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FORWARD

Dear Colleagues,

IJST was a fruitful effort issued by the International Centre for Advancement of Sciences and Technology – ICAST, which tries to take part in both globalization and revolution in information and communication technologies, because S&T development becoming not only the key elements of economic growth and industrial competitiveness, but also essential for improving the social development, the quality of life and global environment. ICAST took then a decision to establish a scientific alliance with TSTC (Tharwa for scientific Training & Consultations) and this alliance comes to support the efforts towards publishing IJST.

Today, we announce a new issue of our journal, that is the third issue from the thirteen volume of IJST, September , 2018.

Finally, I hope that all significant figures of sciences whom joined the editorial board, the researchers, and the readers of our journal will keep IJST between their eyes and contribute in continuing its journey, with their remarks, valuable recommendations and their researching outcomes.

Thanks a lot for all who support IJST.

Editor-in-Chief

IJST

Abdul Jabbar Al- Shammari

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TABLE OF CONTENTS

** Articles in this issue are listed below according to alphabetical order*

8-isoprostane in cord blood of preterm Iraqi neonates and their mothers

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ABSTRACT

Oxidative stress is a phenomenon caused by an imbalance between production and accumulation of oxygen reactive species (ROS) in cells and tissues and the ability of a biological system to detoxify these reactive products. Oxidative stress results as a change from a low oxygen pressure in utero to a high oxygen pressure at birth. Level of oxidative stress is important factor in embryogenesis, as well as in pregnancy and normal birth under conditions of oxygen deficiency in tissues.
This study had focused on a factor that affects preterm delivery which is one of the important determinants of birth weight.
It was concluded that low weight gain in pregnancy was associated with increased risk of preterm delivery.

Keywords: 8-isoprostane , Preterm infants

INTRODUCTION

Oxidative stress can be defined as increased formation of reactive oxygen species or decreased antioxidant defense system (1). High levels of reactive oxygen species can lead to cellular damage and death and extremely cytotoxic resulting from damage to bimolecular including lipid peroxidation, DNA and protein oxidation, which are collectively known as oxidative stress (2). 8-isoprostane is a product of lipid peroxidation that can be used as a measure of free radical exposure or injury. 8-isoprostane has been proposed as good indicator of oxidative stress.

Oxidative stress during pregnancy yields free radicals and other oxidative molecules exceeding the available antioxidant buffering capacity in the mother and growing fetus, which are resulting in cellular damage (3). Causes of oxidative stress activity changes are intensively investigated during experimental modeling of the processes that take place in the uterus and the placenta. Oxidative stress is considered to be responsible for the initiation or development of pathological processes, and has been associated not only with preterm labor and delivery (4), but also several pregnancy-related disorders as preeclampsia, intrauterine growth restriction (IUGR), premature rupture of membrane and gestational diabetes, as well as several serious post-delivery issues for the premature infant (5,6). Study have suggested that an impairment of fetal growth was a consequence of higher maternal oxidative stress as increased oxidative DNA damage was found in women who gave birth to LBW babies when compared to women with babies with normal birth nutritional status during pregnancy has a direct influence upon birth weight of newborn and adequate supply of micronutrient is known to be very important in pregnancy (7).

MATERIALS AND METHODS

The study was conducted at the Department of Obstetrics and Gynecology at Al-Yarmouk Teaching, Al- Imamain Kadhimain Medical City (PBUH), Baghdad in Iraq. Patients studied were admitted between October 2017 to March 2018 on 207 pregnant women with gestational age from 25th to 37th weeks, calculated by either of the first day of last menstrual period or by ultrasound scan obtained before 20 weeks of gestation. Pregnant women age ranged between (18-34) years.

The subjects in this study were divided into three main groups:

1- Extremely preterm includes 65 neonates and their mothers.

2-Very preterm includes 69 neonates and their mothers.

3-Moderate to late preterm includes 73 neonates and their mothers.

Women with an established medical risk factor for having reduced or excessive birth weight of the neonate such as, extremes of age (< 18 or > 34 years). Hypertension, toxemia of pregnancy, renal disease, heart disease, diabetes, urinary tract infection, metabolic disorder, tuberculosis, smoking, and alcohol or chronic drug intake were excluded from each groups. Infants were excluded if they had history of difficult delivery, fetal distress, congenital malformations, birth injury or any complication-requiring special care e.g. sepsis, seizure, respiratory distress, congenital heart disease, hypothermia, hypoglycemia and hyperbilirubinemia. Infants born to mothers who received sedatives /anesthesia within 4 hours prior to delivery were also excluded. All included newborns were delivered by vaginal route, with vertex presentation.

Concentration of 8-isoprostane in serum was determined by specific ELISA Kit for human (8-isoprostane) of US Biological Chemical Company, United States.

Statistical analysis

Data were analyzed by statistical packages of SPSS 18 (statistical packages for social sciences-version 18). All data were presented as a mean ± Sd. Statistical differences among groups were carried out by one-way analysis of variance (ANOVA). Correlation between the variables was performed by spearman correlation coefficient. P values were significant.

RESULTS

Among women investigated in this study, 65 women (31.4%) were in extremely preterm, 69 women (33.4%) in high preterm and 73 women (35.3%) showed moderate to late preterm (Figure 1).

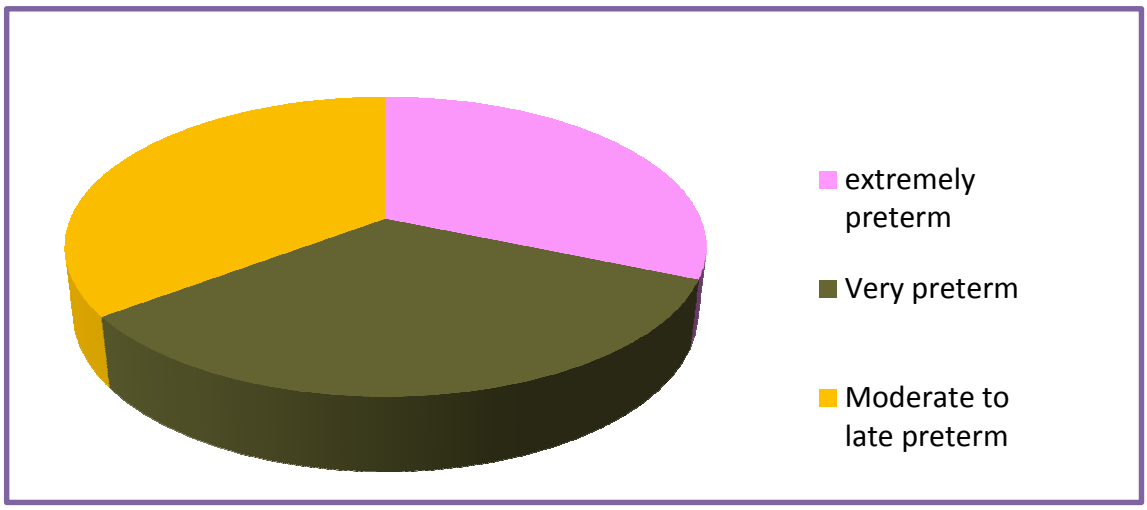

Figure (1): distribution of study cases

Demographic and anthropometric characteristics:

In this study the cases were compared regarding the maternal age range 18-34 year, gestational age ranges25-37 wks and parity ranges 1-3.
Table (1) shows that neonatal was significant difference in mean gestational age, weight, head circumference and length among study groups. While maternal was no significant difference in means age, parity, height among study groups by using one-way ANOVA test.
Table (2) revealed that there significant difference means of Gestational weight gain in Preterm, Term-LBW and Term-NBW, *p* value <0.001 by doing one-way ANOVA test.

Table (1): Demographic and anthropometric characteristics of studied groups

Characteristic	Extremely Preterm (n=65) Mean ±SD	Very preterm (n=69) Mean ±SD	Moderate to late preterm (n=73) Mean ±SD	*P* value
Neonatal				
Gestational age (wks)	26.42± 1.32	30.16 ±1.82	34.49±1.5	0.001
Birth weight (gm)	814.7 ±152.53	1014±564.11	2081 ±289.7	0.001
Birth Head circumference (cm)	24±1.32	28.49 ±0.89	31.73± 0.6	0.004
Birth length (cm)	40.12 ± 1.85	40.13±4.13	44.56± 1.7	0.001
Maternal				
Age (yrs)	24.38±4.43	25.13±4.45	24.8±3.82	0.627
Maternal Height (cm)	157.75±6.01	158.46±8.06	160.4±7.96	0.355
Parity	1.936±0.9	2.04±0.76	1.867±0.9	0.75

Table (2): Comparison of mean gestational weight gain in studied groups

Gestational weight gain (kg) Mean ±SD					
Pre-pregnancy BMI (Kg\m2) Category	**Underweight < 18.5**	**Normal weight 18.5-< 25**	**Overweight 25-< 30**	**Obese >30**	***P* value**
Preterm	9.07±1.38 N=110	8.6±1.0 N=82	5.86±0.9 N=15	----------	<0.001

8-isoprostane and gestational Age:

Table (3) and figure (2) revealed that there were significant difference means of cord and maternal 8-isoprostane in Preterm subgroups.

As shown in table (3), the effect of gestational age was compared on 8-isoprostane, and the result showed that mean of 8-isoprostane in cord and maternal were significant increase in extremely preterm compared with other subgroups of preterm.

Table (3): Comparison of mean 8-isoprostane in preterm subgroups

Parameters	Extremely preterm N=8 Mean±SD	Very preterm N=10 Mean±SD	Moderately to Late preterm N=13 Mean±SD	*P* value
Cord 8-isoprostane (pg/ml)	1013.88±137.95	809.4±157.17	505.3±144.7	0.002*
Maternal 8-isoprostane (pg/ml)	648.25±38.72	541.8±83.8	350.6±135.8	0.001*

**significant using one-way ANOVA test*

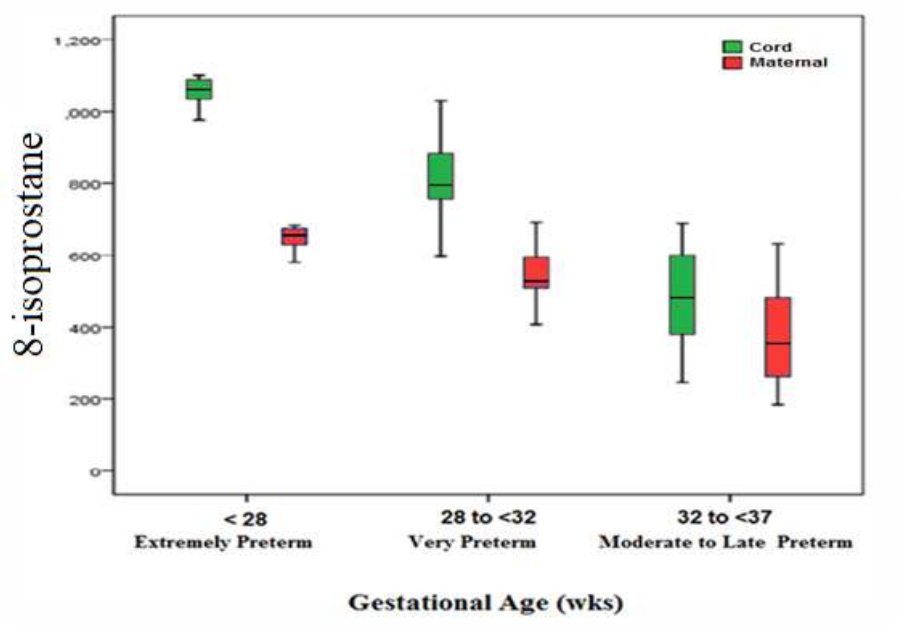

Figure (2): Box plot of cord and maternal 8-isoprostane according to gestational age (preterm subgroups): Box and whisker plot showing relative median cord and maternal of 8-isoprostane levels with 25th and 75th percentiles.

8-isoprostane and birth weight:

The majority of infants born <37 weeks, weighed <2500 grams at birth. Therefore, it was difficult to assess the influence of BW separate from GA. Figure (3) shows the box plot of 8- isoprostane on BW, which also decreased 8- isoprostane with increasing BW.

Table (4) shows the mean of 8- isoprostane that was significant highest value in ELBW, VLBW compared with LBW in studied cases.

However, mean of 8- isoprostane in ELBW, VLBW and LBW in cord blood more than maternal.

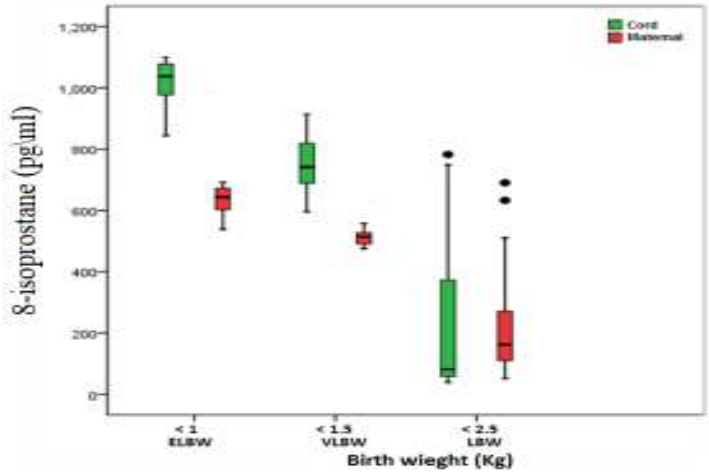

Figure (3): Box plot of cord and maternal 8- isoprostane according to birth weight of studied cases

Table (4): Comparison of means 8-isoprostane between all cases according to birth weight subgroups.

Parameters	ELBW N=53 Mean ±SD	VLBW N=51 Mean ±SD	LBW N=103 Mean ±SD	P value
Cord 8-isoprostane (pg/ml)	1002.1±153.75	664.875±187.5	232.24±256.14	<0.001*
Maternal 8-isoprostane (pg/ml)	651.8±37.169	498.5±116.02	205.44±132.10	<0.001*

**significant using one-way ANOVA test*

DISCUSSION

This study had focused on one of important factors that affect preterm delivery and low birth weight which is maternal nutrition. Extra nutrition is needed during the pregnancy and adequate intake supplement is advisable to decrease the incidence of adverse effects in the mother and the fetus (8,9).

Level of lipid peroxidation is important factor in pregnancy and normal birth Wight (10). In this study, the association of peripartum oxidative stress with gestational age were significant an inverse correlation with increasing gestational age. Cord and maternal serum levels of 8- isoprostane in preterm subgroups which were significant highest in extremely preterm as compared with very preterm and moderately to late preterm as shown in table (3) which were in accordance with another study reported by (11). Therefore, lipid peroxidation in preterm birth, and the relative influence of accompanying peripartum factors, appears to vary according to degree of prematurity.

LBW babies are at high risk of morbidities and mortality. Also their development is affected.

Also this study had described and evaluated the correlation of lipid peroxidation according to birth weight. It was found that cord and maternal levels of 8- isoprostane were significant higher in extremely-LBW as compared with very-LBW, and LBW.

Studies have been suggested that newborn with birth weight < 2500 gm showed relatively greater oxidative stress (12).

Cord serum level of 8-isoprostane were higher than those found in maternal serum and could reflect the oxidative challenge presented at birth, when there is transition from a relatively low intrauterine oxygen environment to a significantly higher extra uterine oxygen environment. Also at delivery, due to the rapidly increasing oxygenation during intensive breathing and labor efforts, shifts of the anti-oxidative system are activated, which in turn are caused by reactive oxygen particles produced in the uterus and placenta tissues (13).

The study had identified a novel physiological role for isoprostanes during postnatal vascular transition and provide evidence that oxidative stress may act on membrane lipids to produce vasoactive mediators that stimulate physiological DA closure at birth or induce pathological patency of the preterm DA (14).

Multiple factors, including BMI before pregnancy is often used as a marker of the mother's nutritional status, and can affect the birth weight of neonates (15-17).

However, in the present study, maternal pre-pregnancy BMI was normal and it was found no significant correlation with low birth weight but maternal gestational weight gain is significant positively influenced with LBW ,similar to the findings of the present study , showed that according to normal pre-pregnancy BMI, gestational weight gain can predict intrauterine fetal weight as well as infant birth weight (18).

It was concluded that low weight gain in pregnancy was associated with increased risk of preterm delivery (19).

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Partial purification of laccase isolated from local fungi degrading Aflatoxin B1

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ABSTRACT

Six fungal isolates have been evaluated for laccase production *Fusarium sp1, Fusarium sp2*, *Pleurotus ostereatu*s, *Pleurotus sapidus*, *Rhizoctonia solani* and *Trichoderma harizanum*. The isolates were subjected to two stages of screening process to select the highest extracellular laccase production. It has been found that *pleurotus sapidus* had the highest productivity of the enzyme. Purification process included two steps dialysis using dialysis back and ion exchange chromatography using DEAE- cellulose, the purified enzyme had fold of purification about 3.14 times with 29.56% recovery.

Aflatoxin B1 degradation study showed that the remaining AFB1 was obtained using crude laccase was 0.208µg/ml while remaining AFB1 using partially purified laccase was 0.662 µg/ml after 72hr incubation and 150 rpm.

The study recommended the following:

1. Investigating other source of laccase producer.
2. Enhancement laccase production using mutation or inducers.
3. Increasing the stability of laccase by immobilization technique.
4. Studying the degradation of other mycotoxin by fungal laccase

Keywords: *Pleurotus sapidus*, *Rhizoctonia solani , Trichoderma harizanum* , *Pleurotus ostereatu*s, AFB1.

INTRODUCTION

Aflatoxins are secondary metabolites produced by the molds *Aspergillus flavus* and *Aspergillus parasiticus.* Aflatoxin-producing molds are widely distributed in nature. Agricultural commodities are susceptible to mold damage during pre- as well as post-harvest stages of production (1). Aflatoxin B1 (AFB1) is considered the most potent, having hepatotoxic, hepatocarcinogenic, mutagenic, and teratogenic effects in many animal species, and classified as a Group I carcinogen in humans (2). Specific enzymes that are capable of degrading aflatoxins have been purified from microbial systems, the detoxification by specific enzymes avoids the drawback of using the microorganism, which may, in addition to its degradation activity, change flavor or impair the nutritional value and acceptability of the product (3). Treatment of Aflatoxin B1 with laccase enzyme produced by white rot fungi in unconcentrated culture filtrates cause its degradation (4).

Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2.) is a multi-copper blue oxidase capable of oxidizing ortho- and para-diphenols an aromatic amines by removing an electron and a proton from a hydroxyl group to form a free radical (5). In most cases laccases are monomeric glycoproteins contain around 500 amino acids with molecular weights in the range 60–85 kDa, depending on the carbohydrate content (6).

Over 60 fungal strains belonging to Ascomycetes, Deuteromycetes and especially Basidiomycetes show laccase activities (7). *Pleurotus spp*., commonly known as oyster fungus, is a common primary decomposer of wood and vegetal residues (8). Oyster mushrooms are found growing in the wild on dead organic matter from tropical and temperate regions (9). *Pleurotus sapidus* is a member of the oyster mushroom family, because of their pleasant flavor and high nutritional value; oyster mushrooms are highly valued as edible fungi all around the world (10).

This study is considered as an attempt to biodegrade Aflatoxin B1 by fungal laccase throughout conducting the following steps:

1. Screening of laccase producing fungi using solid media and submerged liquid media.
2. Purification and characterization of partially purified laccase.
3. Degradation of Aflatoxin B1 using crude and partially purified laccase.

MATERIALS AND METHODS

Primary screening using guaiacol (11):

Five milliliter agar disk plug of fungal isolates were cultivated on guaiacol agar medium and incubated at 25 °C for 7 days, the production of reddish brown zones in the medium indicated positive result.

Secondary screening for laccase producing fungi:

Secondary screening carried out by dispensing 50 ml of laccase production medium (12) in 250 ml Erlenmeyer flasks and inoculated with 5mm agar disc plug of the tested fungal isolates. After incubation on a rotary shaker for 7days at 28 °C and 150 rpm. Laccase-containing culture fluid was separated from the mycelium using a piece of gauze, then centrifuged at 6000 rpm for 15 min. Laccase activity was determined according to the method described by (13).

Enzyme purification:

Crude enzyme was dialyzed against 0.1M of citrate phosphate buffer (pH 5.6) with three times buffer changes, then ion exchange chromatography was done for dialyzed sample using DEAE-cellulose column. DEAE-cellulose exchanger prepared according to the instruction of the Pharmacia Fine Chemical Company. To 1500 ml cylinder 1 liter of distilled water was added to 20 g of exchanger and left to stagnate. Then the upper liquid was removed and the exchanger was washed with distilled water several times until the liquid has become clear and filtrated under vacuum. Sediment was suspended in a solution of 0.25M sodium hydroxide. The filtrate washed several times with distilled water and then with 0.25M solution of hydrochloric acid. After that the filtrate washed several times with distilled water and suspended with 0.01M citrate phosphate buffer pH 5.6 and degassing by vacuum pump. Glass wool was inserted to the bottom of column and the exchanger packed in the glass column with dimensions (2x18 cm). Then 0.01M citrate phosphate buffer pH5.6 added continuously until reaching the equilibrium (pH becomes 5.6). After column equilibrium, 5ml of dialyzed sample was poured gently on the surface of the column, then it was washed by 0.01M citrate phosphate buffer pH5.6 with flow rate of 0.5 ml /min (30 ml / h) and 3ml for each fraction to displace unbinding proteins (wash), fractions were collected from column. After that binding proteins in the column was eluted by linear gradient NaCl (0-1M) dissolved in 0.01M citrate phosphate buffer pH. It was plotted the relationship between the absorption wavelength of 280 nm and fraction which result from washing and elution steps. The activity fractions of two steps were collected separately. The enzyme activity and protein concentration were estimated.

Degradation of Afflation B1 (4):

Crude laccase filtrated through gauze and the laccase activity was determined (U/ml). one ml of crude was added to test tubes separately, each tube contain 0.2ml (8µg/ml) of Aflatoxin B1. The final concentration was 1.33 µg/ml. Then the tubes incubated at different times (24hrs, 48hrs and 72hrs) at 15° C in static conditions. One ml of (crude and partially purified laccase) was added to test tubes separately, each tube contain 0.2ml (8µg/ml) of Aflatoxin B1 and the final concentration was 1.33 µg/ml, then the tubes incubated for 72hr at 15° C in shaking incubator 150rpm. Aflatoxin B1 extracted three times by chloroform. The solvent

evaporated, then dried extract dissolved in 1ml acetonitrale. Then AFB1 was detected using HPLC analytic system.

Analytic system of Aflatoxin B1 by High Performance Liquid Chromatography (HPLC):

HPLC assay. The analyses were carried out *using* by High Performance Liquid Chromatography (HPLC)system (the column type is (250 × 4.6 mm ODS – C 18), mobile phase: water: acetonitrile (60:40) , flow rate: 1ml / min, detector: UV – 365 nm, the CS: 0.5, then AT: 8 , the PT is 1000 , and The retention time of AFB1: 7.6 min.

RESULTS AND DISCUSSION

Primary screening using guaiacol:

A simple screening method was followed in order to detect laccase producing fungi on solid media containing 0.02% guaiacol as indicator compound. Six fungal isolates were screened (*pleurotus sapidus, pleurotus ostreatus*, *Fusarium* sp1, *Fusarium* sp 2, *Rhizoctonia solani* and *Trichoderma harzianum*). Among these only *p. sapidus* and *Fusarium* sp 2 showed positive results when reddish brown zone around colonies was formed (figure 1).

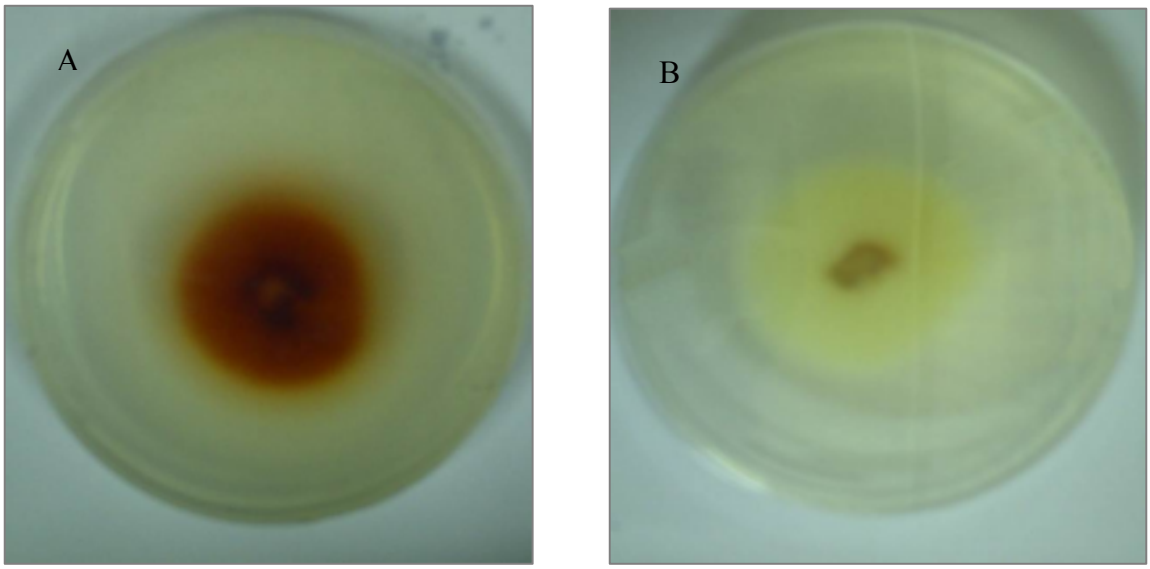

Figure (1): Primary screening of laccase producing fungi using solid media containing 0.02% guaiacol as indicator compound after 4 days of incubation at 25 °C. (A) *p. sapidus* (B) *p. ostreatus* as control negative

Appearance of reddish brown zone in the medium resulted from the oxidative polymerization of guaiacol (14). Artiningish *et al.*, (15) had found that 12 isolates of white rot fungi gave a reddish brown color on a solid media containing 0.5% guaiacol, meanwhile 6 isolates of brown rot fungi were formed very light reddish brown color and 7 isolates gave negative reaction.

Twelve isolates of white rot fungi were tested using 0.02% guaiacol, Positive results for guaiacol oxidation appeared only with six fungal isolates (11).

Secondary screening of laccase producing fungi:

The ability of six fungal isolates for laccase production was tested using submerged culture. Enzyme activity was determined using syringaldazine as substrate. Results in figure (2) indicated that the highest laccase production was observed with *p. sapidus*, laccase activity reached up to (0.064 U/ml), while the activity for the other isolates was ranged between (0.022 - 0.054 U/ml).

According to these results, *P. sapidus* was selected to be used for improving laccase production.

Linke *et al.* (10) demonstrated that the edible fungus *P. sapidus* which grow on submerged liquid medium was selected as the most active laccase producer among Basidiomycota.

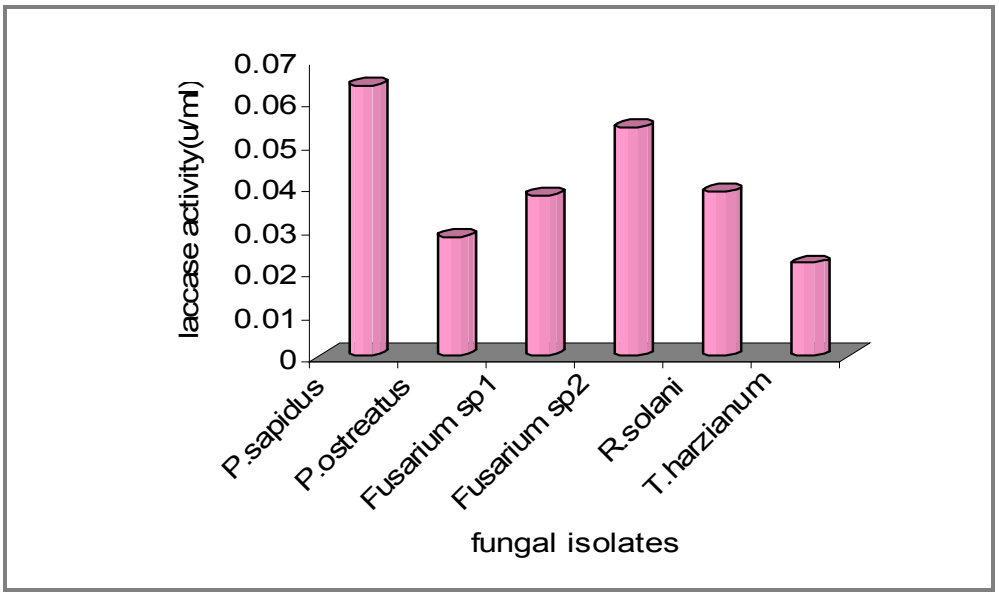

Figure (2): Secondary screening of laccase producing fungi using submerged liquid culture after incubation at 28 ˚C for 7 days and 150 rpm

Laccase purification:

Dialysis: After using dialysis back (10 KDa cut off) with three times buffer changes. The specific activity was 8.7U/mg, purification fold was 2.4 and its yield was 95.5% as shown in table (1). Results of ion exchange chromatography point out that there was no peak of protein in washing step, while four peaks of protein were separated when eluted association proteins, with the appearance of enzymatic activity in two peaks from (120-122 fraction) and (125-129 fractions) as shown in figure (3).

Presence of two activity peaks in elution steps mean that the laccase from *p. sapidus* had isoenzyme forms; in the word the enzyme purified from elution steps had a negative charge adverse to the ion exchanger. The fractions owns the activity of enzyme which was collected for each two peaks; the peaks of elution steps has the specific activity 11.5 U/mg protein, purification fold was 3.14 and its yield was 29.56%. Wood, (16) purified laccase from *Agaricus bisporus* by ion exchange chromatography on DEAE-cellulose after using ultra filtration in amicon membrane (molecular weight cut-off, 10KDa) and ammonium sulphate precipitation 90% saturation, specific activity was 7.20 U/mg, the yield was 60% and the purification fold was 31.4. Sarnthima and Khammuang, (17) purified crude *Pleurotus sajor-caju* laccase using DEAE-cellulose column. Two peaks showing laccase activity were eluted out, and they mentioned that laccase has isozyme form. while *Lentinus polychrous* laccase purified by DEAE – cellulose after using ammonium sulphate precipitation (85% saturation) and Sephacryl S-300 , the specific activity was 14.0 U/mg, yield was 5.6 and purification fold was 5.1(18).

Table (1): Purification steps of laccase from *p. sapidus*

Purification steps	Volumes (ml)	Activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Total Activity (U)	Purification fold	Yield (%)
Crude extract	47	0.132	0.036	3.66	6.204	1	100
Dialysis	52	0.114	0.013	8.77	5.928	2.4	95.5
Ion exchange chromatography by DEAE cellulose	20	0.092	0.008	11.5	1.84	3.14	29.56

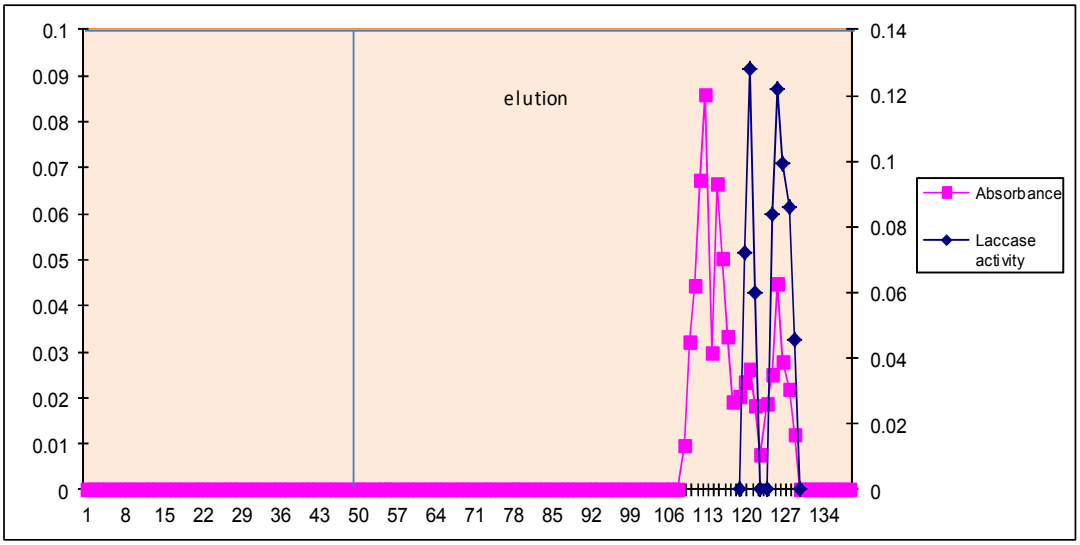

Figure (3): Ion exchange chromatography by using DEAE – cellulose column (2×18 cm), Elution step; enzyme eluted with linear salt gradient 0.01M citrate phosphate buffer – 1 M NaCl pH 5.6 at flow rate of 30 ml /hr (3ml for each fraction)

Degradation of AFB1:

To determine the ability of *p.sapidus* laccase to degrade AFB1, samples containing 0.2 ml of 8 µg/ml AFB1 were supplemented with 1 ml of crude laccase. Result in figure (4) showed that crude laccase was able to degrade AFB1 and its ability of degradation increases with increasing the incubation period, and the most effective degradation of AFB1 was obtained after 72 hrs of incubation, the remaining AFB1 was (0.542µg/ml).

The degradation of AFB1 after 72 hrs by partially purified laccase using shaking incubator was (0.662 µg/ml) while the best degradation obtained by crude laccase (0.208µg/ml) as shown in figure (5).

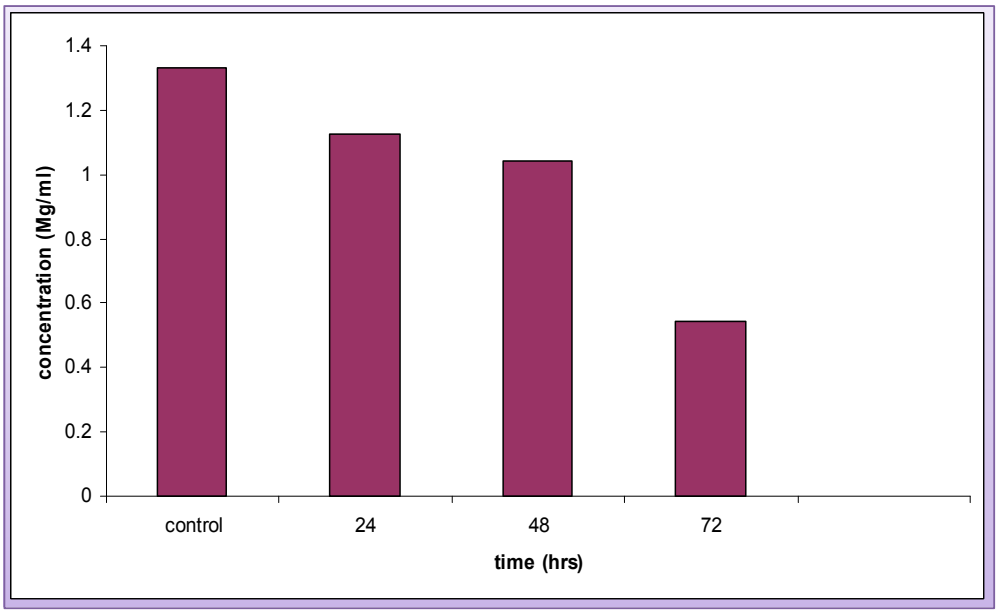

Figure (4): Bio degradation of AFB1 by crude fungal laccase (0.3 U/ml) produced by *p.sapidus* using different incubation period (24, 48 and 72 hrs) at 15°C in static conditions

Degradation of AFB1 by crude laccase was better than partially purified laccase, its may be due to other enzymes that occur with laccase cause the degradation. Baldrian *et al*. (7) reported that *P.ostreatus* degrades a variety of polycyclic aromatic hydrocarbons and the major enzymes involved were manganese-dependent peroxidases and laccases. It is well known that degradation of AFB1 by un-concentrated supernatant of *P. ostreatus* occur by unidentified enzyme, which was not a peroxidase (19).

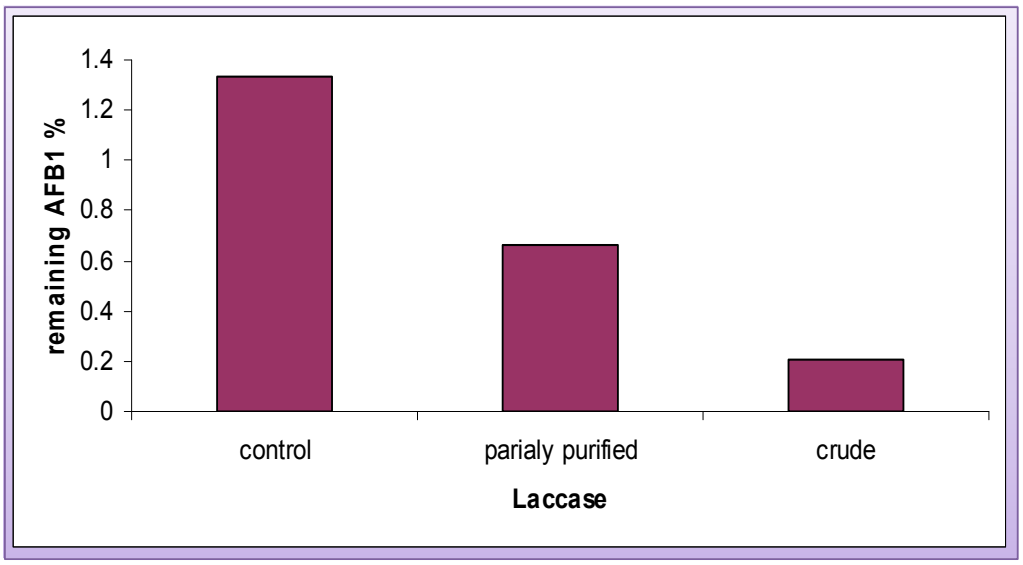

Figure (5): Bio degradation of AFB1 by crude and partially purified fungal laccase produced by *p.sapidus* incubated for 72hr at 15°C and 150 rpm

The treatment of AFB1 with fungal laccase enzymes targets and changes the double bond of the furofuran ring of the AFB1 molecule and as a result influences its fluorescence and mutagenicity properties (20). *Peniophora* sp. produced the highest laccase activity using ABTS as substrate (496 U/L) and AFB1 degradation was 40.45%, while *P. ostreatus* resulted in laccase activity of 416.39 U/L and 35.90% degradation of AFB1 (4).

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Purification and partially characterization of *Echinococcus granulosus* protoscoleces acid phosphatase

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ABSTRACT

Acid phosphatase (E C 3.1.3.2) was purified and characterized from protoscoleces of *Echinococcus granulosus* using ammonium sulfate precipitation , CM-Cellulose column chromatography and gel filtration on Sephadex G-75 .The enzyme was purified by a factor of 36.2 fold with yield 43 % and a specific activity of 103.91 µmole/min/mg protein .The enzyme has a molecular weight of 120000 dalton as determined by gel filtration on Sephadex G-200 .The enzyme has a broad substrate specificity ; it hydrolysed organic phosphates , sugar phosphates and nucleotide phosphates .The km value determined with p-nitrophenylpyrophosphate at pH optimum (4.5) was 0.43mM .The enzyme was inhibited by L-(+)-tartaric acid.

Keywords: purification, acid phosphatase, protoscoleces, *Echinococcus granulosus*

INTRODUCTION

Echinococcus granulosus is a small endoparasitic cestoda of carnivores (dog), has a cosmopolitan distribution, utilizes ungulates (sheep, cattle, goats, horses, etc) as intermediate hosts and causes a form of diseases known as hydatidosis or echinococcosis in human (1,2) .

The acid phosphatase (phosphoric monoester hydrolase, EC 3.1.3.2) comprise a family of isoenzymes involved in the supplement of a usable energy source to the parasite through hydrolysis a broad range of sugar phosphates, phosphoprotein and inositol phosphates (3,4). A number of studies have been performed to elucidate the occurrence of an acid phosphatase in the protoscoleces of *E.granulosus* (5,6). The finding that a tartrate – resistant acid phosphatase purified from promastigote forms of *Leishmania donovani* (7) inhibited the production of oxygen metabolites by neutrophiles (8) raised the possibility that this enzyme may be crucial for survival of the parasite upon entry into macrophages (9).

The current study aimed to investigate the purification of acid phosphatase which could play crucial role in the clinical identification of the human hydatidosis caused by *E .granulosus.*

MATERIALS AND METHODS

Parasites:

Protoscoleces of *E.granulosus* were obtained from liver and lung hydatid cysts of sheep slaughtered in a slaughterhouse in Kirkuk. They were washed three times with medium 199 (HIMEDIA, India, 1.052 gm M 199, 35 mg $NaHCO_3$, 100 ml distilled water, pH 7.2) and stored at -70C° (10).

Preparation of enzyme extraction:

Protoscoleces were homogenized in three volumes of 50mM Tris-HCl , pH 7.2 containing 0.2 mM sucrose and 0.1 mM dithiothreitol by freeze-thawing three times in liquid nitrogen and were sonicated using ultrasonic disintegrator for 10 sec on and 5sec off on ice until no intact protoscoleces were visible microscopically .The crude homogenate was then centrifuged at 10000 g for 30 min at 4C° and the resultant supernatant was stored at -70 C° (11).

Purification of the enzyme;

1. Homogenization: The supernatant resulted as described above was used as source of the enzyme for further purification (4).

2. Ammonium sulfate fractionation: Ammonium sulfate $(NH_4)_2SO_4$ was added slowly to the supernatant with stirring to give 70% saturation. The precipitate was collected by centrifugation at 10000 g for 10 min and dissolved in 50 mM sodium acetate pH 6.0.

3. CM-Cellulose chromatography: The supernatant from the previous step was applied to a 2.6x10 cm CM-Cellulose column equilibrated with 50 mM sodium acetate, pH 6.0. The elution was performed with the same buffer at a rate of 8 ml per hour. The fractions containing most of the enzyme activity were pooled and concentrated.

4. Sephadex G-75 chromatography: The pooled enzyme from the previous step was applied to a 0.5cm x 1cm column of Sephadex G-75 previously equilibrated in 50mM sodium acetate buffer, pH 6.0. The enzyme was eluted using the same buffer. Fractions containing acid phosphatase were pooled, dialyzed against standard buffer and concentrated.

Molecular weight estimation:

The method of Andrews (12) was used to estimate the molecular weight using the following proteins of known molecular weight as standards: Trypsinogen (Tryp22000); malate dehydrogenase (MDH 68000) and Lactate dehydrogenase (LDH 14000).

Enzyme assay:

Acid phosphatase assay mixture contained of 50 mM sodium acetate, pH 4.5, 1mM p-nitrophenylpyrophosphate and enzyme solution in a volume of 0.4 ml .After incubation at 37C° for 30min . 1ml of 0.1 N NaOH was add and the released p-nitrophenol was estimated spectrophotometrically at 410 nm using an extinction coefficient of $14.3x10^{-3}$ M^{-1} cm^{-1} . Acid phosphatase activity towards other substrates was assayed by determining the amount of inorganic phosphate released during incubation for 30 min at 37 C°. Each phosphate compound was tested at pH 4.5 in 0.4 ml of reaction mixture at 1mM concentration. The reaction was terminated and inorganic phosphate estimated as described by (3). Units of enzymatic activity are expressed in micromoles of product per 1 minute. Specific activity is expressed as units per mg protein.

Protein determination:

Protein concentrations were estimated by the method of Lowery *et al*., (13) with bovine serum albumin as standard.

Kinetic studies:

A time-course and substrate-dependent assays were performed and the apparent Km value for substrate was determined by the Lineweaver-Burk method (14). The effect of pH on the activity was determined by using the following buffers, sodium acetate, 50mM (pH 3.5-6.0) and Tris-maleate, 50 mM (pH 6.0-9.0). The effect of inhibitor on enzyme activities was assessed by per-incubating the extract with the inhibitor for 3 min before initiating the assay.

RESULTS

Purification of the acid phosphatase of *Echinococcus granulosus protoscoleces*:

As shown in table (1), approximately 61% of the total acid phosphatase activity in the protoscoleces of *E.granulosus* was present in the high-speed supernatant fraction. The ammonium sulphate treatment of the high-speed supernatant removed the majority of the contaminating proteins. The CM-Cellulose chromatography resulted in 79.8 % of the enzyme and about 91.95% of fractions were eluted from a Sephadex G-75 column chromatography. The overall yield from the purification was 43% and the overall purification achieved was 36 fold with a specific activity of 103.91 µmole/min/mg protein (Table 2).

Table (1): Distribution of acid phosphatase in subcellular fractions of *Echinococcus granulosus* protoscoleces

Fraction	Volume (ml)	Enzyme activity µmole/min	Total activity	Enzyme activity %
Crude homogenate	52	1.34	6968	100
pellet	45	0.61	2745	39
Supernatant	45	0.957	4307	61

Table (2): Purification of acid phosphatase from protoscoleces of *Echinococcus granulosus*

Purification step	Total protein (mg)	Total activity (µmole/min)	Specific activity µmole/min/mg	Purification fold	Yield %
Supernatant	1496	4307	2.87	1	100
Ammonium Sulphate	371	2535	6.83	2.37	59
CM-Cellulose	117	2023	17.29	6.02	47
Sephadex G-75	17.9	1860	103.91	36.2	43

Molecular weight determination:

The molecular weight of the purified acid phosphatase of *E.granulosus* protoscoleces was determined by gel filtration on Sephadex G-200.The elution position of the native enzyme correspond to a molecular weight of 120000 dalton as compared to proteins of known molecular weight (Figure 1).

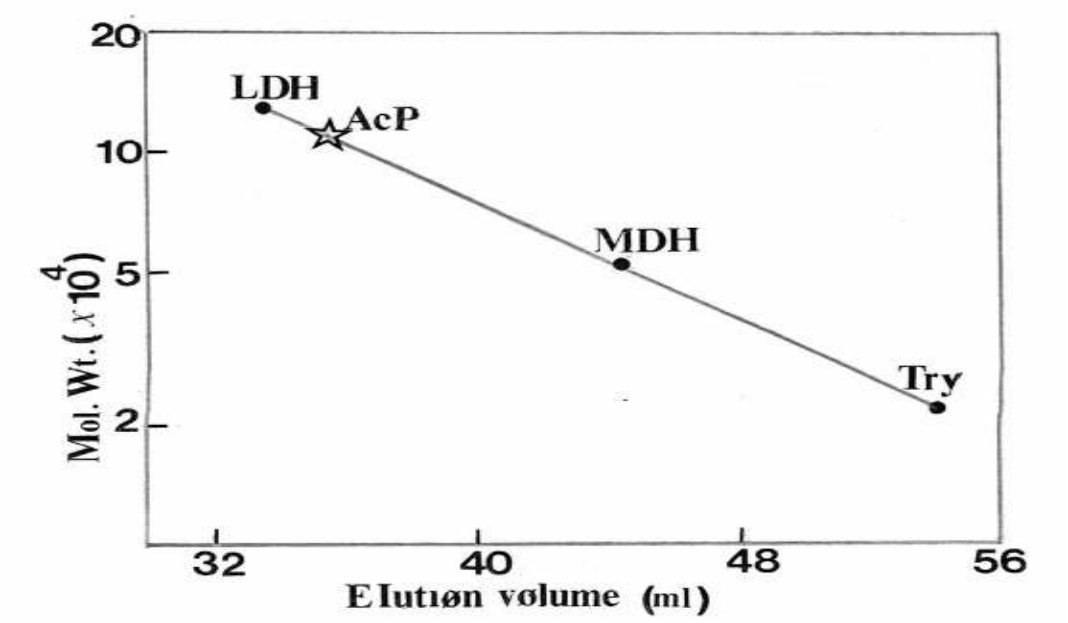


Figure (1): Determination of the molecular weight of the acid phosphatase by gel filtration on Sephadex G-200 . Experimental details are given in the text. The star represents the acid phosphatase (AcP).

Optimum pH and substrate specificity:

The optimum pH of the purified acid phosphatase was at pH 4.5 with P-nitrophenylpyrophosphate as the substrate (figure 2). The purified acid phosphatase of *E.granulosus* protoscoleces was assayed for activity against variety phosphate esters (table 3). The relative activities of the acid phosphatase toward different substrates were expressed as percentage based on the activity for P-nitrophenylpyrophosphate. The most active hydrolysis was directed toward P-nitrophenylpyrophaste. Other physiologically important phosphate including α-naphthyl-1-phosphate and methylumbelliferyl phosphate were hydrolysed at significant rates. On the other hand, glucose-1-phosphate, adenosine-5-monophosphate and glycerol-1-phosphate were degraded to much lesser extents.

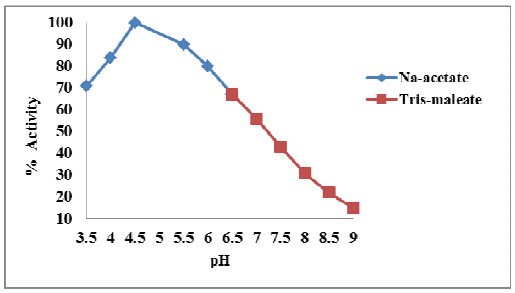


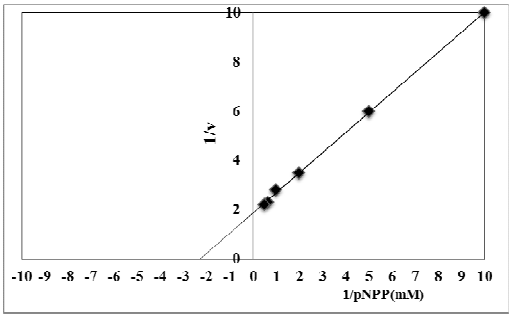
Figure (2): The effect of pH on the activity of the acid phosphatase purified from protoscoleces of *Echinococcus granulosus*

Table (3): Substrate specificities of *Echinococcus granulosus protoscoleces* acid phosphatase

Substrate	Relative rate of hydrolysis
P-nitrophenylpyrophosphate	100
Naphthyl-1-phosphate	64
Glucose-1-phosphate	33
Glycerol-1-phosphate	11
Adenosine monophosphate	22
Methylumbelliferyl phosphate	50

Km value:

The km value for the acid phosphatase of *E.granulosus* protoscoleces was almost 0.43 mM with P-nitrophenylpyrophosphate as substrate (figure 3).



***F*igure (3): Lineweaver- Burk plot for the determination of km value for p-nitrophenylpyrophosphate (pNPP).**

Effect of inhibitors:

The purified acid phosphatase activity of *E.granulosus* protoscoleces was strongly inhibited by sodium potassium tartrate but was resistant to sodium fluoride and EDTA (table 4) . On the other hand , the acid phosphatase activity was inhibited to a considerable degree by N-Ethylmaleimide, ascorbate and p-chloromercuribenzoate (table 5) but there was no inhibition by dithiothreitol and cysteine.

Table (4): Effect of common inhibitors on the acid phosphatase activity of the *Echinococcus granulosus* protoscoleces

Inhibitors	Concentration	% Inhibition
Na k tartrate	0.01	80
Na k tartrate	0.1	82
Na k tartrate	1	86
Na k tartrate	10	88
NaF	0.01	NI
NaF	0.1	NI
NaF	1	NI
NaF	10	NI
EDTA	0.01	NI
EDTA	0.1	NI
EDTA	1	NI
EDTA	10	NI

NI: no inhibitor

Table (5): Effect of various inhibitors (1 mM concentration) on the purified acid phosphatese from *Echinococcus granulosus* protoscoleces

Inhibitor	%Inhibition
N-Ethylmaleimide	54
Glutathione	19
Dithiothreitol	0
Cysteine	0
Ascorbate	66
p-chloromercuribenzoate	67

DISCUSSION

Several of our observations indicate that the partially purified enzyme can be designated as a non-specific acid phosphatase (E C 3.1.3.2). The purified acid phosphatase of *Echinococcus granulosus* protoscoleces closely resembles those of animal, parasitie helminths, parasitic protozoa, bacteria and plant in molecular weight (90000-120000) and optimum pH (4.0-5.0) (15-18). The substrate specificity of the *E.granulosus* protoscoleces acid phosphatase differs somewhat from prostatic acid phosphatase which has been most extensively studied among the animal origin acid phosphatase (19). The prostatic acid phosphatase is true phosphomonoesterase in that it did not hydrolyze terminal pyrophosphate bonds such as ADP or ATP (20). Nonspecific acid phosphatases, which hydrolyze terminal pyrophosphate bonds at the same rate for p-nitrophenylpyrophosphate, have also been found in the induced enzymes of protozoa, bacteria and plants (21-23). It is believed that acid phosphatase of *E.granulosus* protoscoleces would be classified as a non-specific acid phosphatase, since the substrate specificity of this enzyme was rather broad for 4-methylumbelliferyl phosphate, nucleotide phosphate and sugar phosphate and it is able to hydrolyze pyrophosphates. It is noteworthy that (3,4,24) have shown that in the parasitic helminths, the prominent acid phosphatase is a broad specificity enzyme that hydrolyzes adenosine-5-monophosphate and glucose-6-phosphate as well as 4-methylumbelliferyl phosphate.

The acid phosphatase of *E.granulosus* protoscoleces is not stimulated by divalent cations and is inhibited by L-(+)-tartaric acid .In these respect, it resembles lysosomal enzymes of invertebrates (25-27).

Experiments have shown that 39% of the enzyme activity in a crude homogenate of *E.granulosus* protoscoleces is firmly bound to particles; the remaining 61% of the activity is found in the high speed supernatant. Although the relationship of the membrane bound acid phosphatase and cytosolic soluble acid phosphatase to each other is unknown, the possibility exists that *E.granulosus* protoscoleces may contain as many as two or three different enzymes.

Disclosure of the physiological function of the acid phosphatase of *E.granulosus* protoscoleces is of great importance for better understanding of the cell biology of this organism but it remains unknown. It has been thought that the protozoan acid phosphatase would provide inorganic phosphates necessary for cell growth by degrading of phosphate esters present in the culture medium (9). Consequently one would expect that nonspecific acid phosphatase of *E.granulosus* protoscoleces may play a role in providing nutritional inorganic phosphate for cell growth.

Acknowledgements

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Related study between protein tyrosine kinase with some biochemical parameters in diabetes mellitus type2 and diabetic nephropathy Iraqi patients

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ABSTRACT

Diabetes mellitus most important disease can be definition as a metabolic disorder disease and complication of diabetes due to diabetic nephropathy. This study was done *in vitro* to study protein tyrosine kinase enzyme and some biochemical parameters that vital role in diabetes mellitus type 2 such as fasting blood sugar, HbA1C, insulin, c-peptide and find the correlation between this enzyme with the parameters. This study was conducted in The National Diabetes Center, Al-Mustansiriya University - Baghdad and included 150 patients divided in to three groups the first group contain 50 Iraqi patients newly diagnosis type 2 diabetic as group (G2) and the second group contain 50patients diabetic nephropathy as group(G3) and the last group contain 50 healthy as control group(G1). The period times for aggregation the blood sampling July to October 2017. All patients were within (18 to 60) year's age. The present study showed that in newly diagnosis diabetes mellitus appeared elevated levels of serum protein tyrosine kinase (PTK) , FBS, HbA1C, C-Peptide ,Insulin , while elevated levels of serum protein tyrosine kinase (PTK) , FBS, HbA1C, C-Peptide ,Insulin in diabetic nephropathy . Correlation study revealed Non-significant positive correlation between tyrosine kinase with FBG, HbA1C,Insulin,C-peptide in diabetes type2 newly diagnosis as well as in diabetic nephropathy patients correlation study revealed Non - significant positive correlation between tyrosine kinase with FBG, HbA1C. While Non- significant negative correlation between tyrosine kinase with Insulin, C-peptide.

Keywords: C-Peptide, diabetic nephropathy, diabetes mellitus, FBS, HbA1C, Insulin

INTRODUCTION

Diabetes mellitus can be definition is a metabolic disorder disease categorized hyperglycemia resulting from defects in insulin secretion, insulin action or both (1). Signs of hyperglycemia often contain polyuria, polydipsia, weight loss, polyphagia, and unclear vision (2). Chronic symptoms of diabetes include micro vascular and macro vascular complications (3). The classification and diagnosis of diabetes established by the National Diabetes Data Group published in 1979 (4) and classified to Type or juvenile (5) whereas the body does not yield insulin. While type 2 diabetes mellitus results when the body cans no longer enough insulin to requite impaired ability to use insulin. Also to Gestational diabetes (6), its metabolic disorder grows during pregnancy (7). Diabetic nephropathy is one of the most important long-term complications regarding morbidity and mortality in diabetics. The clinical syndrome is recognized by continual albuminuria and developmental decreased in the glomerular filtration rate (GFR) (8). Protein tyrosine kinase (PTK) an enzyme (a class of proteins) with tyrosine kinase activity that catalyzes the transfer of phosphate groups on adenosine tri phosphate (ATP) to the tyrosine residues of many important proteins making proteins phosphorylation, then transferring signal to regulate cell growth differentiation, death and a series of physiological and biochemical processes (9). and classified in two groups: receptor tyrosine kinases and Non - receptor(cytoplasmic) this enzyme have a bio vital role for signal transduction and lead to regulatory major cellular processes such as metabolism, transcription, cell cycle progression, cytoskeletal arrangement, apoptosis, differentiation and cell movement (10) any change in kinase signal transduction via mutation, amplification or alteration may be etiology many diseases (11). Therefore protein tyrosine kinases currently consider the targets use for therapeutic to treated diseases like diabetes, neurological, autoimmune disorders, and cancer (12). Glucose is essential role of fuel for the body, exactly for the brain and red blood cells, the level of glucose is around 90mg/dL (13). Hemoglobin A1c (HbA1c) generally detected glycosylated hemoglobin which measured long term blood glucose (14). Insulin construction is a protein hormone with 2 poly peptide chains A and B composed of 51 amino acid residues split into two molecules one of them is C-peptide another is insulin therefore C-peptide is equal the insulin concentration also c-peptide only minimally extracted by the liver and this reflect the secretion of ß-cells more rightly than insulin (15).

MATERIALS AND METHODS

This study was conducted in The National Diabetes Center, Al-Mustansiriya University - Baghdad and included 150 patients divided into three groups. The first group contained 50 Iraqi patients newly diagnosis type 2 diabetic as group (G2), the second group contained 50 patients diabetic nephropathy as group (G3), and the last group contained 50 healthy as control group (G1). The period time for aggregating the blood sampling was from July to October 2017. All patients were within (18 to 60) years old. Exclusion criteria include alcoholic patients, hypertensive, acute illness or infection at time of sampling. Blood sampling was performed at 8.00-10.00 a.m. in the fasting state for all subjects and Five milliliters of venous blood were obtained by antecubital venipuncture divided into two parts, one of them (2 ml) was put in tube containing anticoagulation [EDTA] to measure HbA1C, while another part (3ml) of blood was put in plan tube to evaluate glucose, by centrifugation at 3500 rpm for 5 minutes. Then the serum was stored in -20c° to measure other parameters by ELISA method (C-peptide, serum insulin and tyrosine kinase).

Fasting blood sugar was determined according to the manufacturer instruction as supplied with Glucose kit from Spain according to Barham and Tinder (16). Hemoglobin A1c HbA1c was determined according to the manufacturer instruction as supplied with HbA1C kit from Korea. This test used a sandwich immune detection method (17) to measure other parameters by ELISA method depending on the quantitative sandwich enzyme immunoassay technique to terminate C-peptide, serum insulin from CUSABUO China also the same tyrosine kinase activity by used protein tyrosine kinase ELISA kit from (SHANGHAI China) (18).

Statistical Analysis:

Data were expressed as Mean ± SD. Statistical analysis was done by ANOVA to compared between three studied groups also used to analysis every pair of study groups by post hoc test variation which considered significant when P- values are ≤ 0.05.

RESULTS AND DISCUSSION

A selected sample of 150 patients were divided into three studied groups, each one contained 50 patients. The mean value of fasting blood sugar in diabetic nephropathy was 217.12 mg/dL and in diabetes patients 215.8 mg/dl compared with control 88.4 mg/dl (significant $P < 0.001$) as shown in table (1) and figure (1). The mean value of HbA1C in diabetic nephropathy patients was 8.79% and in diabetes type 2 was10.26% while in control was 4.69% (significant $P < 0.001$ (table 1, figure 2). The mean of insulin in diabetic nephropathy was 10.44 µIU/mL and in diabetes patients was 7.49 µIU/mL, while in control was 3.72 µIU/mL (significant $P < 0.001$ (table 1, figure 3). The mean C- peptide in diabetic nephropathy was 1.84 ng /mL and in

diabetes patients was 1.74 ng/mL, while in control group was 1.29 ng /mL (significant P < 0.001 (table 1, figure 4). The mean of serum tyrosine kinase in diabetic nephropathy was 15.2 ng /mL and in diabetes type 2 was 21.19 ng/ml while in G1 the mean was 8.86 ng /mL (significant P < 0.001(table 1, figure 5).

Table (1): Comparison of different parameters between three studied groups by ANOVA and Post hoc test

Parameters	Control G1 No.(50) Mean ±SD	DM G2 No.(50) Mean ±SD	NP G3 No.(50) Mean ±SD	P- value	G2 vs.G1	G3vs G1	G3vs. G2
FBG(mg/dl)	88.4±4.97	215.8±62.22	217.12±99.87	HS	HS	HS	NS
HbA1C %	4.69±0.26	10.26±1.62	8.79±2.96	HS	HS	HS	S
Insulin (µIU/mL)	3.72±0.63	7.49±1.25	10.44±0.88	HS	HS	HS	HS
C- peptide (ng/mL)	1.29±0.28	1.74±0.21	1.84±0.16	HS	HS	HS	NS
Tyrosine (ng/mL)	8.86+0.84	21.19+0.53	15.2+0.28	HS	HS	HS	HS

HS=high significant where P<0.001,S=significant whereP≤0.05, NS=No- significant where P>0.05
G1 healthy as control group.
G2 diabetes mellitus type2 newly diagnose patients.
G3 diabetic nephropathy patients

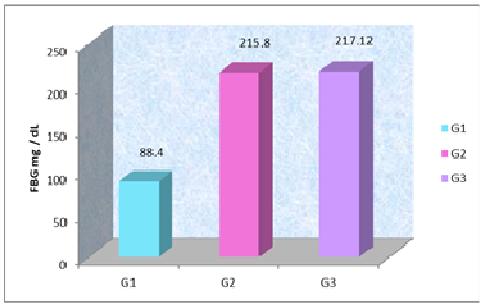

Figure (1): The mean levels of FBG(mg/dl)

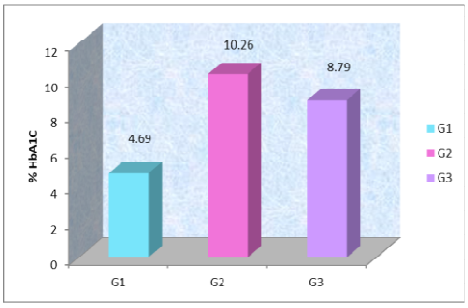

Figure (2): The mean levels of HbA1C%

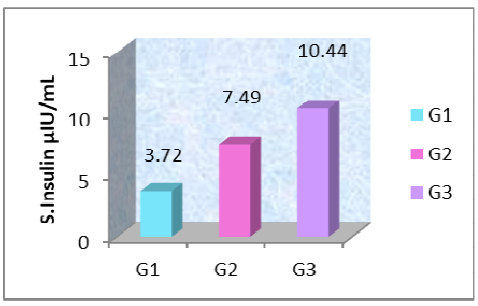

Figure (3): The mean levels of Insulin(µIU/ml)

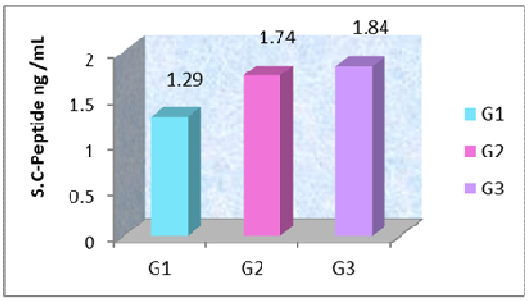

Figure (4): The mean levels of C-peptide(ng/ml)

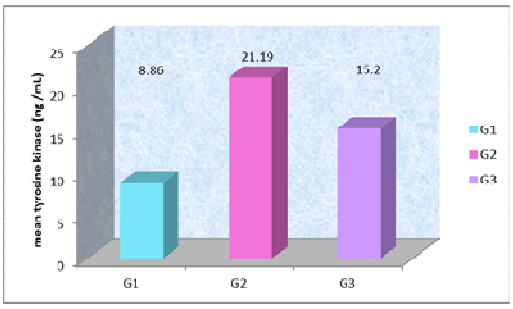

Figure (5): The mean levels of tyrosine kinase(ng/ml)

Correlation study as shown in table (2) and figures (6-9) revealed non-significant positive correlation between tyrosine kinase with FBG, HbA1C, insulin, C-peptide in diabetes type 2 newly diagnosis were (r =0.227,p=0.113), (r = 0.219, P=127), (r =0.236, P=0.098), (r =0.085,P=0.557) respectively, indicating that as tyrosine kinase increases, fasting blood sugar and other parameters also increases, but in diabetic nephropathy patients in table (2) and figures (10,11) correlation study revealed non-significant positive correlation between tyrosine kinase with FBG, HbA1C were (r =0.077, P=0.595), (r =0.149,P=0.300), respectively indicating that as tyrosine kinase increase fasting blood sugar and HbA1C increase, while in figures (12,13) non-significant negative correlation between tyrosine kinase with insulin, C-peptide were (r = -0.085,P=0.559), (r =-0.055,0.704) respectively.

Table (2): The correlation coefficient of tyrosine kinase with HbA1C, FBG, insulin, and C-peptide levels in studied groups.

Correlation parameters	Groups					
	G1		G2		G3	
	r	p	r	p	r	p
Tyrosine vs. FBS	0.237	NS	0.227	NS	0.077	NS
Tyrosine vs. HbA1c	-0.147	NS	0.219	NS	0.149	NS
Tyrosine vs. Insulin	0.133	NS	0.236	NS	-0.085	NS
Tyrosine vs. C- peptide	0.106	NS	0.085	NS	-0.055	NS

Where NS=No- significant where P>0.05

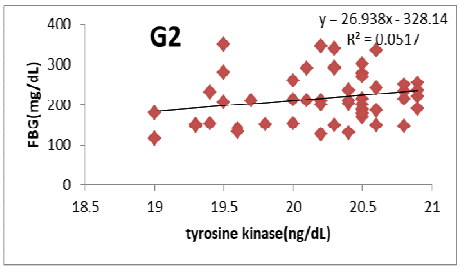

Figure (6): The correlation of tyrosine kinase with FBG inG2

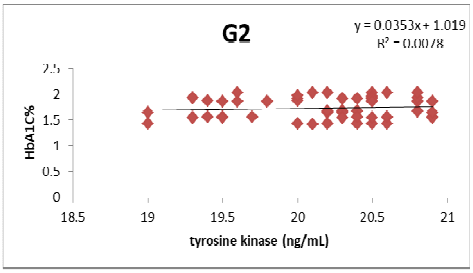

Figure (7): The correlation of tyrosine kinase with HbA1C inG2

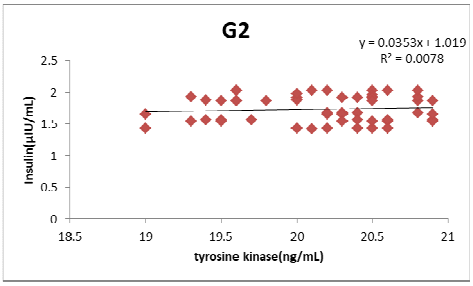

Figure (8): The correlation of tyrosine kinase with Insulin inG2

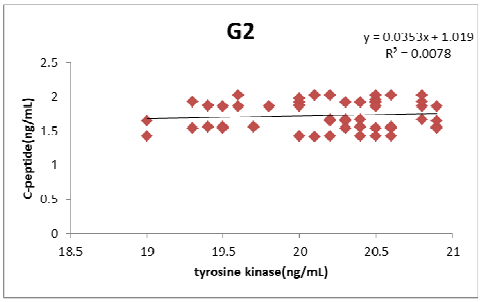

Figure (9): The correlation of tyrosine kinase with C-peptide inG2

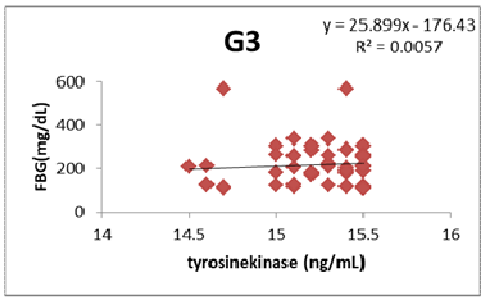

Figure (10): The correlation of tyrosine kinase with FBG in G3

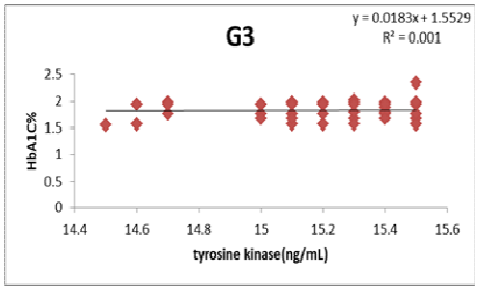

Figure (11): The correlation of tyrosine kinase with HbA1C inG3

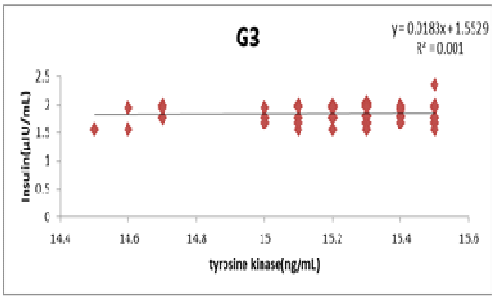

Figure (12): The correlation of tyrosine kinase with Insulin inG3

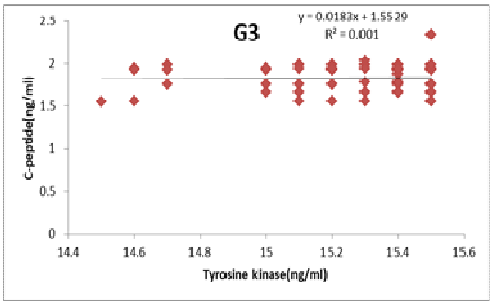

Figure (13): The correlation of tyrosine kinase with Insulin inG3

DISCUSSION

Elevated fasting blood sugar levels in the present study and HbA1Cdue to backs to natural criteria of type 2 DM that is identified by high level than normal range of blood glucose and HbA1C (19, 20). The possible explanation for the elevation of each fasting blood sugar, HbA1C in type 2 diabetic patients might be attributed to use non programmed diet regime for long period which lead to increase HbA1c level (21). The complication of diabetes depends not only by the duration of diabetes mellitus but also by the mean average level of chronic glycaemia. Glycated hemoglobin is a marker for both severity and long term control of disease which reflects the average blood glucose concentration over the preceding 6-8 weeks and is unaffected by diet, exercise ,insulin therapy and other drugs (22). Poor glycemic control indicated by raised mean HbA1c, blood glucose levels in fasting and 2 hours after breakfast were significantly associated with increased prevalence of nephropathy in this study. These findings were consistent with other study (23).

Insulin resistance is characterized by a subnormal response to a given concentration of insulin and can be measured indirectly by a fasting insulin level 2: higher levels of insulin correspond to higher degrees of insulin resistance (24).

Higher levels of insulin in diabetes type 2 newly diagnosis and diabetic nephropathy agreement with previous study (25) that suggested the causes of development type 2 diabetes mellitus result from the combination of genetic factors like (defect in insulin secretion and insulin resistance) and environmental factors like obesity, overweight, eating too much, absence of activity, more stress condition and aging (26). In addition early stages of disease the insulin production is normal or become greater than before in complete conditions (hyperinsulinemia) inappropriate with the degree of insulin sensitivity which is normally reduced, also the capacity of pancreatic β- cell not sufficient to produce hormone in stage with rising glycemia (27).

C-peptide is a useful indicator of beta cell function, allowing discrimination between insulin-sufficient and insulin-deficient individuals with diabetes. C-peptide has been shown to correlate with diabetes type, duration of disease, and age of diagnosis also c-peptide has been demonstrated to be associated with microvascular complications (28). C-peptide as a proinsulin cleavage product released from the pancreas at amounts equimolar to insulin therefore the resistance to insulin in DM type 2 patients catalytic pancreas to produce excess amount of insulin (29). Elevated levels of C-peptide have been found in patients with insulin resistance and early type 2 diabetes, reflecting increased insulin secretion as well as in patients with chronic kidney disease elevated levels of C-Peptide due to impaired renal elimination (30). Protein tyrosine kinase elevated in diabetes mellitus type 2 and diabetic nephropathy, this result is in agreement with pervious study (31). This difference in the groups may be due to treatment patients in group G3 with a long duration of disease but group G2 patients was newly diagnosis, so that the levels of tyrosine kinase was increased in G2 as compared with G3 this hypothesis may be for. Other study suggested that insulin is a peptide hormone and maintain the normal levels of glucose in blood and regulates carbohydrate, lipids and protein metabolism (32) low insulin levels increase the production of glucose by promoting the hepatic gluconeogenesis and glycogenolysis in the fasting state. Glucagon stimulate the gluconeogenesis and glycogenolysis.in type 2 DM is a heterogeneous group of disorders and is characterized by the resistance and impaired secretion of insulin and high levels of glucose production. Insulin binding to its receptor results in receptor activation and the recruitment of a family of downstream signaling molecules. Activation of phosphoinositide 3-kinase (PI-3K) through binding to phosphorylated IRS is a critical step in the translocation of glucose transporters to the cell membrane to facilitate glucose uptake (33).

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Some adipokines neuregulin4 C1q-TNF-related protein-3 biomarkers in prediabetic and type 2 diabetic patients

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ABSTRACT

Type 2 diabetes (T2DM) mellitus is caused by a combination of pancreatic β-cell failure and insulin resistance in the target tissues; liver, muscle, and adipose tissue. Insulin resistance is characterized by an impaired effect of insulin to reduce hepatic glucose production and promote glucose uptake in peripheral tissues. Furthermore, the development of T2 DM may be partially mediated by altering secretion of some adipokines by adipose tissues, such as neuregulin 4 and C1qTNF.

This study was conducted at the National Diabetes Center (NDC) located at Alkindy Hospital/ Baghdad/Iraq, during the period from March to June 2017. One hundred and fifty patients were investigated in this study, half of them male and the other half female. The patients were distributed into 3 groups equally; prediabetes, diabetes mellitus type 2 and healthy individuals. Blood samples were collected from all patients and serum samples were extracted. The following serological and biochemical tests were conducted: serum Neuregulin 4 , C1qTNF, insulin, fasting plasma glucose, HbA1c, lipid profile, Urea, Serum creatinine and serum uric acid and BMI were measured. The results found the Serum Neuregulin 4 concentration to be significantly higher in prediabetes patients and patients with T2 DM compared with the control group ($P<0.01$). While the C1qTNFis was significantly lower in prediabetes patients and patients with T2 DM compared with the control group ($P<0.01$).

Keywords: C1q-TNF- Neuregulin4, Related Protein-3, FBS, HbA1C, Insulin

INTRODUCTION

Diabetes mellitus is a global disorder, characterized by an elevated blood glucose level (hyperglycemia) for an extended period of time, resulting from absolute or relative insulin deficiency (1). Features of hyperglycemia often include polyuria, polydipsia, weight loss, occasionally by polyphagia, and unclear vision (2). Chronic complications of diabetes include microvascular and macrovascular complications that cause visual damage, blindness, kidney disease, nerve loss, amputation, heart illness, and stroke (3). Prediabetes or "intermediate hyperglycemia" is determined on the basis of glycemic parameters which are above normal but below diabetes thresholds a high-risk state for diabetes with an estimated annual conversion rate of %–10%; a similar proportion is converting back to normoglycemia (4). Prediabetes is associated with the presence of insulin resistance and β- cell dysfunction. These abnormalities starting before glucose changes are detectable (5). Adipokines are protein molecules released by the adipocytes and are molecules that are biologically active and that have been shown to mediate various stages of metabolism. Adipokines help to mediate the regulation of food consumption, the metabolism of glucose, protein, lipids, blood pressure regulation modulation of inflammation and immunity, and they work together to regulate inflammation, insulin action, and glucose metabolism locally and systemically (6). In an obese person, the adipokine/cytokine network is altered leading to a state that encourages inflammation and the impairment of adipocyte metabolism (7). Adipokines that stimulate inflammation such as pro-inflammatory cytokines, chemokines, molecules associated with thrombosis, and hypertension are produced in several pathways and these are initiated by stressors from both within and outside the cells. The leading stressor from within the cell are the free fatty acids (FFA) which are chronically increased in obesity as a result of insulin becoming limited in its ability to inhibit lipolysis and consumption of dietary lipids in excess (8). Neuregulin 4 (NRG4) is a member of the neuregulin protein family (NRG1-NRG4) and serves as an adipokine that is synthesized in many tissues, especially in brown adipose tissue (BAT). Similarly to other neuregulin members, NRG4 activates epidermal growth factor receptors (EGFR), also known as erbB receptors, and binds specifically to ErbB3 and ErbB4 receptors initiating cell-to-cell signaling via tyrosine phosphorylation (9). NRG4 has been shown to have an effect in the development of obesity and metabolic disorders including T2DM and non-alcoholic fatty liver disease (NAFLD) in animals and humans (9). NRG4 reduces hepatic lipogenic signals and it maintains glucose and lipid homeostasis in obesity (10). C1qTRP3 was first discovered in 2001 (11) in C3H10T1/2 mice mesenchymal stem cells treated to induce chondrogenic differentiation. Because of its size and 23 Gly-X-Y repeats in the N-terminal collagen domain, it was originally named CORS 26 Collagenous repeat-containing sequence 26 kDa protein later (12).

MATERIALS AND METHODS

This study was conducted at The National Diabetes Center NDC/ Alkindy Hospital and included 150 patients divided into three groups. The first group included 50 Prediabetic patients, the second group included 50 patients with diabetic type 2 and the third group included 50 healthy as control group. The period times for aggregating the blood samples was from March to June 2017. All patients were within (32 to 59) years old. Exclusion criteria included alcoholic patients, hypertensive, acute illness or infection at the time of sampling. Blood sampling was performed at 8.00-10.00 am. in the fasting state for all subjects and 5 ml of venous blood were obtained by antecubital vein puncture divided into two parts; one of them (2ml) was put in tube containing anticoagulation [EDTA] to measure HbA1C, while the another part (3ml) of blood was put in plan tube to evaluate glucose, by centrifugation at 3500 rpm for 5 min., then serum were stored in the freezing -20c° to measure (Neuregulin4, C1q TNF, and insulin) by ELISA method.

Fasting blood sugar was determined according to the manufacturer instruction as supplied with Glucose kit from Spain according to Barham and Tinder (13). HbA1c was determined according to the manufacturer instruction as supplied with HbA1C kit from Korea this test used a sandwich immune detection method (14). Neuregulin4 C1qTNF and insulin were measured by ELISA method.

RESULTS

The range of BMI was (19.2-24.4) kg/m^2 with mean of 22.97± 0.20 for the control group, while the range of BMI was (19.2-25.0) kg/m^2 with mean± SE of 23.37± 0.59 for prediabetes patients group and the range of BMI was (22.7-25.6) kg/m^2 with mean± SE of 24.41±0.62 for the newly diagnosed diabetic patients. There were no significant differences between the control and prediabetes, while results showed significant differences between control and newly diagnosed and newly diagnosed with prediabetic as the shown in the table (1). Results revealed a significant elevation in mean± SE of glucose levels in patients with T2 DM (174.12±0.92 mg/dl) and significant elevation in prediabetics with mean± SE (114.04±0.20 mg/dl) as compared with mean± SE of control group (88.25±0.23 mg/dl) ($p< 0.001$). The mean± SE HbA1c was significantly increased (9.97±0.34%) in patients with T2 DM and prediabetics with mean± SE (6.06±0.13%) compared with the mean± SE (

5.10±0.2%) healthy control group. Blood urea showed significant differences in T2 DM with mean± SE (33.79±0.43mg/dl) and prediabetic with mean± SE (30.25±0.28 mg/dl) compared with mean± SE (26.99±0.19 mg/dl) of control group. Serum creatinine showed significant difference of T2 DM with mean± SE (1.07±0.08mg/dl) and prediabetics with mean± SE (0.95±0.04mg/dl) compared to control group with mean± SE (0.84±0.06 mg/dl). There was no significant difference in serum of uric acid in T2 DM with mean± SE (5.05±0.14mg/dl) and prediabetes with mean± SE (5.03±0.12 mg/dl) compared with mean± SE (5.02±0.1 mg/dl) of control group.

Table (1): The case-control differences in mean values of selected biochemical serum parameters related to BMI glucose control and kidney function

	Control group (1) N 50 mean±SE	Prediabetes group (2) N 50 mean±SE	Type 2 DM group (3) N 50 mean±SE	P value ANOVA	P value t test G1vsG2	P value t test G1vsG3	P value t test G2vsG3
BMI (kg/m^2)	22.97±0.20	23.37±0.59	24.41±0.62	P<0.001	0.48	P<0.001	P<0.001
FBS mg/dl	88.25±0.23	114.04±0.20	174.12±0.92	P<0.001	P<0.001	P<0.001	P<0.001
HbA1c	5.10±0.2	6.06±0.13	9.97±0.34	P<0.001	P<0.001	P<0.001	P<0.001
Blood urea mg/dl	26.99±0.19	30.25±0.28	33.79±0.43	P<0.001	P<0.001	P<0.001	P<0.001
Serum creatinine mg/dl	0.84±0.06	0.95± 0.04	1.07±0.08	P<0.001	P<0.001	P<0.001	P<0.001
Serum uric acid mg/dl	5.02±0.1	5.03±0.12	5.05±0.14	0.884	0.93	0.80	0.91

Table (2) shows that mean serum cholesterol, triglyceride, and LDL were significantly increased in the T2 DM and prediabetes compared to control group with mean± SE values of serum cholesterol, Triglyceride, and LDL: (185.52±0.6mg/dl), (168.82±0.30mg/dl),(114.22±0.39mg/dl) respectively, while the mean± SE values of serum cholesterol, triglyceride, and LDL, prediabetes were (172.60±0.22mg/dl), (130.34±0.39 mg/dl), (101.8±0.13mg/dl) respectively, and the mean± SE values of serum cholesterol, Triglyceride, and LDL of control group were (160.80±0.94 mg/dl), (97.34±0.03 mg/dl), (90.38±0.21 mg/dl). The mean Serum HDL value showed significant decrease in the T2 DM and prediabetic group compared to the control group. The mean± SE of HDL was (34.24±0.23 mg/dl) in the diabetic group, mean± SE (41.06±0.08mg/dl) compared to 46.80.±0.80 mg/dl) in control group. Statistical analysis showed a significant increase in serum total cholesterol, triglyceride, and LDL, in T2 DM patients and prediabetes compared to serum of control group (P<0.001), while there was a significant decrease in serum HDL of T2 DM patients and prediabetes compared with control group (P<0.001).

Table (2): The case-control differences in mean of selected blood lipid profile parameters

	Control group (1) N 50 mean±SE	Prediabetes group (2) N 50 mean±SE	Type 2 DM group (3) N 50 mean±SE	P value ANOVA	P value t test G1vsG2	P value t test G1vsG3	P value t test G2vsG3
Cholesterol mg/dl	160.80±0.94	172.60±0.22	185.52±0.6	P<0.001	0.48	P<0.001	P<0.001
Triglycerides mg/dl	97.34±0.03	130.34±0.39	168.82±0.3	P<0.001	P<0.001	P<0.001	P<0.001
LDL mg/dl	90.38±0.21	101.18±0.13	114.22±0.34	P<0.001	P<0.001	P<0.001	P<0.001
HDL mg/dl	46.80±0.8	41.06±0.08	34.24±0.23	P<0.001	P<0.001	P<0.001	P<0.001

Table (3) represents the means of serum insulin, neuregulin 4 and C1qTNF in T2 DM patients, prediabetics and control groups. The mean±SE Values of serum insulin, nuruglin 4 and C1qTNF in type 2 DM were (9.21±0.04 mIU/ml), (8.44±0.58 ng/ml) and (3.95±0.23ng/ml) respectively and in prediabetes were (6.22±0.56 mIU/ml) (7.22±0.65ng/ml) and (5.76±0.37 ng/ml) while in control group were (5.10±0.69mIU/ml) (2.90±0.84ng/ml) and (8.21±0.59ng/ml) statistical analysis showed significant increase in mean of insulin and neuregulin 4 (P<0.001) and decreased in mean of serum C1qTNF (p<0.001).

Table (3): The case-control difference in mean serum Insulin Neuregulin4 and C1q TNF

	Control group (1) N 50 mean±SE	Prediabetes group (2) N 50 mean±SE	Type 2 DM group (3) N 50 mean±SE	P value ANOVA	P value t test G1vsG2	P value t test G1vsG3	P value t test G2vsG3
Insulin (mIU/ml)	5.10±0.69	6.22±0.56	9.21±0.14	P<0.001	0.48	P<0.001	P<0.001
Neuregulin 4(ng/ml)	2.9±0.84	7.22±0.65	8.44±0.58	P<0.001	P<0.001	P<0.001	P<0.001
C1q/TNF (ng/ml)	8.21±0.59	5.76±0.37	3.95±0.23	P<0.001	P<0.001	P<0.001	P<0.001
Insulin (mIU/ml)	5.10±0.69	6.22±0.56	9.21±0.14	P<0.001	P<0.001	P<0.001	P<0.001

DISCUSSION

In the present study, it was found that serum Nrg 4 concentrations were notably increased in patients with diabetes. The higher serum Nrg4 levels were independently associated with a higher prevalence of diabetes. The notion that brown adipose tissue (BAT) regulates whole-body energy balance by uncoupling mitochondria fuel oxidation from ATP production and then generating heat, thereby enhancing energy expenditure has been widely acknowledged (15). In fact, activation of BAT is indeed linked to reduced adiposity and lower plasma lipid and glucose (16). The results were agreed with other results obtained by (17), who found that circulating Nrg4 levels had elevated in T2 DM and showed positive correlation with glucose parameter. Similar results were reported by (18), who found that circulating Nrg4 levels were significantly higher in patients with diabetes mellitus compared with controls without diabetes and were correlated with the serum glucose level and insulin resistance. In contrast, Pijun *et. al.* (19) demonstrated that circulating Nrg4 was inversely associated with fasting glucose and body fat mass in obese adults. Recently, C1q/TNF-related protein-3 (CTRP-3) is considered a secreted hormone that plays a role in the hepatic glucose and lipid metabolism. Several researches had reported the association of circulating CTRP-3 with obesity and T2 DM in rodent models and in human (21). Studies conducted by (21) showed that both infusions of recombinant CTRP-3 protein and transgenic overexpression of CTRP-3 in mice were involved in regulating the hepatic gluconeogenesis and lipid metabolism. Recombinant CTRP-3 administration overtly lowered the blood glucose levels in C57BL/6 mice and leptin-deficient obese (ob/ob) mice (21). In our study, it was found that serum CTRP-3 concentrations were significantly lower in subjects with pre-DM and nT2DM compared with the NGT group A multiple linear regression analysis showed the plasma CTRP3 levels were independently associated with insulin resistance HbA1c, and HDL-C. Further multiple logistical analyses indicated that serum CTRP-3 concentrations were significantly correlated with pre-DM and nT2DM these data manifest that CTRP-3 is an independent and strong predictor for prediabetes and diabetes.

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