

EFFECT OF URSOLIC ACID, HYPERINSULINEMIA AND VITAMIN B COMPLEX ON SOME BIOCHEMICAL PARAMETERS AND SKELETAL MUSCLE HISTOLOGY OF ALLOXAN INDUCED DIABETIC RABBITS

* Shaymae' H. Shabaa Abdul Razzaq N. Khudair

* Physiotherapy Department, College of Health and Medical Technology,
Middle Technical University, Baghdad, Iraq.

** Physiology and Pharmacology Department, College of Veterinary Medicine,
Basrah University, Basrah, Iraq.

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Correspond Author e .mail:d.abdulrazak79@gmail.com

ABSTRACT

This study aimed to evaluate the effect of ursolic acid, hyperinsulinemia and vitamin B complex in skeletal muscle, which represented by Gastrocnemius muscle after sciatic nerve crush in diabetes mellitus male rabbits, determine their effects on some biochemical parameters which include insulin, total serum protein (TSP) and skeletal muscle protein percentage and detect their effects on histological structures of the skeletal muscle represented by Gastrocnemius muscle. The results of our study showed that there is a significant increase of insulin in diabetic rabbits and this increase is continued in all treatment groups with a higher level range of (3.56-8.4 $\mu\text{u}/\text{ml}$); the lowest significant increase is with vitamin B complex treated group. Our study illustrated that Induction of diabetes has no significant effect on skeletal muscle protein percentage but has a significant effect on total serum protein, while all treatment groups caused a significant increase in skeletal muscle protein percentage and total serum protein. Histological sections of the Gastrocnemius muscle represent some changes happened due to sciatic nerve crush injury, such as some distraction in skeletal muscle fibers, splitting and atrophy of others as well as there is an increase in thickness of fibrous tissue separating them. There is a good regeneration of muscle fibers with little splitting phenomena; also there is a clear formation of muscle cell nuclei, which indicate an improvement of muscle after different treatment.

INTRODUCTION

Diabetes Mellitus (DM) is a metabolic alteration characterized by the incidence of chronic hyperglycemia associated with a different degree of alterations in the metabolism of protein, lipids and carbohydrate (1) as an effect of disorder on insulin excretion, insulin activity or both of them. The diabetes type depends on its etiology. It consists of two types of diabetes mellitus, commonly established which are diabetes mellitus type 1 and type 2 (2). The traditional signs of diabetes are polyuria, polydipsia, and polyphagia (3).

Ursolic acid (UA) ($C_{30}H_{48}O_3$) is a pentacyclic triterpenoid that exists in numerous plants and is a component of several herbal medicines (4). It is the main active component among the other triterpenoids in apple peels (5). Ursolic acid has antifungal, insecticidal, antibacterial, complement inhibitor, diuretic, anti-HIV, gastrointestinal system adjusting actions and antidiabetogenic (6,7). It has antitumor action (8), and produces anti-inflammatory influence in cells with definite inflammation, but in normal cells, it can be pro-inflammatory (9).

Hyperinsulinemia is a situation with a level of blood insulin greater than normal level in individuals without diabetes. Although hyperinsulinemia is not diabetes, it is usually accompanied with diabetes type 2 (10, 11).

Skeletal muscle has a serious function in locomotion functioning, posture maintenance, breathing and production of heat during cold environment (12). Skeletal muscle is quantitatively the greatest essential tissue participating in preserving glucose homeostasis, as it accounts for $\approx 75\%$ of glucose utilization after insulin stimulation (13). Those with type 2 diabetes may have hastened muscle atrophy and loss of strength (14).

MATERIALS AND METHODS

Animals:

Fifty healthy, mature male local rabbits (*Lepus cuniculus*) collected from the local marketplaces of Basrah, their age ranged from (5–8 months) were adapted for one week in an animal house of the College of Veterinary Medicine/ Basrah University. The experimental period lasts for seven weeks included (one week–adaptation period, three

weeks–DM induction, one week–sciatic nerve crush surgery and two week treatment period). The animals were distributed into five groups (ten animals in each group). Diabetes Mellitus was induced in all groups by 200 mg/ kg of Alloxan injected in the marginal ear vein (15); each group divided into two subgroups without and with sciatic nerve crushes operation by an incision made over the lateral aspect of the hind limb. The sciatic nerve of the left limb was crushed at the mid-thigh level using a small hemostatic forceps for a period of 60 Second (16); then each group except (G1+ve control/ diabetic rabbits) treated with different treatment for two weeks, the treatments include extracted ursolic acid (50 mg/ kg) (17), standard ursolic acid (50 mg/ kg) (17), hyperinsulinemia (1.2 IU/ kg Insulin) (18) and vitamin B complex (2 mg/ kg) (19), respectively.

Biochemical Parameters:

- **Determination of Serum Insulin:**

The insulin levels were measured by ELISA according to the manufacturer's instructions. Insulin levels were estimated by means of colorimetric measurement at 450 nm with (Reader Automated ELISA system; Human ELISA; German) through interpolation from a standard curve (20).

- **Determination of Total Serum Protein (TSP):**

The measurement was done by the (Biomaghreb; Tunisia) Kit depending on the enzymatic method of (21).

- **Determination of Skeletal Muscle Protein:**

The Kjeldahl method was used for analysis of the total protein content of the rabbit gastrocnemius muscle by direct nitrogen measurement and subsequent multiplication by a conversion factor (22).

Histological Parameters:

Skeletal muscle, which represented by Gastrocnemius muscle were isolated and fixed in 10% formalin. The fixed tissues were prepared routinely for paraffin embedding; the sections were deparaffinized using xylene and dehydrated in a gradient of alcohol solutions. Tissue sections were prepared for examination.

- **Hematoxylin and Eosin stain:**

Gastrocnemius Muscle stained with Hematoxylin and Eosin. Hematoxylin and Eosin (H&E) stain as a routine histological stain are used to demonstrate the general composition of the tissue (23).

Statistical Analysis:

All data were expressed as means \pm SD. Significant differences among the experimental groups were determined by one way ANOVA method analysis of variance. Statistical significance was considered significant at $p < 0.05$ (24).

RESULTS

Biochemical Parameters:

Biochemical Parameters include determination of serum insulin, total serum protein (TSP) and skeletal muscle protein; these results illustrated in the (Tables 1 and 2).

Table (1): Effect of ursolic acid, hyperinsulinemia and vitamin B complex on serum insulin of alloxan induced diabetic male rabbits.

Groups	Subgroups	Parameter	
		Insulin	$\mu\text{u}/\text{ml}$
G1 +Ve control (Diabetes only)	With Crush	4.920 \pm 0.311	B
	Without Crush	3.560 \pm 0.673	C
G2 Extracted UA	With Crush	8.320 \pm 0.277	D
	Without Crush	8.400 \pm 0.387	D
G3 Standard UA	With Crush	5.700 \pm 0.158	E
	Without Crush	5.660 \pm 0.114	E
G4 Hyperinsulinemia	With Crush	7.160 \pm 0.114	F
	Without Crush	8.060 \pm 0.114	G
G5 Vitamin B complex	With Crush	3.460 \pm 0.305	H
	Without Crush	3.560 \pm 0.279	H
LSD		0.259	

Means bear different letters differs significantly at the 5% level.

It is clear from the table, there is a significant increase ($p < 0.05$) of insulin in diabetic rabbits and this increase is continued in all treatment groups with a higher level with a range of (3.56-8.4 $\mu\text{u}/\text{ml}$). The lowest significant increase is with vitamin B complex treated group.

Table (2): Effect of ursolic acid, hyperinsulinemia and vitamin B complex on skeletal muscle protein percentage (Gastrocnemius muscle) and total serum protein (TSP) of alloxan induced diabetic male rabbits.

Groups	Subgroups	Parameters			
		Skeletal Muscle Protein %		TSP g/ dl	
G1 +Ve control (Diabetes only)	With Crush	40.780 ± 0.130	A	4.824 ± 0.023	C
	Without Crush	40.960 ± 0.114	A	5.470 ± 1.436	D
G2 Extracted UA	With Crush	41.700 ± 0.158	C	5.653 ± 0.002	D
	Without Crush	42.700 ± 0.158	D	5.541 ± 0.001	D
G3 Standard UA	With Crush	41.580 ± 0.130	E	5.552 ± 0.002	D
	Without Crush	41.080 ± 0.130	F	5.323 ± 0.002	D
G4 Hyperinsulinemia	With Crush	41.400 ± 0.158	G	6.076 ± 0.057	E
	Without Crush	41.000 ± 1.000	H	4.080 ± 0.130	F
G5 Vitamin B complex	With Crush	41.180 ± 0.130	J	4.982 ± 0.001	G
	Without Crush	41.480 ± 0.130	K	5.051 ± 0.001	H
LSD		0.267		0.345	

Means bear different letters differs significantly at the 5% level.

It is clear from the table that induction of diabetes has no significant effect on skeletal muscle protein percentage but has a significant effect on total serum protein. It is also clear from the table that all treatment groups (ursolic acid either extracted or standard, hyperinsulinemia and vitamin B complex) caused a significant increase ($p < 0.05$) in skeletal muscle protein percentage and also the same effect of treatments are noticed on total serum protein.

Histological parameter:

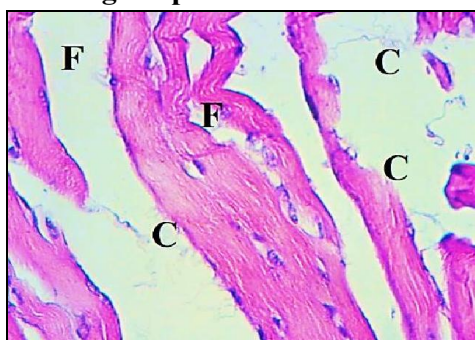


Figure (1): Longitudinal section of rabbit's Gastrocnemius muscle of G1-positive control group (diabetic) after 14 days of sciatic nerve crush injury, showing very clear Cloudy (C) and Fatty (F) degenerations in addition to the clear splitting of myofibrils and pyknosis of muscle cell nuclei (H&E 400X).

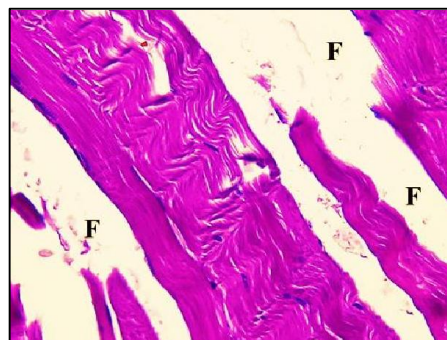


Figure (2): Longitudinal section of rabbit's Gastrocnemius muscle of G1-positive control group (diabetic) without sciatic nerve crush injury, showing muscular Cloudy (C) and Fatty (F) degeneration still detected, but there is less splitting of myofibrils and less pyknosis of muscle cell nuclei (H&E 400X).

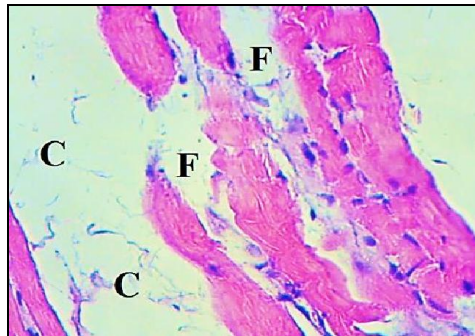


Figure (3): Longitudinal section of rabbit Gastrocnemius muscle of G2-diabetic treated with extracted UA group after 14 days of Sciatic Nerve crush injury, showing the less regenerative process of the muscle fibers, there is a splitting of muscle fibers and the large amount of Fibrous tissue (F) still present (H&E 100X).

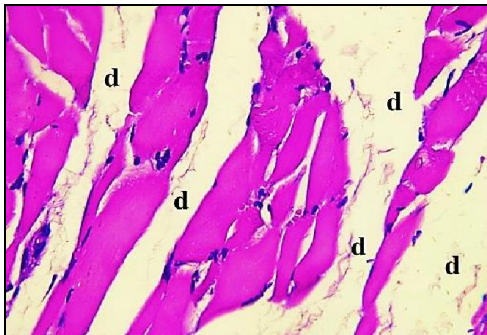


Figure (4): Longitudinal section of rabbit's Gastrocnemius muscle of G3-diabetic treated with standard UA group after 14 days sciatic serve crush injury, showing partially good muscular fiber regeneration multinuclei formation, no muscular fiber splitting but there are cloudy and fatty degenerated areas (d) still existence (H&E 100X).

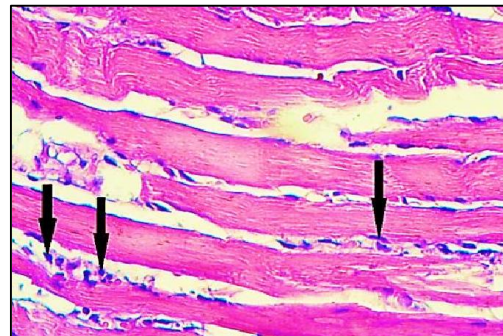


Figure (5): Longitudinal section of rabbit's Gastrocnemius muscle of G4-diabetic treated with hyperinsulinemia group after 14 days sciatic nerve crush injury, indicating some degrees of regeneration of muscle fibers; also there is increased the number of irregularly located muscle cell nuclei (Black arrows). Muscle fibers are characterized by crimping and waving (H&E 400X).

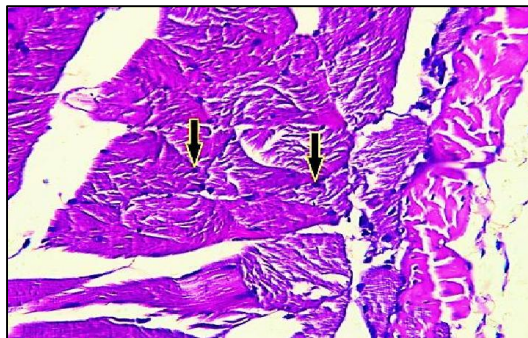


Figure (6): Transverse section of rabbit Gastrocnemius muscle of G5-diabetic treated with Vit. B complex group after 14 days of Sciatic Nerve crush injury, showing good normal size of muscle fibrils with formation of dark well stained muscle cell nuclei (Black arrows) but the contents of most myofibrils are still loose and not densely compacted in spite of some myofibrils which began to be dense and compact (H&E 400X).

DISCUSSION

Biochemical parameters were investigated in our study in order to examine the effect of diabetes and different treatment on the rabbit's serum insulin, total serum protein (TSP) and skeletal muscle protein.

Results in the (Table 1) indicated a significant increase ($p < 0.05$) of insulin in diabetic rabbits treated with an extracted ursolic acid with a higher level compared with the other groups. These results are assisted by those found by (25,26,27,28) who illustrated that natural components can enhance the release of insulin and/ or stimulate translocation of glucose transporter type 4 (GLUT4) and glucose uptake, they explained that ursolic acid represented a powerful antihyperglycemic activity, increased insulin secretion, insulin vesicle translocation, and increased glycogen content.

It seems from (Table 2) that alloxan induced diabetes did not influence skeletal muscle protein percentage, but increased total serum protein (TSP) significantly. It appeared also that treatment by ursolic acid either extracted or standard and hyperinsulinemia caused a significant increase ($p < 0.05$) in skeletal muscle protein percent and total serum protein (TSP) when compared with the positive control (diabetic group) either with or without crush injury of the sciatic nerve.

These results resemble that found by (29) when illustrated that ursolic acid has been identified as a powerful stimulator of muscle protein anabolism and increase muscle hypertrophy and maintaining of muscle protein by preventing proteolysis. Also, our results of the muscle protein percentage rise by hyperinsulinemia are in agreement with findings of (30) when explained that the physiologic raise of insulin enhance net muscle protein anabolism primarily by inhibiting protein breakdown rather than by stimulating protein synthesis.

After crush injury of alloxan induced diabetes rabbits and after treatment used in this study; numerous histological changes of Gastrocnemius muscle observed in slides taken from all groups of the experimentation those changes are demonstrated in histological figures of the Gastrocnemius muscle of experimental groups indicated in (Figures 1-6) which shows the destruction of skeletal muscle fibers and splitting fibers in addition to atrophy and increased thickness of fibrous tissues. Those changes happened to Gastrocnemius muscles of alloxan diabetic animals subjected to crush injury, but after treatment with ursolic acid either extracted or standard there were a good regeneration and muscle fibers regain their normal size after being atrophied before the treatment with ursolic acid these results come with confinement of what is found by (29) when found that ursolic acid extracted from apple peels can prevent muscular atrophy caused by muscle damage by poor peripheral innervation or by malnutrition. (29) also have been stated that ursolic acid increases brown fat and decreased diet induced obesity.

CONCLUSIONS

From the results of this experiment, we can conclude that alloxan induced diabetes causes degeneration of the peripheral nerve represented by the sciatic nerve. Treatment with ursolic acid specially extracted from apple peels can overcome degenerative processes in the Gastrocnemius muscle and have a good effect on biochemical parameters such as serum insulin, total serum protein (TSP) and skeletal muscle protein. Physiologic hyperinsulinemia has less effect on studying parameters compared to ursolic acid. Treatment with vitamin B complex gave very good results and can overcome bad results which occur due to diabetes and crush injury to the sciatic nerve and Gastrocnemius muscle.

تأثير حامض الاورسوليك، فرط الانسولين وفيتامين ب المركب على بعض القياسات الكيميائية الحيوية والتركيب النسيجي للعضلات الهيكلية في الارانب مستحدثة السكري بالالوكسان.

عبد الرزاق نعيم خضير , شيماء حسين شبع *

* قسم تقنيات العلاج الطبيعي، كلية التقنيات الصحية والطبية، الجامعة التقنية الوسطى، بغداد، العراق.
** فرع الفلسفة والادوية، كلية الطب البيطري، جامعة البصرة، بصرة، العراق.

الخلاصة

تهدف هذه الدراسة إلى تقييم تأثير حامض الأورسوليك، فرط الأنسولين وفيتامين ب المركب في العضلات الهيكلية والتي تمثلها عضلة الساق بعد سحق العصب الوركي في ذكور الأرانب مستحدثة السكري بالالوكسان، تحديد آثارها على بعض القياسات الكيميائية الحيوية التي تشمل الأنسولين، البروتين الكلي في مصل الدم (TSP) ونسبة البروتين في العضلات الهيكلية والكشف عن آثارها على التركيب النسيجي للعضلات الهيكلية ممثلة بعضلة الساق. حيث أظهرت نتائج دراستنا أن هناك زيادة معنوية في الأنسولين في الأرانب المستحدثة السكري بالالوكسان، وتستمر هذه الزيادة في جميع مجموعات العلاج وبمستوى تراوح بين (٣.٥٦-٨.٤ $\mu\text{g/ml}$) بينما أقل زيادة معنوية كانت في المجموعة المعالجة بفيتامين ب المركب. كما أظهرت دراستنا أن استحداث مرض السكري ليس له تأثير معنوي على نسبة البروتين في العضلات الهيكلية ولكن له تأثير معنوي على البروتين الكلي في الدم، في حين أن جميع مجموعات العلاج سببت زيادة معنوية في نسبة البروتين في العضلات الهيكلية والبروتين الكلي في مصل الدم. كما أظهرت المقاطع النسيجية لعضلة الساق بعض التغييرات التي حدثت بسبب عملية سحق العصب الوركي، مثل تدمير بعض ألياف العضلات الهيكلية، انقسامها وضمور الأخرى، كذلك هناك زيادة في سمك الأنسجة الليفية الفاصلة بينها. وظهر في المقاطع النسيجية لمجاميع المعالجة وجود إعادة بناء جيد للألياف العضلية مع القليل من الانقسامات فيها، أيضا هناك تشكيل واضح من نوى الخلايا العضلية، التي تشير إلى تحسن في العضلات بعد أنواع العلاج المختلفة.

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