl)	r	0.129	0.267	0.388*
	P	0.538	0.198	0.034
Triglycerides(mg/dl)	r	0.292	0.083	0.325
	P	0.157	0.692	0.079
HDL(mg/dl)	r	-0.125	-0.012	0.065
	P	0.551	0.953	0.732
LDL(mg/dl)	r	0.185	0.168	0.068
	P	0.375	0.422	0.723
VLDL(mg/dl)	r	0.208	0.131	0.254
	P	0.318	0.532	0.176
FasL(ng/dl)	r	0.563*	0.067	-0.044
	P	0.003	0.749	0.815
hs-CRP(mg/l)	r	0.429*	-0.076	-0.036
	P	0.032	0.718	0.851
*. Correlation is significant at the 0.05 level.				

Table (8): The correlation of FasL other parameters in three studied groups.

Parameter		FasL (ng/ml)		
		Diabetic foot	Diabetic	Control
Age (years)	r	-0.453*	0.133	-0.066
	p	0.023	0.525	0.728
BMI (kg/m^2)	r	-0.157	0.072	-0.296
	p	0.446	0.731	0.112
Waist (cm)	r	-0.145	-0.116	-0.313
	p	0.490	0.582	0.093
Duration (years)	r	0.062	-0.356	-
	p	0.764	0.081	-
FBS (mg/dl)	r	0.297	0.039	-0.326
	p	0.153	0.853	0.078
Cholesterol(mg/dl)	r	0.059	0.072	-0.219
	p	0.779	0.732	0.246
Triglycerides(mg/dl)	r	0.364	0.548**	0.041
	p	0.073	0.005	0.828
HDL(mg/dl)	r	-0.139	-0.294	-0.065
	p	0.507	0.154	0.732
LDL(mg/dl)	r	0.031	0.087	-0.133
	p	0.882	0.678	0.483
VLDL(mg/dl)	r	0.191	0.087	-0.287
	p	0.361	0.680	0.124
hs-CRP(mg/l)	r	0.471*	0.041	-0.197
	p	0.018	0.847	0.296

*Correlation is significant at the 0.05 level. **Correlation is significant at the 0.01 level.

Discussion

Type 2 diabetes is characterized by hyperglycemia and dyslipidemia which associated with a cluster of risk factors forming the metabolic syndrome [32]. And leads to serious complications. High levels of glucose (and cholesterol) leads to macro and micro vascular diseases. Cardiovascular diseases are the cause of death up to 80% of patients with type 2 diabetes [4, 22]. Retinopathy is damage of small blood vessels, in the retina of eyes, can cause blindness. The high hyperglycemia (carbohydrate metabolic disorder) in diabetic patients leads to neuropathy. How the nerves are injured is not entirely clear but research suggests that high blood glucose changes the metabolism of nerve cells and causes reduced blood flow to the nerve. Neuropathy and vascularopathy alone or together can cause diabetic foot ulcerasions [4]. In this study, the levels of blood glucose was significantly higher in the diabetic foot patients than blood glucose of diabetic patients without diabetic foot and control(fig.1), and that showed that the diabetic foot patients often with very poor(bad) glycemic control. Also lipids (except HDL) were significantly higher in diabetic patients (with and without diabetic foot) as compared with control subjects (Fig.

2), but the levels were with no significant different between diabetic foot patients and diabetic patients without diabetic foot, this may attributed to, there is no other reasons can increase lipids in diabetic foot patients over without diabetic foot patients. The dyslipidemia which observed in the present diabetic patients elucidated by significant elevation in plasma total cholesterol, triacylglycerol, LDL-c and vLDL-c, while the plasma level of HDL-c was not chanched markedly in diabetic groups compared to control one. These results agree with those of [33,34]. These lipoprotein abnormalities which are due to reduction of lipoprotein lipase activity are held to be responsible for considerable cardiovascular diseases related morbidity and mortality [35]. In the diabetic state, the generation of ROS is enhanced through the processes of glucose autooxidation and protein glycation [36,13]. ROS cause lipid peroxidation and damage protein by chemical modifications through cross-linking and fragmentation. Therefore oxidative stress has been considered to contribute to the pathological processes of diabetic complications. The long duration of diabetic type 2 is contribute to increased peroxidation of lipid in plasma membranes [37]. These results demonstrated that diabetes mellitus produces significantly more free radicals depletes antioxidant enzymes causes chromosomal aberration and DNA damage. Several reports have demonstrated that diabetes increases oxidative damage to DNA [38,39], it increases with aging [40], and in patients with diabetes [39] (most of patients in the study were with age near to sixteenth fig.1). Lorenzi *et al*. (1986) reported that hyperglycemia causes glycosylation of DNA and decreases the DNA repair process human endothelial cells [41]. Nishikawa *et al*. (2003) suggested that damage DNA and produce urinary 8-OHdG (8-hydroxydeoxyquanosine) is a useful marker of early micro- and macro vascular complications in type 2 diabetic patients [42]. Oxidative stress which induces by diabetic disease is a critical part of the apoptotic agent. Dysregulation of apoptosis has been implicated as an important factor mediating tissue turnover [21]. The Fas/FasL pathway may be involved [43]. In the current study, the sFas level was higher in a diabetic population which and without diabetic foot than in subjects without diabetes and was higher diabetic foot patients than without foot patients. sFasL was also found to be higher in the diabetic foot patients than without diabetic foot patients and control subjects, this findings agreed with this of Sibel *et al*.(2009)[44]. Cosson *et al*. (2005) demonstrated in his study that sFas levels were higher in the diabetic patients than in the control subjects,and sFasL was found to be undetectable in more than half the patients with diabetes versus of the controls[22]. In this line, sFas was found to be increased at the acute phase lesions(wagner3,4,5) more than (wagner lesions of stage0,1,2),this also agreed whith findings of Sibel *et al.(2009)*[44]. This high level of sFas appeared to be an independent predictor of vascularopathy and neuropathy events in this population [45] especially in diabetic foot patients group.High levels of CRP(C-reactive protein) in diabetic foot patients agreed with fact that most of lesions are infected because wounds are an ideal place for bacteria to colonize and proliferative since raw tissue and serous exudate provide an excellent medium for bacterial growth.

Conclusion

In type 2 diabetic patients, sFas/FasL could be a sensitive biomarkers for evaluating diabetic foot ulcerations, because we assessed that the apoptotic pathway in the development of diabetic foot increases by

means of Fas/Fas ligand in diabetic foot. Apoptosis, as expressed by enhanced sFas/FasL levels, suggesting that these markers may be helpful for the early diagnosis of type 2 diabetic foot patients, and we consider that the development of new treatment strategies against apoptosis may play important role in the management of diabetic foot lesions.

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