CONTENTS

A Study on the Stability of Different Frusemide Liquid Dosage Formulas: Oral Solution, Syrup, Elixir, Suspension and Emulsion

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Abstract

The present study aim at preparing frusemide in liquid form suitable for oral use. This is achieved through preparing different liquid forms of frusemide. The frusemide liquid is prepared in the following forms: oral solution, syrup and elixir with intensity of 1, 0.4 and 0.8% weight /volume respectively and in combination with potassium carbonate, polysorbate 80, alcohol and phosphate buffer solution of pH8 to dissolve the frusemide in the above mentioned forms. The different forms of the prepared medicine have been stored in glass bottles that can provide protection against light and at 40, 50, 60^{0}C for four months. Besides the pH has been checked to decide the period of validity. The results show that the expiration date of frusemide have lasted for 1.8, 1.07 and 1.22 years respectively for the oral solution, the syrup and the elixir. The suspensions of frusemide are formulated in combination with the following: polyvinyl pyrolidine, xanthan gum, the combination of (xanthan gum and sodium carboxymethyl cellulose), the combination of (xanthan: methyl cellulose) and chitosan. The formulas which give suitable release of the drug are chosen for assessment according to the following considerations: The rat of sedimentation and apparent zero order degradation constant at 25^{0}C. In conclusion, it is found the best formula is that which includes poly vinylpyrolidine, tween20, glycerol, sorbitol, cocoa syrup and parabens at pH7. the fluidity of this chosen formula is psendoplastic type and its validity has lasted for about three years. The emulsion of frusemide is also prepared extemporaneously by using the commercial frusemide tablets in combination with acacia and olive oil. This should be consumed within 45 days of the date of production.

Key word: frusemide, elixir, suspension, emulsion.

الخلاصة

تهدف الدراسة تحضير سائل فموي مقبول للفروسيمايد من خلال تصنيعه بمختلف الاشكال السائلة الفموية. حضر الفروسيمايد كمحلول فموي وشراب والكسير بقوة 1، 0.4 ، 0.8 % وزن/حجم على التعاقب مع كاربونات البوتاسيوم والبولي سوربات 80 والكحول ومحلول الفوسفات الدارئة رقم 8 لاذابة الفروسيمايد في الاشكال المذكورة اعلاه. خزن الدواء المحضر باشكاله المختلفة في قناني زجاجية مضادة للضوء بدرجات حرارة 40، 50، 60 درجة مئوية لمدة أربعة أشهر مع ضبط الاس الهيدروجيني بحدود 8 لتحديد الفترة الزمنية للصلاحية. اظهرت النتائج بأن مدة صلاحيات الفروسيمايد كانت 1.8 ، 1.07 ، 1.22 سنة في المحلول الفموي والشراب والكسير بالتعاقب. ثم تصنيع معلقات الفروسيمايد مع بولي بايرولدين وصمغ الزانثان ومؤتلف (صمغ الزانثان والصوديوم مثيل سليلوز) ومؤتلف (صمغ الزانثان: المثيل سليلوز) والكيتوسان. التراكيب التي اعطت تحرر مناسب للدواء تم اختيارها للتقييم من خلال قياس سرعة الترسب وثابت التفكك لمرتبة الصفر الظاهرية الحركية بدرجة حرارة الـ 25 مئوية وقد وجد بان احسن تركيبة هي التي كانت تحتوي على بولي فينل بابرولدين وتوين 20 وكليسرول وسوربتول وشراب الكاكاو والبرابينات عند اس هيدروجيني 7. وشكل جريان هذه التركيبة المختارة سلوك سيدوبلاستيكي اما صلاحياتها كانت بحدود ثلاث سنوات. حضر مستحلب الفروسمايد انياً من اقراص الفروسيمايد التجارية مع الصمغ العربي وزيت الزيتون على ان يتم تداوله خلال 45 يوم من تحضيره.

Introduction

Frusemide which belongs to the group of loop diuretic is very effective indraining all kinds of oedemas (of cardiac, hepatic or renal origin), in mild or moderate hypertension or used in greater doses in acute and chronic renal failure, oliguria. [(1)] Commerrically available as tablets (20, 40, 80 and 500mg) and injection (10 and 20 mg/ml) and frusemide oral solution which mentioned in USP. [(2)] Many studies concered frusemide to prepare it in defferent pharmaceutical dosage forms as frusemide containing rectal suppositories to increase the drug liberation with the use of non-ionic surfactants (solutol of HS 15, cremophor RH60 and montanox 60DF).[(3)] Frusemide adhesive micro-spheres in hard gelatin capsules, frusemide granules with dika fat with maize starch and microcapsules of frusemide with Acrycoat E30 acrylic polymer were prepared and evaluated in man resulting in sustained release. [(4)] To the patients who have difficulty in swallowing the oral liquid dosage forms (syrup, elixir, suspension and emulsion) and rectal are offen supplied.

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Frusemide is week organic acid such as barbiturates and the sulfonamide. Its solubility in water is increased as the pH is increased by addition of a base. Therefore syrups which are sweet viscous oral solutions can be prepared as well as elixir which is sweet hydroalcoholic solution containing flavoring materials. [5] Also frusemide as insoluble materials can be prepared in liquid media by means of appropriates suspending agents or mix with oil which dispersed as small globules in water in presence of emulsifying agent to form emulsion. [6] In hospitals because the absence of commercial liquid dosage forms solutions and suspensions of frusemide are prepared extemporaneously from injections and tablets respectively may be susceptible to sedimentation of insoluble frusemide, chemically degraded by gastric acid and impractical in case of injection due to many ampoules required.[7] The aim of this study is to prepare frusemide in different liquid dosage forms (syrup, elixir, suspension and emulsion) because these forms are not commercially available. Then test its stability and compatibility to decide on an appropriate formulation and assigne an expire date.

Materials, Instruments and Methods

Frusemide (USP), xanthan gum, cherry flavor and sorbitol supplied by SDI, Iraq; sodium carboxy methylcellulose, methylcellulose, methyl and propyl paraben from Hopkin and Williams LTD, England; Tween20 and 80 (Merck-Schanchard Germany); Ethanol GCC Gainland chemicals company, U.K.; polyvinyl pyrrolidin (PVP) and potassium carbonate from BDH chemical LTd. Pool, England. Sodium saccharin and aspartam (BDH limited pool, England); sodium hydroxide (Fluka-AG); Disodium hydrogen phosphate and potassium dihydrogen orthophosphate (Atlas chemie, W-Germany); frusemide 80 mg tablets (Hoechst Marion Roussel) and date of production is 9-2007. Sartorius balance AG Gottingen, BL210S, CE, Germany; pH meter, Orchidis Labrotaries, France and Hanna instruments type, France; Dissolution apparatus type II, Dis 6000, Copley scientific, Nottingham, U.K.; UV. Visible spectrophotometer, Gitra 5,GBC scientific equipment, U.S.A.; Oven 50, 40^0C Memmert 854 Schwabach, W. Germany; Oven 60^0C Gallenkamp, B5 size one, England.

Experimental

Formulas I, II and III which are summarized in table (1) were prepared according to the following methods.

Table (1): Different formulas of frusemide prepared as solution, syrup and elixir (I, II and III respectively)

Matarials	formulas		
	I	II	III
Frusemide (gm)	1.0	0.4	0.8
Potassium carbonate (gm)	0.5	--	--
Tween 20 (ml)	--	--	5
Tween 80 (ml)	--	6	--
Sodium carboxy methyl cellulose (1%w/v)	--	5 ml	--
Aspartam (gm)	--	0.054	--
Sodium saccharin (gm)	--	--	0.08
Glycerol (ml)	30	--	--
Sorbitol 70% w/w (ml)	--	32.4	10
Alcohol 95% (ml)	--	10	40
Citric acid (gm)		0.1	
Cherry flavor (gm)		0.05	
Phosphate buffer 8 (ml) QS	--	100	100
Purified water (ml) QS	100	--	--

Frusemide oral solution (formula I)

0.5 gm potassium carbonate was dissolved in 45 ml purified water. Then frusemide 1gm and citric acid 0.1gm were added with stirring. Glycerine 30 ml was measured by graduated cylinder and added to previous mixture. Before the volume was completed to 100ml, the cherry flavor was added. Finally the pH was adjused by using pH-meter. [6]

Frusemide free sugar based syrup (formulaII)

0.4 gm of frusemide was mixted with *6m1 tween80 and ethanol 10ml with stirring*. Aspartame and citric acid were added to

previous mixture. Sorbitol 32.4ml and 5ml dispersion of sodium carboxy methyl cellulose (1% w/v) were measured in graduated cylinder and added to resulting product with stirring. After the product was filtered by cotton the cherry flavor was added. The volume was completed to 100ml by phosphate buffer 8. finally the pH was adjusted by using pH-meter.[8]

Frusemide elixir (formula III)

0.8gm of frusemide was dissolved in 10ml sorbitol plus 40ml ethanol. Then citric acid 0.1 gm and sodium saccharin 0.08gm were mixed in 20 ml purified water puls 5 ml tween 20. Then the aqueous solution was added to the alcoholic solution to maintain the highest possible alcoholic strength at all times so that the minimal separation occurs when the two solutions were completely mixed, the cherry flavor was added. Then the volume was completed to 100ml by phosphate buffer 8. The elixir was permitted to stand for a few hours to ensure saturation of alcoholic solvent. The product was filtered by using talc as filter aid to prevent cloudy appearance. Finally the pH was adjusted by using pH-meter. [6] Phosphate buffer pH8 was prepared by mixing 50ml of a solution of 0.2 M potassium dihydrogen orthophosphate with 46.8ml of 0.2M sodium hyproxide then diluted to 200ml with water.[2]

Formulation of frusemide suspension

Table (2) shows 6 formulas of frusemide suspension prepared by the following method: frusemide, methyl plus propylparaben, sorbitol and glycerol were Levigated in the mortar with tween20 and part of prepared dispersions of suspending agents (PVP, xanthan gum, sodium carboxy methyl cellulose, methylcellulose and chitosan) in different concentration as summarized in table (2). The remaining amounts of the dispersions were added in divided portions to the mixture. The mortar was rinsed several times with purified water and the rinsed volume of dispersion was added to cylinder, cocoa syrup was added before the volume was completed to 100ml by adding purified water.

[5]***Comparison studies of formulas A, B, C, D and E***

The following parameters were used to compare the prepared formulas A, B, C, D and E.

Table (2): Different formulas of frusemide prepared as suspension (A, B, C, D and E). and emulsion (F).

Matarials	formulas					
	A	**B**	**C**	D	E	F
Frusemide (gm)	2.5	2.5	2.5	2.5	2.5	-
Frusemide tablet (80mg)						5
PVP (gm)	10					
Xanthan gum (gm)		0.5	0.5	0.5		
Sodium carboxy methyl cellulose (gm)			0.5			
Methyl cellulose (gm)				0.25		
Chitosan (gm)					1.5	
Acacia (gm)						6
Olive oil (ml)						18
Tween 20 (ml)	1					
Glycerol (ml)	10					
Sorbitol (ml)	5					
Cocoa syrup (ml)	20					
Methyl + propyl paraben (gm)	0.18 +0.03					
purified water Qs(ml)	100					90

Dissolution rate measurement

The dissolution medium was 900ml of phosphate buffer 6.8. The temperature of study was 37^0C and the rotating velocity was 100 rev. min^{-1}. 5 ml of each formulas A, B, C, D and E was transferred to the jar bottom using a syringe. At appropriate intervals samples of 5ml were taken from the jar and analyzed for total content of frusemide by UV-spectrophotometer. Detection was done at 330nm. 5ml of fresh phosphate buffer was added to the jar with each time intervals to keep the volume constant.[10]

Sedimentation volume

100 ml of each formulas (A, B, C, D and E) was transferred to the stoppered graduated cylinder. The suspension were shaken vigorously to ensure uniformly then left undistributed. The sedimentation volume was measured at selected time intervals during storage without agitation for a period of 8weeks and was calculated in terms of the ratio of ultimate settled height (Vu) to the original height (Vo).[11]

Extemporaneous preparation of frusemide emulsion (formula F)

Acacia was triturated in mortar to be in powder form. 12ml water was added to get primary emulsion. 18ml olive oil was added

drop by drop with continuous trituration in same direction until clickuing sound was heard. Spread frusemid powder from grinded (5) tablets of 80 mg strength. The primary emulsion was diluted to 90ml by purified water. The contents of formula F are showed in table (2) as well as the dissolution rates of formula F were measured as described previously.[8]

Stability study

2ml samples of formulas I, II and III were stored in closed tubes at 40, 50 and 60 ^{0}C measurement on: 0, 7, 15, 30, 60, 90, and 120 day.Sample of 100ml suspension was inspected for change in color, odor, pH and precipitant. Analysis for remaining frusemide was carried by diluting 2ml of sample with distilled water to 200ml. The 5 ml of resultant solution was taken and completed to 50 ml with 0.1 N NaOH. The absorbance of later solution was detected by a UV-method at 271 nm.[9] The accelerated stability test were also carried out on the suspensions showing the heighest sedimentation volume which were formulas A and D. the suspension of each formula was centrifuged to get supernatant solution. 1 ml samples of the resultant solution were stored in closed tubes at 40, 50 and 60 ^{0}C measurement on: 0,7, 15, 30, 60, 90 and 120 day. Samples of 100ml suspension were inspected for change in color, odor, pH and precipitant. Analysis of remaining frusemide was carried out by diluting 1ml of supernatant solutions with phosphate buffer 6.8 to 25 ml. The absorbance of latter solutions were determined by a spectrophotometer at 330 nm.[9,12] The shelf life calculated from the initial concentration [A_0] and the apparent zero-order rate of degradation (k_0) accordings to the following equations.[13]

$$K_0 = Kx[\,frusemidesolubility\,]$$

$$t_{10\%} = \frac{0.10[A_0]}{K_0}$$

The stability of extemporaneous frusemide emulsion was done as those of suspension which mentioned previously.

Rheogram

Rheogram was obtained for the selected formula at 37^{0}C with Brook field DV-II+Pro viscometer which read shear stress versus shear rate.

Results and Discussion

Oral frusemide solution was claimed to produce agreater diuretic with congestive heart failure than tablet so formula I prepared as oral solution containing potassium carbonate which added to increase pH up to 7. The effect of pH on solubility is critical in the formulation of liquid dosage forms. The solubility of frusemide (PKa=3.9) is often pH dependent. Furthermore, the pH control is at least as important to fully control the crystallize habit and the stat of agglomeration to ensure quality, efficacy and safety of the drug.[10,14] Also frusemide prepared as syrup(formula II) which containing tween 80 to increase solubility of frusemide. Alcohol is present in formula II to serve as a solvent and preservative. While benzoates and parabens was excluded from this formula (pH8) because they are ineffective as preservative in alkaline solutions which frusemide freely soluble in it. Sorbitol is compatible with alcohol as much as 10 percent (v/v) before crystallization is observed. [8, 15] Formula III having a high alcoholic content (elixir) contains saccharin which is required only in small amount rather than sucrose which is only slightly soluble in alcohol and required greater quantities for equivelant sweetness. This formula is self preserving and don't require the addition of antimicrobial agent because it contains more than 10-12% of alcohol. The carboxymethylcellulose, a derived gum function as viscosity builder agent.[6] The presence of glycerine and sorbitol in formula I, II and III contributes to solvent effect, assists in the dissolution of the solute and enhance the stability of the preparation. However, the presence of these materials also adds to the viscosity. [6] The effectiveness of cherry flavour in masking the taste of frusemide is enhanced by presence of weak acid (citric acid). [8] The accelerated studies applied on formulas I, II and III at higher temperatures (40, 50, and 60^{0}C) were employed to predict the expiration date of these formula using UV-spectrophotometer. The degradation of frusemide in these formulas shows first order kinetics since straight lines were obtained by plotting the logarithm of percent remaining of frusemide versus time as shown in figures (1,2 and 3) according equation (1).

$$Log\ C = Log\ Co - \frac{kt}{2.303} \qquad \text{---------- } 1$$

In which Co is the initial concentration; C is the remaining undecomposed concentration at time t; and k is the first order rate constant and –k/2.303 is the slope of the line from which the value of the rate constant is obtained. [12]

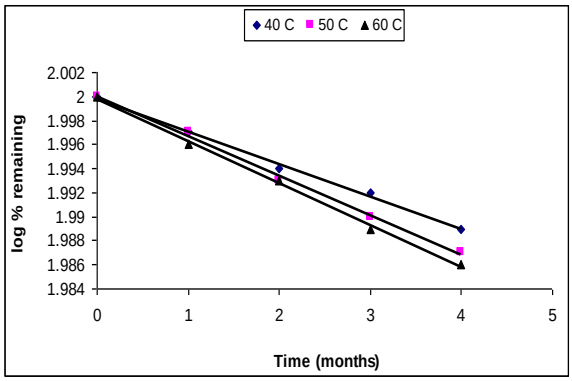

Figure (1): Degradation curve of frusemide oral solution (formula I) at 40, 50, 60 ^{0}C

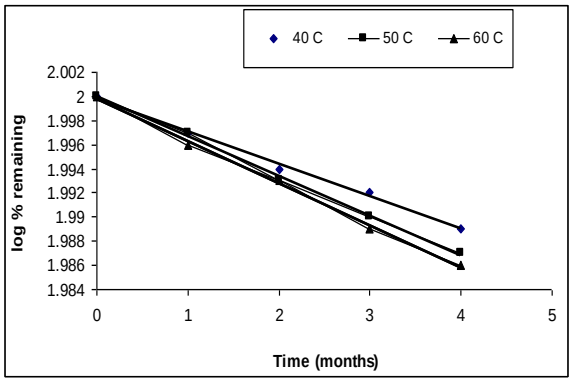

Figure (2): Degradation curve of frusemide syrup (formula II) at 40, 50, 60 ^{0}C

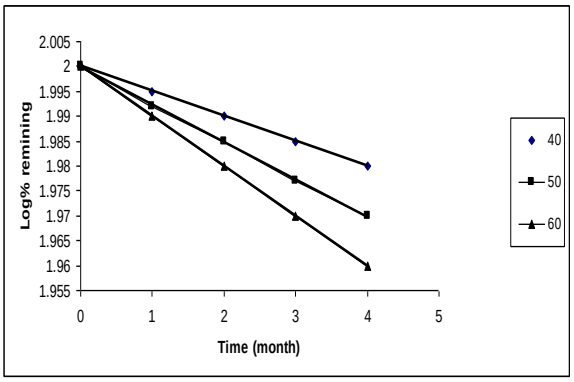

Figure (3): Degradation curve of frusemide elixir (formula III) at 40, 50, 60 ^{0}C

Table (3) summarized the degradation rate constant of formulas I, II and III. Arrhenious plots were constructed to predict the degradation rate constant of frusemide at 25^{0}C as shown in figures (4, 5 and 6). The results indicate no significant differences (P>0.05) between K_{25^0C} for formulas (I, II and III). The expiration data of frusemide was calculated according to first order reaction equation:

$$t10\% = \frac{0.104}{K_{25^0\ C}} \quad \text{----------2}$$

Table (3): Degradation rate constants of frusemide in formulas I, II, III at 40, 50 and 60 ^{0}C.

Formulas	$K_{40}(x10^{-3})$ month^{-1}	$K_{50}(x10^{-3})$ month^{-1}	$K_{60}(x10^{-3})$ month^{-1}
I	5.9	7.1	8.0
II	10.7	26	35
III	10.1	15	20

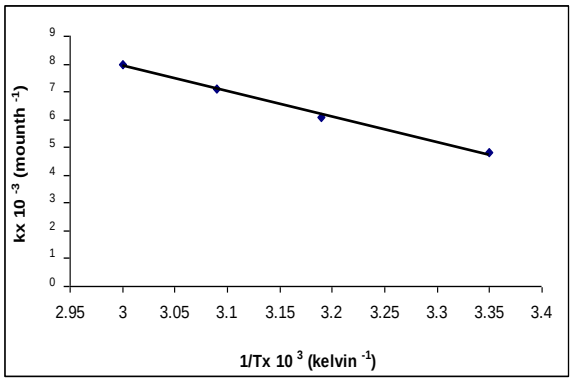

Figure (4): Arrhenious plot for estimation of the expiration date of formula I at 25 ^{0}C.

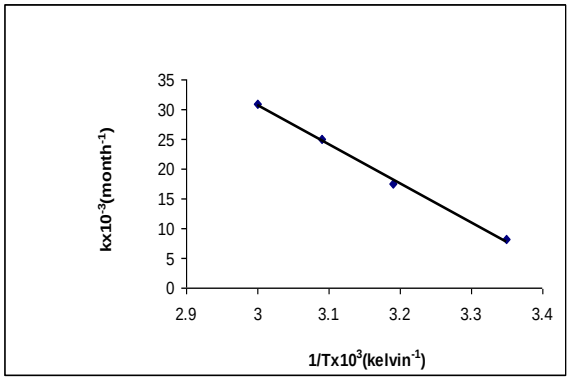

Figure (5): Arrhenious plot for estimation of the expiration date of formula II at 25 ^{0}C

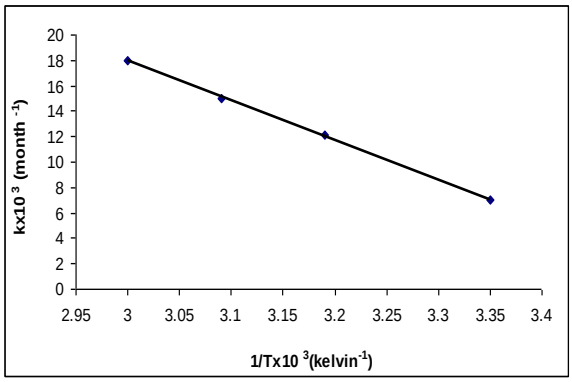

Figure (6): Arrhenions plot for estimation of the expiration date of formula III at 25 ^{0}C

The expiration dates were found to be equal to 1.8, 1.07 and 1.22 years for formulas I, II and III respectively as shown in table (4).

Table (4): Degradation rate constants at 25^{0}C and the corresponding expiration dates of the prepared formulas.

Formulas no.	$K_{25}(x10^{-3})$ month^{-1}	$t_{10\%}$ (years)
I	4.8	1.8
II	8.09	1.07
III	7.0	1.22

Figures (7 and 8) show the dissolution rate profile of frusemide for formulas A, B, C, D, E and F. The results showed that frusemide amounts released increases in the following order:
F<E<C<D<B<A. The differences was significant (P<0.05). Formula A showed the highest dissolution rate for a short periods of time (10 minutes), this could be due to the wetting effect of water soluble polymer (10%PVP) solution with intrinsic rapid dissolution properties especially in the practice of the presence solubilizer glycerol and sugar alcohol such as mannitol and mixture thereof . Optionally a surfactant such as tween 20 may be added to facillate wettability within formulation. [16] Figure (9) shows the sediment volume of formulas A, B, C, D and E during 8 weeks after 48 hours undisturbed.

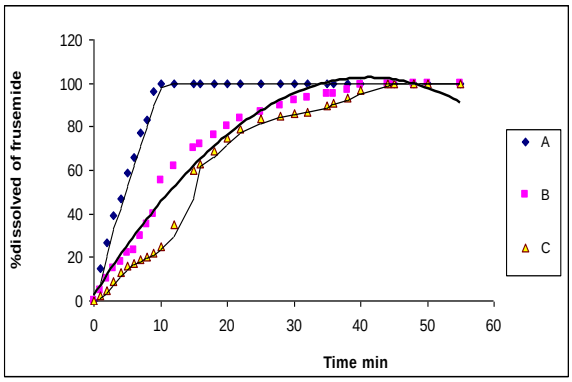

Figure (7) : Dissolution rate profile of frusemide formula (A, B and C)

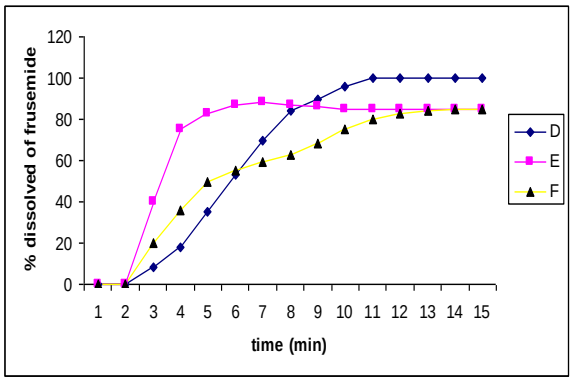

Figure (8) : Dissolution rate profile of frusomide formulas (D, E and F)

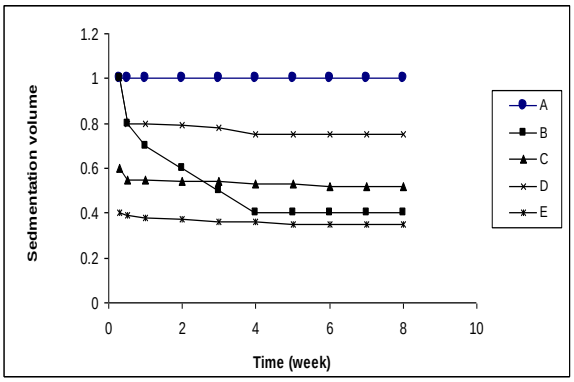

Figure (9): Sedimentation volume of formulas (A, B, C, D and E)

The sediment volume measured according to the ratio of the final height of the sediment after settling (Vu) to the initial height of the total product (V_0).

$$F = \frac{Vu}{Vo} \quad \text{-------- (3)}$$

The results show that the sediment volume increases in the following order: E<B<C<D<A

The sedimentation volume depends on the types and concentrations of suspending,

wetting and viscosity enhancing agents used. As a result, a high sediment volume observed in formula A could be a result of the increase in the viscosity of the PVP dispersion in alkaline media which may be caused by the expansion resulting from electrostatic repulsion between negatively charged carboxylate group. [17, 18] In formula D the increase in viscosity due to strong cross-linking between the nonionic gum (methylcellulose) and anionic gum (xanthan gum).[19] The presence of glycerol, sorbitol and cocoa syrup adds to the viscosity of suspension especially cocoa syrup (chocolate syrup) which is a suspension of cocoa powder in vehicle containing liquid glucose, glycerine, vanillin and sucrose. It is effective because of its high viscosity and enhance the palatability by coating the tongue and thus it tends to inhibit diffusion of the frusemide to the taste buds. [8] The resultant solutions from centrifuging of formulas A and D suspension was with no reservoir of frusemide to replace that depleted so frusemide degradation in them follows the first-order expression as in equation (4).

$$-\frac{d[c]}{dt} = k[c] \quad \text{--------} (4)$$

In which C is the concentration of frusemide remaining undecomposed at time and k is known as a first-order rate constant. When the concentration [c] is rended constant as in the case of a suspension, the equation (5) is applied.

$$k[c] = k_o \quad \text{-------} (5)$$

In which K_0 is apparent zero order rate constant, [c] is the solubility of frusemide at 25^0C which equal 0.086 gm/100ml at pH 7 and K is first order rate constant at 25^0C The first order rate constant for frusemide degradation in supernatant centrifuged solution of formula (A and D) were calculated from slopes of straight lines which result from plotting log% remaining of frusemide in these solutions versus time at elevated temperatures (40, 50 and 60 ^{0}C) as shown in figure (10). Then by plotting the log of these rate constants versus reciprocal of the absolute temperature. First order rate constant K_{25^0C} was obtained by extrapolating the straight line in Arrhenious plot as shown in figure (11).

$K_0 = K_{25^0C}$ x [frusemide in solution] as in equation (5)

$K_0 = (8.07 \times 10^{-2}\ \text{month}^{-1}) \times (0.086\ \text{g/100ml})$

$K_0 = 6.94 \times 10^{-5}$ g/ml. month^{-1}

Then, the expiration date of frusemide suspension formula (A and D) which showed the best release and sedimentation volume than other formulas was calculated according to apparent-zero order reaction equation

$$t10\% = \frac{0.10[A_0]}{K_O} \quad \text{----------} (6)$$

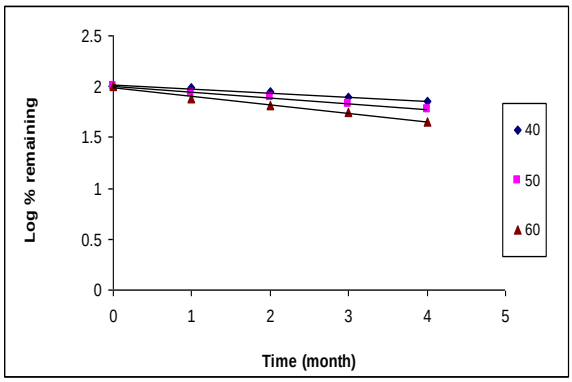

Figure (10): Degradation curve of centrifuged frusemide solution (formula A and D) at 40, 50, 60 ^{0}C

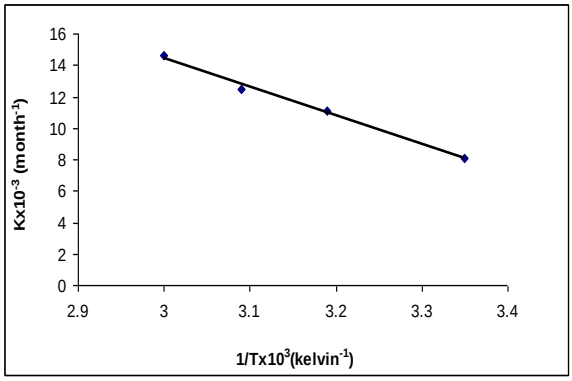

Figure (11): Arrhenenius plot of centrifuged frusemide solution (formula AandD)

The expiration dates were found to equal 3 years for both formula A and D. The shelf life of extemporaneously frusemide prepared emulsion (formula F) was calculated from the initial concentration and the apparent zero-order rate of degradation (K_0) as the steps followed by estimation of expiration data of formulas A and D frusemide suspensions. The shelf life of formula F was 45 days. Acacia used in preparation of formula F because it is preferred in the formulation of the most extemporaneous prepared products. As well as the experimental studies in animal and clinical studies in humans showed that acacia gum has a urea lowering effects so this preparation is useful for patients suffering from hypertension

with chronic renal failure. [20] Formula A was selected to study the rheology as shown in figure (12) because of it's highest release, sediment volume and shelf life than other formulas. The rheogram of formula A has a high viscosity at low shear stress while at higher shearing stress it has low viscosisty due to the flow behavior of PVP at concentration above 5% witch is typical pseudoplastic and thixotropic characteristics.[18] Therefore formula A which containing PVP, tween20, glycerol, sorbitol, cocoa syrup and parabens considered a well formulated suspension because it was physically and chemically stable due to PVP is compatible with many drugs such as frusemide, paracetamol, salicylic acid and testosterone. [16]

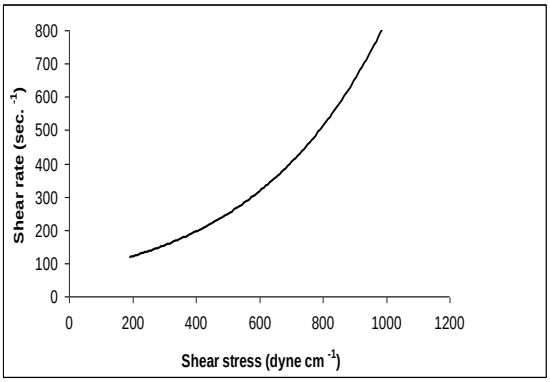

Figure (12): Rheogram of the selected frusemide suspension formula (A)

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Effects of Abuse of Anabolic Androgenic Steroids on Iraqi Athletes

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Abstract

Anabolic androgenic steroids (AAS) are man-made derivatives of the male sex hormone testosterone, originally designed for therapeutic uses to provide higher anabolic potency with lower androgenic effects. Increasing numbers of young athletes are using these agents illicitly to enhance physical fitness, appearance, and performance despite their numerous side effects and worldwide banning. Today, their use remains one of the main health problems in sports because of their availability and low price. The present study focuses on investigating the adverse effects of anabolic androgenic steroid abuse on sex hormones, liver and renal function tests, fasting glucose levels and lipid metabolism in Iraqi male recreational bodybuilders. We have recruited fifteen male bodybuilders (age 19-32 years) and an equal number of healthy non-obese, non-AAS-using sedentary controls. Serum hormones (luteinizing hormone (LH), follicle stimulating hormone (FSH), total testosterone, and prolactin), liver function indices (serum alanine aminotransferase (ALT), aspartate aminotransferase(AST), alkaline phosphatase(ALP), total and direct bilirubin), renal function parameters (serum creatinine and urea), lipid profile and serum glucose levels were measured. Abuse of AAS was associated with significant decreases ($p< 0.005$) in serum levels of LH (66.9%), FSH (49.8 %) and testosterone (63.7%) together with significant increases ($p< 0.05$) in prolactin concentrations (49.8%) in AAS-using bodybuilders compared to sedentary controls. Anabolic androgenic steroids-using athletes had significantly higher ($p< 0.05$) circulating levels of total bilirubin (116.3%), direct bilirubin (127.6%), aspartate (1752.9%) and alanine (263.1 %) transaminases than those of sedentary control subjects. Serum alkaline phosphatase levels were not significantly different ($p> 0.05$) between the two groups. Concerning renal function, AAS-using athletes had significantly higher serum concentrations of creatinine (28.6%) and urea (21.3%) than sedentary controls. Meanwhile, AAS abuse was accompanied by atherogenic lipid profile. Anabolic androgenic steroids -using athletes had significantly higher ($p< 0.05$) serum levels of triglycerides (TG) (45.6%), low density lipoprotein-cholesterol (LDL-C) (26.0%) and very low density lipoprotein-cholesterol(VLDL-C) (45.6%) together with significantly lower serum concentrations of high density lipoprotein-cholesterol (HDL-C) (31.3%) than sedentary controls. Serum total cholesterol (TC) and fasting glucose concentrations were not significantly different ($p> 0.05$) between the two groups. The results presented in the study confirm that abuse of AAS induces unfavorable body functions and undesirable side effects. Therefore, efforts should be sought against use of these compounds outside the therapeutic frame.

Key words: anabolic steroids, athletes, bodybuilding, exercise.

الخلاصة

الستيرويدات البنائية هي مشتقات الهرمون الذكري المعروف بـ (Testosterone) صُنعت خصيصاً للتغلب على عيوب و مساوئ الـ (Testosterone) كمستحضر دوائي. تذكر الدراسات أنَّ أعداد الرياضيين الذين يقبلون على تعاطي مثل هذه المركبات بدون تصريح طبي في تزايد ملحوظ بمرور السنين على الرغم من التحذيرات المتكررة حول أعراضها الجانبية و على الرغم من وضع المنظمات الاولمبية و الجهات الحكومية عقوبات صارمة على من يتعاطى هذه المواد أو يتاجر بها. يعتبر الاستخدام الخاطئ لهذه المركبات من قبل الرياضيين من أهم مشاكل الرياضة في العصر الحديث. أجريت هذه الدراسة و صممت لتقييم تأثيرات تعاطي هذه الستيرويدات البنائية على مستوى الهرمونات في مصل الدم و وظائف الكبد و الكلى و مستوى توزيع الشحوم و مستوى السكر في مصل الدم لمجموعة من ممارسي رياضة كمال الأجسام. اشتملت هذه الدراسة على (15) رياضياً يتعاطون الستيرويدات البنائية و (15) متطوعاً لا يمارسون رياضة كمال الأجسام و لا يتعاطون الستيرويدات البنائية. إعتمدت طرق التقييم على قياس مستوى الهرمونات في مصل الدم (LH, FSH, Testosterone, and prolactin) و وظائف الكبد (Total and direct bilirubin, SAST, SALT, and alkaline phosphatase) و وظائف الكلى (S creatinine and S urea) و مستوى توزيع الشحوم في مصل الدم (TC, TG, HDL-C, LDL-C, and VLDL-C) بالإضافة إلى تركيز السكر في الدم. أظهر تحليل البيانات تأثيراً ذو قيمة معنوية واضحة لتعاطي الستيرويدات البنائية على تركيز الهرمونات في مصل الدم .

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حيث لوحظ إنخفاض معنوي في تراكيز الـ (LH, FSH, and total testosterone) و إرتفع مستوى (prolactin) بصورة معنوية لدى متعاطي الستيرويدات البنائية، ترافق ذلك مع زيادة معنوية واضحة في مستويات الـ (Total and direct bilirubin, SAST and SALT) مع عدم وجود أي فرق معنوي في مستوى الـ (alkaline phosphatase) بين المجموعتين. أظهرت الدراسة أيضاً إرتفاع مستوى الـ (creatinine and urea) في مصل الدم لدى متعاطي الستيرويدات البنائية بالإضافة إلى إرتفاع معنوي في مستوى الـ (TG, LDL-C, and VLDL-C) و انخفاض معنوي في مستوى (HDL-C) في مصل الدم. لم يتم ملاحظة أي فرق معنوي في مستوى (TC) و السكر في مصل الدم بين المجموعتين. في ضوء النتائج التي أفرزتها هذه الدراسة، يمكننا استنتاج أن إستخدام الستيرويدات البنائية خارج إطار العلاج يؤدي إلى حصول مضاعفات تؤثر سلباً على وظائف جسم الإنسان. واجبنا يكمن في بذل قصارى الجهود من أجل تقليل أو منع استخدام هذه المركبات خارج الاطار العلاجي.

Introduction

Self-administration of large doses of anabolic androgenic steroids (AAS) by athletes to obtain a well-shaped body and to increase muscular strength has been increasingly noticeable since the 1950s.[1,2] Anabolic androgenic steroids are widely used by professional and recreational athletes as well as nonathletes.[3] Abuse of AAS is not limited to male adults but also reported in female adults as well as adolescents of both sexes.[1] Every tissue in the body, including the brain, has androgen receptors; therefore, AAS exert systemic as well as psychological effects.[4] Anabolic androgenic steroids have been linked with a wide range of unwanted adverse effects. These effects may range from physically unattractive, such as acne and gynecomastia in males, to serious and life threatening, such as cardiovascular diseases and hepatic carcinoma. Most effects are reversible upon withdrawal.[2,5] Because of their widespread use, many side effects may turn out to be significant risk factors when considering public health. Increased risk of violent death was reported among AAS abusers from impulsive, aggressive behavior, or depressive symptoms.[6] Anabolic androgenic steroids have been taken in cycles. Traditionally, AAS users combine two or more different drugs, mixing oral and injectable AAS. They begin a cycle with a low dose of AAS and slowly increase the dose and then the dose is tapered to zero.[3] Doses taken by abusers can be 10-100 times higher than those used for medical purposes. The aim of the present study was to evaluate the changes in serum sex hormones, liver and renal function indices, glucose level and lipid metabolism in Iraqi male anabolic androgenic steroid abusers.

Material,Subjects and Methods

Participants

Fifteen non-obese male bodybuilders aged 19-32 years (mean 23.27 ± 3.73) were recruited at local gyms in Baghdad city . Bodybuilders were interviewed concerning their health (current diseases and family diseases), consumption of high protein diet, regular exercise, lifetime steroid abuse, pattern of use, and whether other supplements and drugs being used. Exclusion criteria included smoking, alcohol consumption, presence of chronic medical conditions (diabetes mellitus, liver or kidney disorders) and the use of growth hormone. Anabolic steroid abusers were selected if they were currently using AAS. Table (1) summarizes AAS used with their doses and duration of use prior to sample withdrawal. . All of the participants took androgens in cycles and none was taking AAS in a continuous pattern. Cycles were 4-8 weeks in duration separated by suspension periods of 4-12 weeks. A control group of healthy sedentary males (n=15) with a mean age of 22.1 ± 3.65 years were recruited from the community and historically had not ever used anabolic steroids. Unfortunately, absence of bodybuilding controls was evident because, as was found, persons who continue to exercise regularly use AAS routinely. The two groups of volunteers were comparable with respect to their age and height. However, the study group taking AAS had significantly greater mean weight and BMI.

Sample collection

Subjects' weight and height were measured using a balance beam and a vertical ruler. Participants were asked to fast for 12 hours and avoid heavy physical exercise before attending for sample collection. One blood sample was collected from each volunteers by venipuncture between 08:00-10:00 *AM*. A total of 15 ml blood was obtained and placed in EDTA-free tubes to be centrifuged for 5-10 minutes at 3000 rpm. Serum was then divided into several 1.5 mL Eppendorf tubes and stored at (-20^{o}C) until time for the assay.

Laboratory measurements

Serum concentrations of LH, FSH, prolactin and total testosterone were determined by immunofluorometric assays on a mini VIDAS analyzer [7,8] . Liver and renal function indices were measured by colorimetric methods using the commercially available kits[9,10,11,12,13,14,15]. Serum total cholesterol, triglyceride, high-density lipoprotein cholesterol and fasting glucose concentrations were determined by

routine autoanalyzer methods[16,17,18,19]. Serum low- and very low-density lipoprotein cholesterol concentrations were determined through the Friedwald formula[20].

Table 1 : Dose and duration of AAS use for fifteen body builders

Subject no,	AAS used	Route of administration	Dose (mg/kg)	Duration of prior sampling(wk)	Total dose received (mg)
1	Methandrostenolone	O	175	6	1350
	Nandrolone decanoate	P	50		
2	Methandrostenolone	O	140	4	660
	Nandrolone decanoate	P	25		
3	Methandrostenolone	O	140	4	760
	Nandrolone decanoate	P	50		
4	Methandrostenolone	O	245	4	980
5	Methandrostenolone	O	210	4	840
6	Testodterone proponate	P	50	3	450
	Nandrolone decanoate	P	100		
7	Mthenolone	O	20	3	660
	Oxymetholone	O	150		
	Nandrolone decanoate	P	50		
8	Methandrostenolone	O	175	6	1350
	Nandrolone decanoate	P	50		
9	Methandrostenolone	O	140	4	760
	Nandrolone decanoate	P	50		
10	Methandrostenolone	O	175	6	1650
	Nandrolone decanoate	P	100		
11	Methandrostenolone	O	245	4	2780
	Sustanon	P	250		
	Nandrolone decanoate	P	200		
12	Methandrostenolone	O	175	4	1800
	Nandrolone decanoate	P	25		
	Sustanon	P	250		
13	Methandrostenolone	O	175	4	1100
	Nandrolone decanoate	P	100		
14	Methandrostenolone	O	245	6	2670
	Nandrolone decanoate	P	200		
15	Methandrostenolone	O	245	6	2670
	Nandrolone decanoate	p	200		

Statistical analysis

Data were expressed as mean ± SD (standard deviation). Unpaired t-test was employed to examine the difference in means of the AAS-using group and sedentary controls. Pearson correlation (r) was used to analyze the relationships between total dose of AAS used prior to sample withdrawal and the hormonal and biochemical changes. A level of p value < 0.05 was considered statistically significant difference.

Results

Serum hormone levels

Serum LH, FSH, and total testosterone levels in AAS-using bodybuilders were significantly lower ($p < 0.005$) than those in the sedentary controls (66.9%, 49.8%, and 63.7% respectively). However, AAS-using bodybuilders had significantly higher ($p< 0.05$) prolactin concentrations (49.8%) than sedentary controls (table 2).

Table 2: Effects of anabolic androgenic steroids on serum hormones in AAS-using bodybuilders compared to sedentary controls.

Variable	Sedentary Controls N=15 (mean ±SD)	AAS-using BB N=15 (mean ± SD)
LH (mIU/mL)	3.11 ± 0.94	1.03 ± 1.09**
FSH (mIU/mL)	2.93 ± 1.30	1.47 ± 0.87**
Testosterone (ng/mL)	7.47 ± 1.95	2.71 ± 1.75**
PRL (ng/mL)	14.44 ± 6.19	21.63 ± 8.88*

Values expressed as mean ± SD
The P- values refer to the differences from the control group.
* : P < 0.05 significant difference between the two groups.
** : P <0.005 highly significant difference between the two groups.
N = no. of subjects.

Table 3: Effects of anabolic androgenic steroids on liver function tests in AAS-using bodybuilders compared to sedentary controls.

.Variable	Sedentary Controls N=15 (mean ± SD)	AAS-using BB N=15 (mean ± SD)
Total bilirubin (mg/dL)	0.49 ± 0.63	1.06 ± 0.74*
Direct bilirubin (mg/dL)	0.29 ± 0.41	0.66 ± 0.47*
ALP (U/L)	80.20 ± 20.26	93.20 ± 37.19 ^{ns}
AST (U/L)	1.21 ± 4.69	22.42 ± 27.02**
ALT (U/L)	4.69 ± 6.52	17.03 ± 17.02*

Values expressed as mean ± SD
The P- values refer to the differences from the control group.
* : P < 0.05 significant difference between the two groups.
** : P <0.005 highly significant difference between the two groups.
^{ns} : non significant (P ≥ 0.05) significant difference between the two groups
N = no. of subjects.

Liver function parameters

Anabolic androgenic steroids-using bodybuilders had significantly higher (p< 0.05) circulating levels of total and direct bilirubin (116.3% and 127.6% respectively) than sedentary controls. Anabolic androgenic steroids -using bodybuilders had significantly higher serum AST (1752.9%, p< 0.005) and ALT (263.1%, p< 0.05) activities than sedentary controls (table 3). Serum alkaline phosphatase levels were not significantly different (p> 0.05) between the two studied groups.

Renal function tests

(Table 4) demonstrates that AAS-using bodybuilders had significantly higher circulating levels of creatinine (28.6%) and urea (21.3%) (p< 0.005 and p< 0.05, respectively) than sedentary control group.

Table 4: Effects of anabolic androgenic steroids on renal function tests in AAS-using bodybuilders compared to sedentary controls.

Variable	Sedentary Controls N=15 (mean ± SD)	AAS-using BB N=15 (mean ± SD)
Cr (mg/dL)	0.84 ± 0.19	1.08 ± 0.21**
Urea (mg/dL)	38.07 ± 8.58	46.18 ± 14.37*

Values expressed as mean ± SD
The P- values refer to the differences from the control group.
* : P < 0.05 significant difference between the two groups.
** : P <0.005 highly significant difference between the two groups.
N = no. of subjects.

Lipid profile and fasting serum glucose

Circulating levels of HDL-C were significantly lower (p< 0.005) (31.3%) in AAS-abusing bodybuilders than sedentary controls. Table (5) indicates that AAS-using bodybuilders had significantly higher (p< 0.05) serum levels of triglycerides (45.6%), LDL-C (26.0%) and VLDL-C (45.6%) than sedentary controls. Serum total cholesterol and glucose concentrations were not significantly different (p> 0.05) between AAS-using bodybuilders and sedentary control subjects.

Table 5: Effects of anabolic androgenic steroids on lipid profile and serum glucose in AAS-using bodybuilders compared to sedentary controls

Variable	Sedentary Controls N=15 (mean ± SD)	AAS-using BB N=15 (mean ± SD)
Cholesterol (mg/dL)	153.80 ± 21.62	171.20 ± 37.19 ^{ns}
TG (mg/dL)	74.93 ± 42.84	109.13 ± 57.50*
HDL-C (mg/dL)	44.60 ± 7.15	30.67 ± 7.64**
LDL-C (mg/dL)	94.21 ± 21.13	118.71 ± 34.76*
VLDL-C (mg/dL)	14.99 ± 8.57	21.83 ± 11.50*
FSG (mg/dL)	83.20 ± 17.63	89.93 ± 9.16^{ns}

Values expressed as mean ± SD
The P- values refer to the differences from the control group.
*: P < 0.05 significant difference between the two groups.
**: P <0.005 highly significant difference between the two groups.
^{ns} non significant (P ≥ 0.05) significant difference between the two groups
N = no. of subjects.

Adverse effects

Participants were asked questions about unusual adverse effects that would be felt during an AAS cycle and the most common reported side effects were aggression, changes in libido, acne formation, headaches, and premature hair loss as summarized in table 6.

Table 6: Adverse effects reported by AAS-using bodybuilders.

Adverse effect	No. of Subjects N=15	%
Unusual aggression	8	53
Changes in libido	6	40
Acne	3	20
Headaches	4	27
Hair loss	2	13

Discussion

Subjects of this study use independently anabolic androgenic steroids mainly to enhance external physique. Besides being an unethical practice, abuse of AAS has been associated with several health risks and various adverse effects which affect almost all organs and systems of the human body. Anabolic androgenic steroids-using bodybuilders had significantly lower ($p< 0.005$) serum levels of LH, FSH and total testosterone than sedentary controls (table 2). The results were consistent with those reported by Holma et al[21] who observed reduced serum levels of LH, FSH and testosterone in athletics during a course of oral intake of methandrostenolone (15 mg/day). Exogenously administered anabolic androgenic steroids exert a negative feedback on the secretion of gonadotrophins, mostly attributed to a direct effect on the hypothalamus to decrease secretion of GnRH. This in turn causes a corresponding decrease in secretion of both LH and FSH and eventually biosynthesis and release of testosterone from the testes.[22] In addition, anabolic androgenic steroids may produce local suppressive effects on the testes and on adrenal androgen production.[23] Serum prolactin levels in AAS-using bodybuilders were significantly higher than those in sedentary controls ($p < 0.05$) (table 2). Data reported by Stoffel-Wagner et al[24] and Leibenluft et al[25] were consistent with the interpretation that testosterone and/or its metabolites facilitate the secretion of prolactin. Estrogen is known to stimulate prolactin release from the anterior pituitary.[26] Non-aromatizable AAS (stanozolol and methandrostenolone) were reported to activate estrogen receptors through interaction of either the parent compound or its metabolites indicating a possible mechanism for increased prolactin secretion.[27] The available data in the corresponding literature on the influence of exogenously administered androgens on prolactin serum level were found controversial. Serum total and direct bilirubin levels in AAS-using bodybuilders were significantly higher ($p< 0.05$) than those in sedentary controls (table 3). Androgens can selectively interfere with bile excretion by the liver. Canalicular bile plugs were observed after treatment with methyltestosterone, oxymetholone, mestranol, and norethandrolone.[28] Cases of cholestatic jaundice have been recorded in patients therapeutically using or athletes abusing AAS (especially 17□ alkylated agents).[29,30] Serum AST and ALT levels in AAS-using bodybuilders were significantly higher ($p< 0.005$ and $p< 0.05$, respectively) than those in sedentary controls (table 3). Canalicular cholestasis is characterized by mild hepatocellular injury and release of transaminases leading to mild elevations in serum levels of these enzymes.[29] However, since sustained weightlifting alone can result in mild elevations in serum transaminase

activities[31,32], the increase in serum transaminases may be attributed to mild hepatocellular damage, muscle injury, or both. Urhausen et al[33]reported that serum transaminase levels were significantly higher ($p< 0.001$) in anabolic androgenic steroid-abusing athletes than bodybuilders who stopped using anabolic steroids for at least a year. A non-significant difference in serum ALP levels was found between the two studied groups ($p > 0.05$) (table 3). These results are consistent with those reported by O'Sullivan et al[34] who observed no significant difference in alkaline phosphatase activities between anabolic steroid users and potential or past users. Anabolic androgenic steroids can induce cholestasis without elevating alkaline phosphatase levels. ALP activity is usually less than threefold elevated and often is normal.[35] Anabolic androgenic steroids -using athletes had significantly higher serum creatinine ($p< 0.005$) and urea ($p< 0.05$) levels than sedentary controls (table 4). Studies in rat models provide evidence that, compared with females, aging males exhibit decreased glomerular filtration rate and develop glomerular injury, glomerulosclerosis and proteinuria.[36] In addition, cases of acute renal failure had been reported in clinical patients or bodybuilders administering anabolic steroids.[29,37] However, in the present study, we cannot ignore other factors that may have participated in deteriorating renal function parameters in anabolic androgenic steroid-using athletes *e.g.* consumption of high protein diet. Serum concentrations of triglycerides, VLDL-C and LDL-C were significantly higher in AAS-useres ($p< 0.05$) than those in controls (table 5). The rise in serum levels was positively correlated with the intake of AAS. Anabolic androgenic steroids can elevate serum levels of triglycerides by 40-50% in bodybuilders and other power-training athletes.[38] Kiraly[39]in 1988 reported similar results while studying the effects of large doses of testosterone and other anabolic androgenic steroids on serum lipids during a 12 week strength-training period. Elevated serum triglyceride ($p< 0.05$) levels were found with decreased serum HDL-C ($p< 0.005$). Serum LDL-C levels were significantly higher ($p< 0.05$) during steroid intake in studies reported by Fröhlich et al[40] and Palatini et al[41]. Anabolic androgenic steroids -users had elevated levels of apolipoprotein B, a component of both LDL and VLDL.[42,43] Conversely, circulating levels of LDL-C and VLDL-C were not significantly different while using AAS in studies reported by Sader et al[44] and Singh et al[45], respectively. A non-significant difference in serum total cholesterol levels was found between the two studied groups ($p > 0.05$) (table 5). Our results confirm those reported by many studies[40,42] . Anabolic androgenic steroids effects on plasma lipids have been reported to be unpredictable and depend on the dose, route of administration, and type of AAS (aromatizable or not).[46] Low dosages have been associated with hypolipemic response, while high doses have had opposite effects.[47,48] Serum HDL-C levels in AAS-using bodybuilders were significantly lower than those in sedentary controls ($p < 0.005$) (table 4). The postulated mechanism to explain anabolic steroid-induced alteration in serum HDL-C levels is an increase in hepatic triglyceride lipase activity, an enzyme responsible for catabolizing HDL with its phospholipase activity.[49] In addition, apolipoprotein A-1, a major component of HDL particle, was reported to be decreased by AAS.[42,45] The results obtained in the present study showed absolute consistency with the available data. A non-significant difference in serum glucose levels was found between the two studied groups ($p > 0.05$) (table 5). The influence of testosterone and anabolic steroids on glucose metabolism was found controversial. Results of the present study agree with those reported by Friedl et al[50] who observed no alterations in fasting serum glucose in normal men treated with testosterone enanthate or nandrolone decanoate for 6 weeks. On the other hand, Cohen and Hickman[51] concluded that power lifters taking high dose (mean 200 mg/day) of anabolic androgenic steroids had diminished glucose tolerance compared to non-steroid using athletes, obese sedentary men, or non-obese sedentary men. Such controversy in the corresponding literature may be explained to be due to differences in doses used. Higher AAS doses reduce insulin sensitivity and impair glucose tolerance.[46] Although AAS doses used by subjects in the present study were considered to be high; they were much smaller than those used by athletes in Cohen and Hickman[51] study. The most common side effects reported by our subjects were unusual aggression (53%), changes in libido (40%), acne formation (20%), headaches (27%) and premature hair loss (13%) (table 6). Perry et al[52] reported that anabolic steroid using weightlifters were more aggressive than nonusers according to different psychiatric scores. Changes in libido appear to be the most common adverse effect reported in a group of present and past AAS users (approximately 61%).[34] Reports do indicate that toward the end of AAS cycle, some males may experience loss of libido.[53] Acne was also found very

common side effect among anabolic steroid users as reported by O'Sullivan et al.[34] Increases in acne formation is related to stimulation of sebaceous glands resulting in a more oily skin.[34] Premature hair loss does not appear to be very common. It is likely that androgenic alopecia as a result of AAS use is more prevalent in males who are genetically predisposed to balding.[54] Headaches are also not very common among AAS abusers. O'Sullivan et al[34] reported only 9% of AAS using athletes may develop headaches. However, the exact mechanism is unknown.

Conclusion

In conclusion, anabolic androgenic steroid abuse lowered serum concentrations of pituitary gonadotrophins, LH and FSH, and testosterone. Increased levels of prolactin were also manifested. Abuse of anabolic steroids probably causes cholestasis, however, with mildly elevated liver enzymes. In addition, effect of anabolic androgenic steroids on renal function indices was not well established indicating that other factors, such as high protein diet, may have contributed in elevation of blood urea and creatinine levels. Finally, lipid profile was impaired toward evidenced dyslipidaemia.

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Some Variables Affecting the Formulation of Oral Loratadine Suspension

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Abstract

Loratadine is a long acting non-sedating anti-histaminic agent that was developed for the treatment of seasonal allergic rhinitis, whose anti-histaminic action is more effective than the other anti-histaminic drugs available commercially. This project was carried out to prepare an acceptable suspension through studying the release of drug in presence of different types and concentrations of suspending agents such as polysorbate 40, xanthan gum, sodium carboxymethylcellulose (NaCMC), aluminum magnesium silicate (veegum) and sodium alginate. The effects of these suspending agents were studied at pH 1.2 (0.1N HCl) and 37 ℃. The results showed that the release rate of loratadine in the presence of these suspending agents was dependent on their types and concentrations. The results showed that loratadine release from the formula prepared from xanthan gum is more than that prepared from other polymers in the following order: Sodium alginate < NaCMC < veegum ≤ xanthan gum. However, elegancy of suspension was better on using xanthan gum in a concentration of 0.5%. The obtained results were utilized to formulate 0.1% suspension of loratadine which is physically stable with an optimum drug release. The rheology, sedimentation volume, resuspendability and expiration date were evaluated for the selected formula. The formula that contains loratadine, xanthan gum, glycerol, sorbitol, methyl paraben, propyl paraben, sodium edetate, raspberry flavor at pH 5.0 appears to be a promised formula to be present with estimated shelf life of about 3.8 years.

Key word: loratadine, suspension, suspending agent, xanthan gum.

الخلاصة

اللوراتادين عقار مضاد للهستامين طويل المفعول و غير مسبب للنعاس , اكتشف لعلاج حساسية الانف الموسمية و ان مفعوله المضاد للهستامين اكثر فاعلية من الانواع الاخرى المتوفرة تجاريا. تم إجراء هذا البحث لتحضير معلق مقبول من الناحية الصيدلانية من خلال دراسة تحرر الدواء بوجود انواع مختلفة من المواد المعلقة و بعدة تراكيز مثل: البولى سوربات 40, صمغ الزانثان, كاربوكسى مثيل سليلوز الصوديوم, فيكام (سليكات المغنسيوم و الالمنيوم) و الجينات الصوديوم. تمت دراسة هذة التاثيرات بمحلول حامض الهيدروكلوريك عيارية ٠,١ و بدرجة حرارة 37 ْم. اشارت النتائج الى ان تحرر اللوراتادين من التركيبة التى تحتوى على صمغ الزانثان اسرع من التراكيب التي تحتوى على الانواع الاخرى من المواد المعلقة و بالشكل التالى: الجينات الصوديوم > كاربوكسى مثيل سليلوز الصوديوم > فيكام ≥ صمغ الزانثان. تم الاستفادة من هذه النتائج فى تحضير صيغ تركيبية تحتوى على مادة اللوراتادين بتركيز ٠,١٪ . اختيرت الصيغ التى اعطت احسن تحرر للدواء و افضل استقرارية للتقييم من خلال قياس جريان المعلق و حجم ترسب و تجانس المعلق بعد تحريكه و تاريخ الصلاحية لافضل تركيبة. لقد وجد بان احسن تركيبة هى التى تحتوى على اللوراتادين , صمغ الزانثان, كليسيرول, سوربيتول, مثيل بارابين, بروبيل بارابين, ايديتيت ثنائى الصوديوم و نكهة التوت عند الاس الهيدروجينى 5 . اما مدة صلاحية المعلق المحضر فكان بحدود ٣,٨ سنوات.

Introduction

A coarse suspension is a dispersion of finely divided, insoluble solid particles (the dispersed phase) in a fluid (dispersion medium or continuous phase) [(1)].A conventional suspension may be readily prepared in an aqueous solution with small percentage of hydrophilic polymers (like methyl cellulose, hydroxypropylcellulose and xanthan gum), as well as small percentage of surfactant like polysorbate 80. The suspending agent was used to achieve homogeneity of redispersed suspension while surfactants are used for wetting and dispersing of insoluble particles [(2)].For many patients, the liquid dosage form is preferred over the solid dosage form of the same drug because of the ease of the swallowing liquids and flexibility in the administration. The disadvantage of disagreeable taste of certain drugs (loratadine bitter taste) is overcome when the drug is administered as undissolved particles [(3)]. In general, aqueous suspension gives more extended effect than aqueous solution [(4)]. Insoluble or poorly soluble drugs in suitable solvents could be formulated as flavored suspension as an ideal choice, example; nalidixic acid suspension [(5)] and fluconazole suspension [(6

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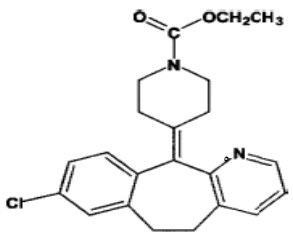
Commercially available loratadine solution (syrup) products contain excipients that are inappropriate for pediatric population, particularly the neonatal and infant age groups. To improve the solubility of loratadine, most of the syrups contain propylene glycol which may put the infants at risk of displacement of bilirubin and consequently hyperbilirubinemia [(7)]. Excessive intake of propylene glycol and subsequent metabolism to lactic acid may cause the development of hyperosmolality and lactic acidosis [(8)]. Also the high concentration of sucrose over 60% w/v made the syrup to be administered with care to patients with diabetes mellitus [(9)]. In addition the stability of the drugs when prepared as insoluble particles is higher compared to that of dissolved drug in syrup, as shown by the aminophylline suspension whose chemical and physical stability showed minimum rate of degradation after 91 days period [(10)]. Loratadine is a tricyclic antihistamine, which has a selective and peripheral H_1-antagonist action. It has a long-lasting effect and does not normally cause drowsiness because it does not readily enter the cerebro spinal fluid (CSF) [(11)].It is white to off-white powder, practically insoluble in water, but very soluble in acetone, alcohol and chloroform [(12)]. Loratadine rapidly absorbed after administration, peak plasma concentration being attained in about 1 hour. It is extensively metabolized; the major metabolite is desloratadine, which has potent antihistamine activity. The elimination $t_{1/2}$ of loratadine and desloratadine are 8.4 and 28 hours respectively [(13)].

Chemical formula $C_{22}H_{23}Cl\,N_2O_2$
Mol.wt 382.8 gm
Melting point 134-136 C



There are different types of suspending agents used in suspension can be classified as [(14)]:

1. Naturally occurring suspending agents like xanthan gum, sodium alginate, that forms multilayer film around the drug particles.
2. Semi synthetic suspending agents like NaCMC, which form multilayer also.
3. Synthetic suspending agents (surfactants) like polysorbate 40 (tween 40), that form monomolecular film.
4. Finely divided solids like veegum which form solid particle film.

The goal of this study is to formulate the loratadine as a suspension instead of syrup to mask its bitter taste, to improve its stability and to decrease side effects of the excipient by decreasing their use in the suspension formula.

Experimental

Materials and Equipments

Loratadine powder (supplied by Philadelphia pharmaceutical, Jordan). Veegum, xanthan gum, methyl paraben, propyl paraben, raspberry flavor (supplied by SDI, Iraq). Sorbitol, hydrochloric acid (Riedel-de haen hannover, Germany). Disodium EDTA (BDH chemical Ltd.pool, England). Polysorbate 40 (Merck-Scanchard, Germany). Sodium carboxymethylcellulose, sodium alginate (Hopkin and Williams Ltd, England). UV spectrophotometer (Carrywin UV, Varian, Australia). USP dissolution apparatus (Erweka). Viscometer (Brookfield Copley Scientific, England). pH meter (Hanna instrument pH 211, Italy).

Method of Preparation

Preparation of stock dispersion of suspending agents:

Stock dispersion of each suspending agent used (polysorbate 40, xanthan gum, NaCMC, veegum and sodium alginate) was prepared by dispersing (10, 1, 1.5, 5 and 3 gm) of the suspending agents, respectively in 75 ml of distilled water (D.W) using an electrical mixer at 150 r.p.m. The volume of the dispersion was made up to 100 ml with D.W, the resultant dispersions were allowed to hydrate for 24 hours [(15)].

Preparation of suspension:

Loratadine suspension was prepared by levigating 0.1 gm loratadine in a mortar with the prepared dispersion of suspending agent. When smooth paste was formed, the remaining of the vehicle was added and the volume completed to 100 ml with shaking [(16)].

Effect of type and concentration of suspending agent on the drug release:

The dissolution pattern of loratadine was studied in the presence of different types and concentrations of suspending agents including: polysorbate 40, xanthan gum, NaCMC, veegum and sodium alginate.

Formulation of loratadine suspension:

Five different formulas were prepared using different suspending agents as shown in table (1)

Table (1): Different formulations of loratadine as suspension dosage form represented as % (w/v)

Materials	Formula number				
	A	B	C	D	E
Loratadine	0.1	0.1	0.1	0.1	0.1
Xanthan gum	0.5		0.3	0.3	
SCMC		0.5	0.5		
Veegum				2	0.5
Polysorbate 40					1.25
Methyl paraben	0.2				
Propyl paraben	0.02				
Glycerol	5				
Sorbitol	7				
Disodium edetate	0.1				
Raspberry flavor	0.05				
Final volume	100 ml				

Each formula was prepared as follows: Loratadine, methyl paraben, propyl paraben were levigated in a mortar with glycerol and the prepared dispersion of suspending agent. The mixture was triturated with a pestle until a smooth paste was formed. With continuous trituration, the paste was diluted with the remaining amount of the dispersion of the suspending agent then transferred to graduated cylinder. Finally, the required amount of disodium EDTA and sorbitol were dissolved in a small portion of water and added to the graduated cylinder and raspberry flavor was added and water to make the final volume. The suspension was shaken and the pH was adjusted to 5 with few drops of 5M sodium citrate.

In Vitro Evaluation of Suspension

Dissolution rate profile:

The dissolution rate of loratadine suspension was studied using USP dissolution apparatus type II which was provided with stainless steel stirrer connected to electrical motor and rotated at 50 r.p.m. with 900 ml of 0.1N HCl placed in the dissolution jar and equilibrated at 37 ̊C. This was followed by transferring 5 ml of the prepared suspension to the jar bottom using a syringe. Then a sample of dissolution medium was withdrawn at different time intervals (2, 5, 15, 30, 45 and 60 minutes) through a pipette fitted with a filter paper. Fresh dissolution medium was added to the jar each time to replace withdrawn samples. Each sample was suitably diluted and assayed spectrophotometrically at 278 nm for loratadine content.

Measurement of rheograms:

Rheograms was obtained at 25 ̊C using Brookfield viscometer.

Sedimentation volume measurement

Fifty milliliters of each suspension was diluted with distilled water to a volume of 100 ml in a stoppered graduated cylinder. The suspensions were shaken vigorously to insure uniformity, and then left undisturbed. The sedimentation volume was measured every 4 hours for period of 48 hours [17].

Resuspendability of suspension

The efforts required to convert the sedimented system to homogenous suspension upon shaking the cylinder manually, were rated with a ranks as follows: resuspendable, resuspendable with difficulty or not resuspendable [18].

Stability study

The accelerated stability study was done in order to determine the expiration date of the selected formula. The suspension was centrifuged to get the supernatant solution; 5ml samples of the resultant solution were stored in several closed amber glass containers at 35, 45 and 55 ̊C for 90 days. Samples were inspected for change in color, odor, pH, precipitant and assayed for drug content at suitable time intervals (0, 10, 20, 30, 45, 60 and 90) days.

Results and Discussion

Effect of type and concentration of suspending agent on the drug release:

The effect of various types of suspending agents (surfactants and polymers) on the dissolution rate of loratadine was investigated.

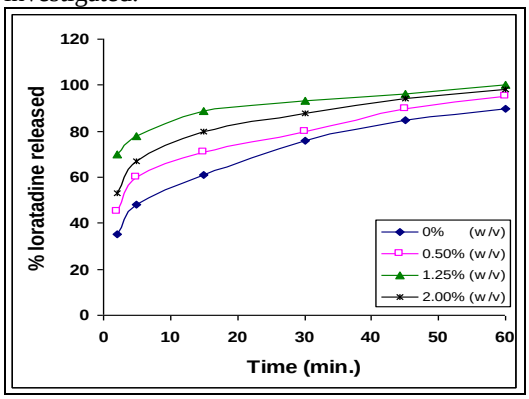


Figure (1): Effect of polysorbate 40 concentration on the percent of loratadine released in 0.1 N HCl pH (1.2) at 37 °C.

Figure (1) indicated that polysorbate 40 at all concentration used increases the amount of drug released in the following order: 0.0% < 0.5% < 2.0% 1.25% It was seen that increasing polysorbate 40 concentration from 0% to 0.5% and 1.25% (w/v) enhance the drug release, this may be attributed to the wettability and micellarization effect of polysorbate 40 at the interface between insoluble drug particles and the vehicle [19]. Meanwhile increasing polysorbate 40 concentration to 2% (w/v) enhance the release of loratadine to a lesser extent compared with 1.25% (w/v), this odd behavior may referred mainly to the formation of micelle macromolecules of drug with polysorbate 40 that hinder the excessive drug release from insoluble loratadine particles, this behavior give an impression of critical micelle formation with this range of polysorbate 40 concentration (2% w/v) [20]. From the other hand figures 2, 3 and 4 illustrate the effect of anionic hydrophilic polymers including xanthan gum, NaCMC and sodium alginate on the dissolution rate of loratadine using different concentrations of these polymers. The results indicated that the percent of drug release were as follows:

Xanthan gum 0.7% < 0.0% < 0.3% < 0.5%
NaCMC 0.0% < 1.0% < 0.25% < 0.5%
Sod. Alginate 2.0% < 1.0% < 0.5% < 0.0%

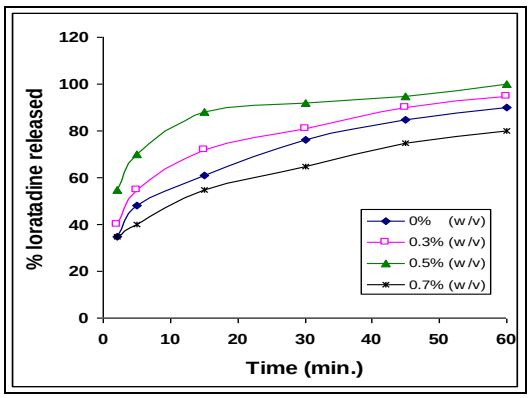


Figure (2): Effect of xanthan gum concentration on the percent of loratadine released in 0.1 N HCl pH (1.2) at 37 °C.

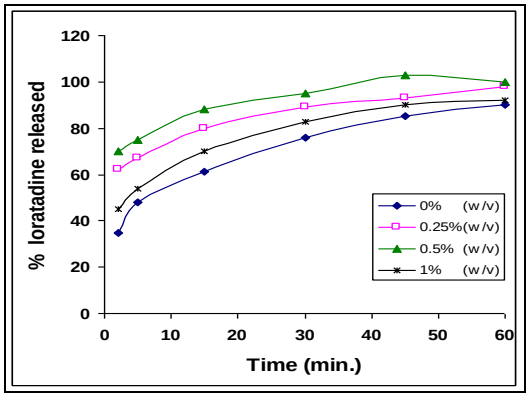


Figure (3): Effect of sodium carboxymethylcellulose concentration on the percent of loratadine released in 0.1 N HCl pH (1.2) at 37 °C.

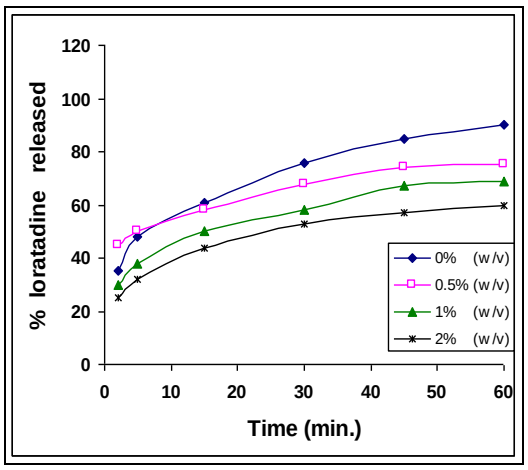


Figure (4): Effect of sodium alginate concentration on the percent of loratadine released in 0.1 N HCl pH (1.2) at 37 °C.

The hydrophilic polymers xanthan gum and NaCMC enhanced the dissolution rate of loratadine, since these polymers behave as protective colloid by coating the solid hydrophobic particles with multimolecular layer. This imparts hydrophilic character to the solid and thus promotes wetting [1], but to certain limits, then the dissolution rate of loratadine decreased by increasing xanthan gum and NaCMC above 0.5% (w/v), this result may be attributed to the increase in viscosity of the prepared formula [21, 22]. Moreover the results showed that the presence of sodium alginate retarded the dissolution rate of loratadine at all concentrations used. This result is related to the formation of higher viscosity regions due to the hydrated polymer surrounding drug particles which encounter high resistance to the dissolution [23]. On the other hand, the effect of veegum on the dissolution rate of loratadine is shown in figure(5). 0.0% < 2.0% < 1.5% < 0.5%

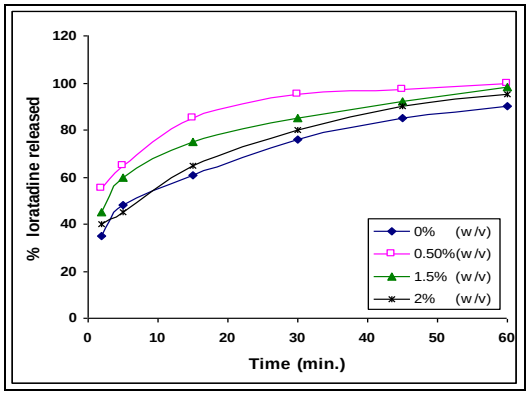


Figure (5): Effect of veegum concentration on the percent of loratadine released in 0.1N HCl pH (1.2)

This retardation in dissolution of loratadine as the concentration of veegum increases may be attributed to adsorption of loratadine on veegum or solid particle films formed around the drug particles [24].

Formulation of the loratadine suspension:

Xanthan gum was used as a suspending agent in concentration of 0.5% to prepare formula A. This concentration gave the best release as mentioned previously. It is an effective flocculating agent at relatively low concentration and has excellent suspending properties to suspend solid [25]. The rheological stability of xanthan gum toward pH changes encountered during transit through the gastrointestinal tract in addition to large sedimentation volume presumably by polymer bridging phenomena providing reasons for its use [26]. NaCMC was incorporating in formula B in concentration of 0.5% and enhanced the dissolution rate of loratadine, but its aqueous dispersion has low viscosity and produced sediment layer that was easily redispersed by shaking. Farther more, a combination of xanthan gum and NaCMC (formula C) resulted in too viscous suspension which has less flexibility when was pouring. Veegum was chosen in a concentration of 0.5% (formula D), since this concentration gave the best release but this suspension had low viscosity. The linear branched chain molecules of veegum form a gel-like network within the system and became adsorbed on to the surface of the dispersed particles, thus holding them in flocculated state [27]. So formula E was prepared with 2% veegum to get higher viscosity. The non-ionic surfactant polysorbate 40 was incorporated as a wetting agent and to increase the dissolution rate of loratadine. This dispersion exhibit thixotropy band plasticity with high yield value. It remained in the flocculated state (i.e., no hard sediment or caking was formed) and poured easily. The following excipients were added to the prepared suspension; sorbitol and glycerol as a sweetening agent. They produce pleasant taste, less viscous suspension and are better than sucrose in producing structure in vehicle suspension [28]. Disodium edetate was involved in the formulation to protect loratadine from deterioration [29]. Raspberry flavor as flavoring agent, methyl paraben and propyl paraben were added as preservatives. The pH of the formula was adjusted to 5 using 5M sodium citrate.

In vitro evaluation of suspension:

dissolution rate profile:

Table (2) and figure (6) showed the dissolution rate profiles of the prepared loratadine suspensions. The data indicated that formula A had the highest dissolution rate constant as compared with the other formulas, since the rate constant of formula A was 24.3×10^{-3} ($mg^{1/3}$ /min) using Hixson-Crowell root equation, that expresses the dissolution rate of solid particle based on the cube root of the weight of the particles [30], this equation was as follows: $W_o^{1/3} - W^{1/3} = Kt$

Since: W_o=the original mass of the drug particles

W = the mass of drug dissolved

K = the cube root dissolution rate constant

T = the time required to dissolve (W) mass of drug particles

Table (2): The calculated dissolution rate constants of loratadine in the prepared formulas.

Formula	$K \times 10^{-3}$ ($mg^{1/3}$ / min)
Formula A	24.3
Formula B	15.0
Formula C	21.3
Formula D	21.2
Formula E	21.5

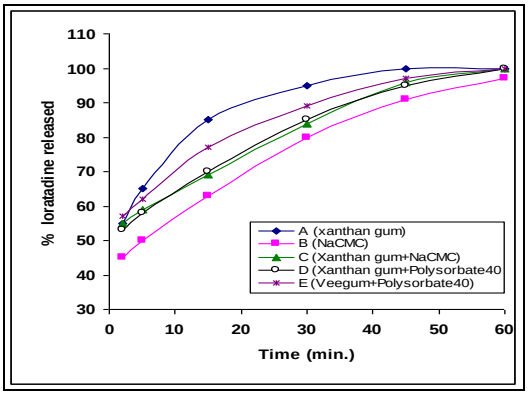


Figure (6): Dissolution rate profile of loratadine in the prepared formulas in 0.1N HCl at 37°C.

Measurement of rheograms:

The rheograms of the prepared formulas are represented in figure (7). The profile showed that the viscosities of loratadine suspensions were shear rate dependent and it increased in the following order:

Formula C > D > A > B > E

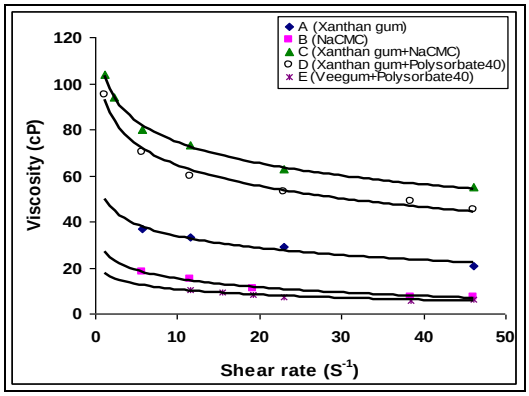


Figure (7): Shear rate dependency of the viscosities of the prepared formulas of loratadine suspension at 37 ̊C.

The results also illustrated that all the prepared formulas exhibited pseudoplastic flow which evidenced by shear thinning and increase in shear stress with increased angular velocity. This behavior due to the suspending agent used, because as a general rule, the pseudoplastic flow is exhibited by polymer in solution[31]. The rheological properties of the prepared formulas revealed that formula A was the best one which offered ease of pouring and swallowing compared with formula B and E which they are less viscous and could not obtain the structured suspension with them. On the other hand, higher viscosity was obtained with formula C and D with a difficulty in the pouring and swallowing from the bottle orifice. This was a result of using a combination of two polymers (xanthan gum + NaCMC) and (xanthan gum + veegum) respectively which leads to rheological synergism to be occurred due to stronger cross-linking between the two polymers used, where the presence of carboxyl groups on NaCMC and xanthan gum promote stronger hydrogen bonding between them [32].

Sedimentation volume and resuspendability:

The sedimentation volume (F) is the ratio of the ultimate height of the sediment as suspension settles in a cylinder (H_μ) to the initial height of total suspension (H_o).

$$F = H_\mu / H_o$$

While the Resuspendability is a quantitative test to evaluate the ease of redispersion of a suspension after a long period of standing [30].

Table (3): Sedimentation volume and Resuspendability of the prepared formulas.

Formula	Sedimentation volume $F = H\mu / H_o$	Resuspendability
A	1	No sedimentation
B	0.6	Easily resuspended
C	1	No sedimentation
D	0.9	Easily resuspended
E	0.4	Resuspendable with difficulty

Table (3) shows the sedimentation volume and Resuspendability of the prepared formulas. The data indicated that the formulas prepared with xanthan gum (formula A, C and D) had sedimentation volume almost equal to 1 i.e., no sedimentation was occurred during the test period. The obtained results attributed to the network of flocs formed in the suspension which is so loose and fluffy that can extend through out the extra vehicle [31]. Xanthan gum is often used as flocculating agent to achieve non-sedimenting suspension of drugs with no need to other adjuvant[18]. However, formula B had a sedimentation volume equal to 0.6 and was easily resuspended by shaking. This low sedimentation volume may be due to the type of the suspending agent used, since NaCMC may form homogenous network in all concentrations used [23]. On the other hand, formula E, which showed sedimentation during the test period, had low sedimentation volume of 0.4 and was resuspendable with difficulty, so it is pharmaceutically unacceptable.

Stability study:

Formula A was chosen for the stability study as the promising formula since it gave the optimum physical stability and remarkable release profile. The stability study carried at moderate exaggerated temperatures (35, 45 and 55 ̊C) to predict the expiration date of the promised formula. The degradation of loratadine followed apparent zero-order kinetics, since the concentration in solution depends on the drug solubility. As loratadine decomposes in solution, more drug is released from the suspended particles so that the concentration remains constant [30]. The resultant solution from centrifuging formula (A) was with no reservoir of loratadine to replace that depleted so loratadine degradation

in them followed first-order expression as in equation (1):

$$-d[A]/dt = K[A] \quad \ldots\ldots\ldots (1)$$

in which A is the concentration of loratadine remaining undecomposed at time t, and K is the first-order rate constant. When the concentration [A] is rendered constant, as in case of a suspension, equation (2) is applied:

$$K[A] = K_0 \quad \ldots\ldots\ldots\ldots (2)$$

were K_0 is the apparent zero-order rate constant, [A] is the solubility of loratadine at 25 ̊C which is equal to 0.096 gm/100ml and K is the first–order rate constant at 25 ̊C. The first-order rate constant for loratadine degradation in supernatant centrifuged solution of formula A was calculated from the slopes of straight lines which resulted from plotting log percent remaining of loratadine in the solution versus time at elevated temperatures (35, 45 and 55 ˚C) as shown in figure (8) and listed in table (4).

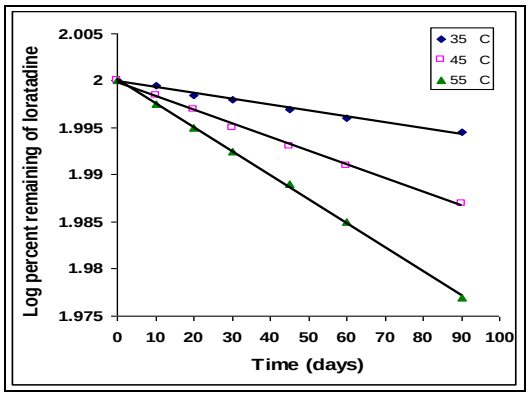


Figure (8): Accelerated stability study of loratadine in the prepared suspension (formula A) at elevated temperatures.

Table (4): Degradation rate constants (K) of loratadine in formula A at different temperatures.

Temperature	K $(day)^{-1}$
35 ºC	0.155×10^{-3}
45 ºC	0.33×10^{-3}
55 ºC	0.691×10^{-3}
25 ºC	0.0749×10^{-3}

Then by plotting the log of these rate constants versus the reciprocal of the absolute temperatures, the first-order rate constant obtained was equal to 0.0749×10^{-3} (day^{-1}) as shown in figure (9).

$K_0 = K[A]$

$K_0 = 0.0749\times10^{-3}$ day^{-1} . 0.096 gm/100ml

$K_0 = 0.71\times10^{-5}$ gm /100 ml. day^{-1}

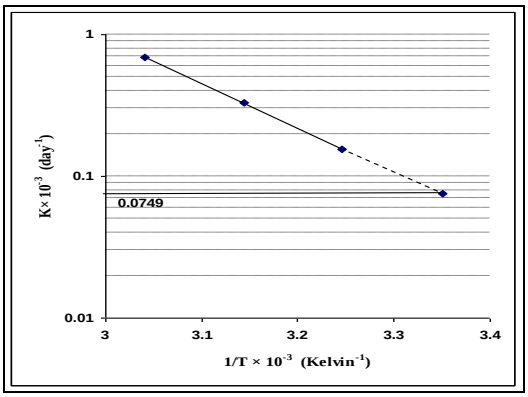


Figure (9): Arrhenius plot for shelf life estimation of loratadine in the prepared suspension (formula A).

Then the expiration date of loratadine suspension (formula A) was calculated as follows: $t_{10\,\%} = 0.1\,[A]_o / K_0$

The expiration date was found to be equal to 3.8 years. The formula show good physical stability, as there was no discoloration, precipitation or any other physical changes after the storage period. The pH of the formula was 5.0 for whole the period.

Conclusions

A stable suspension of loratadine could be prepared and used efficiently using xanthan gum as a suspending agent (Formula A), since it provided an easily pourable suspension with no sedimentation with expiration date of 3.8 years.

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Determination of Enzymatic Antioxidant in Iraqi Patients with Chronic Gastritis

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Abstract

Infection of the gastric mucosa with *Helicobacter pylori* is strongly associated with chronic gastritis, peptic ulcer and gastric cancer. *Helicobacter pylori* virulence factors include a variety of proteins that are involved in its pathogenesis, such as VacA and CagA. Another group of virulence factors is clearly important for colonization of *H.pylori* in the gastric mucosa. These include urease, motility factors (flagellin), and Superoxide dismutase (SOD). Because of this organism's microaerophilic nature and the increased levels of reactive oxygen in the infected host, we expect that other factors involved in the response to oxidative stress are likely to be required for virulence. Superoxide dismutase is a nearly ubiquitous enzyme among organisms that are exposed to toxic environments. In this study, we measured the SOD in serum of 80 patients complain from chronic gastritis and infected with *H.pylori*. 37 patients infected with *H.pylori* have the CagA gene, and 13 patients are not and also measured the SOD in 30 control groups that not infected with *H.pylori*. Serum level of SOD was significantly ($p<0.05$) higher in patients with chronic gastritis compared to controls. Also significantly higher ($p<0.001$) in patients with chronic gastritis infected with *H.pylori* positive CagA than patients infected with *H.pylori* negative CagA.

Key words: chronic gastritis, *H.pylori*, CagA, SOD

الخلاصــة

إصابة الغشاء المخاطي للمعدة ببكتريا ال *Helicobacter pylori* تترافق مع التهاب المعدة المزمن والقرحة المعدية وسرطان المعدة. عوامل الضراوة الخاصة بهذه البكتريا تتضمن أنواع من البروتينات التي تشارك في أحداث في المراضية وهي ال CagA و VagA . مجموعة أخرى من عوامل الضراوة تكون مهمة في تكوين المستعمرات في الغشاء المخاطي المعدي منها اليوريز و عامل الحركة وأنزيم ال Superoxide dismutase (SOD) الذي يتواجد في الكائنات التي تتعرض إلى وسط سام. في هذه الدراسة تم قياس مستوى الـ SOD في مصل 80 مريض كانوا يعانون من التهاب المعدة المزمن ومصابين ببكتريا ال *H.pylori* منهم 37 مريض مصابين ال *H.pylori* الحاملة لل CagA و13 مريض مريض مصابين ال *H.pylori* لكن غير حاملة لهذا الجين . وكانت مجموعة السيطرة تتكون من 30 شخص بحالة صحية سليمة و غير مصابين بالبكتريا. وجد أن مستوى الـ SOD يزداد معنوياً ($p<0.001$) في المرضى المصابين بالتهاب المعدة المزمن مقارنة بمجموعة السيطرة ويزداد معنويا في مرضى التهاب المعدة المزمن المصابين ببكتريا ال *H.pylori* والحاملة للـ CagA ($p<0.05$) مقارنة بمرضى التهاب المعدة المزمن المصابين بالبكتريا ولكن غير حاملة للـ CagA.

Introduction

The gastric pathogen *Helicobacter pylori* is a curved, microaerophilic proteobacterium that has been implicated as a causal agent of peptic ulcers and a risk factor for adenocarcinoma (1,2, 3,4,). During the infections, disease symptoms may or may not occur, though gastric inflammation is apparently ubiquitous. The pathogenesis of *H.pylori* relies on its persistence in surviving a harsh environment, including acidity, peristalsis, and attack by phagocytic cells and their released reactive oxygen species (5). Several potential virulence factors derived from *H.pylori* are considered to attract or activate neutrophils and mononuclear cells., an immunodominant 120-140 KDa antigen termed cytotoxic associated antigen (CagA), the CagA positive strain cause more server inflammations(6).

The stomach gastritis associated with *Helicobacter pylori* infection stimulates the generation of reactive oxygen species (ROS) by the inflammatory cells present in the mucosa (7, 8, 9). An increase in ROS directly correlated with bacterial load (10). In addition to internally generated reactive oxygen species, the successful pathogen must also deal with reactive oxygen species that are generated by phagocytic cells of the host immune response. (11, 12). Protection of cells against ROS is accomplished through the activation of oxygen-scavenging enzymes such as SOD, catalase and glutathione peroxidase have been identified (13). Superoxide dismutase is a nearly ubiquitous enzyme among organisms that are exposed to toxic environ

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The single SOD of *Helicobacter pylori*, encoded by the *sodB* gene, has been suspected to be a virulence factor for this pathogenic microaerophile, but mutations in this gene have not been reported previously (14). The mechanisms for the detoxification of reactive oxygen species are of particular interest in *H.pylori*. Despite the fact that this organism is an obligate aerobe, it is unable to grow in atmospheric concentrations of oxygen. microaerophilic organisms, like *H.pylori*, are particularly vulnerable to the detrimental effects of oxygen and oxidative stress (15). Nevertheless, they do possess some of the enzymatic machinery needed to eliminate or minimize toxic oxygen-derived products. Organisms that grow in toxic environments must have mechanisms to handle reactive oxygen species (e.g., superoxide anions, peroxides, and hydroxyl radicals) that are by-products of oxygen metabolism (16, 17). Of these genes, only *katA* (catalase) mutants have been characterized (18). Although catalase-defective mutants are no different from the parent in their binding to epithelial cells, this enzyme may be important in detoxification of reactive oxygen species produced by the host immune response. Genes encoding an akyl hydroperoxide reductase, a thiol-specific peroxidase, and other potential detoxification enzymes were identified, but mutations in these genes have not been reported or characterized (14, 19). Impairment in this important host cell defense mechanism would greatly reduce the ability of the gastric to epithelial cells to tolerate an environment high in ROS, such as would be present with the chronic gastritis associated with *H.pylori* infection. Disturbance of the oxidant-antioxidant balance in the stomach might greatly increase the risk of cell death or DNA damage, from ROS (20, 21). The aim of this study to investigate the relation between the *H.pylori* with CagA- positive and CagA - negative strains and the production of SOD in patients with chronic gastritis (HP+) and healthy control group (HP-).

Materials and Methods

Eighty subjects (48 male and 32 female; mean age 51.7), were referred to the gastrointestinal endoscopy unit at Al-Yarmook Teaching Hospital, non of whom had received non steroidal anti- inflammatory drugs, within previous three months, participated in this study. Endoscope fining in the patients were as follows: normal mucosa and no *H.pylori* infected (30 subjects) and 50 patients with chronic gastritis without ulcer. Biopsy specimens were taken from the antrum of all subjects in this study, by using the same size forceps, from similar topographical sites at each endoscopy; biopsies were fixed in 10% formal buffer saline for histological examination. Blood samples were taken from all subjects and the serum were stored at -20 Ċ until be used.

Histology

The biopsy specimens were embedded in paraffin and stained with haematoxylin – eosin (HandE) and Giemsa stained for *H.pylori* determination and diagnostic as chronic gastritis. In situ hybridization (ISH) for detection of *H.pylori* / CagA gene. In situ hybridization (ISH) is a technique makes use of the high specificity of complementary nucleic acid binding to detect specific DNA or RNA sequence in the cell. For detection of this markers , the biotinylated DNA probe hybridize to the target sequence (*H.pylori* / CagA mRNA sequence) then a streptavidin-AP (streptavidin-alkaline phosphatase) Conjugate is applied followed by addition of the substrate promo-chloro – indolyl – phosphatel / nitro-blue tetrazolium (BCIP/NBT) which yield an intense blue – black signal appears at the directly specific site of the hybridized probe. This strepteividin – Ap conjugate like the biotinylated probe provides raid and highly sensitive detection method. The use of Biotin – Labeled DNA probe for *H.pylori* / CagA (8 μg/10015 ML) litter dd H2O . Probe size: 349 bp (Maxim Biotech, Inc., U.S.A).

Scoring

Hybridization /Detection System will give an intense blue –black color at the specific sites of the hybridization probe in both positive test tissues. A scoring system that includes evaluation of the staining percentage of stained gastric cells was employed for the expression of CagA gene of *H.pylori*. Counting the number of the positive cells in the gastric tissue which gave a blue-black nuclear staining under the light microscope. The extent of the ISH signaling the cells of the examined tissue was determined in 10 fields under high power microscope (40X). In each field, the total of examined cell was about 100 cells per field and this gives a total number.

Measurement of superoxide dimidiate (SOD)

For the quantitative determination of superoxide dismutase in whole blood. This produce is suitable for manual use RANDOX, Cat. No. SD 125 Mixed substrates, Buffer, xanthine oxidase and standard. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react

with 2 (4- iodophonyl) – 3- (4- nitrophenol) -5- phanyltetrazolium chloride (I.NT) to form a red fermazan dye. The SOD activity is then measured by the degree of inhibition of this reaction. One unit of SOD is that which causes 50% inhibition of the rate of reduction of INT under the conditions of the assay.

Xanthine $\longrightarrow$ **Uric acid + O_2**

I.N.T $\longrightarrow$ **formazan dye**

$O_2 + O_2 + 2H \longrightarrow O_2 + H_2O$

Statistical analysis

Statistical analysis was performed using ANOVA test to determine whether the means were equal among three groups – i.e. CagA-, CagA+ and controls, p value of < 0.05 was considered statistically significant.

Results

The expression of CagA was detected by in situ hybridization technique. From 50 patients complaining chronic gastritis and infected with *H.pylori* who were tested for CagA, 37 (74%) were found to be positive CagA and 13 (26%) patients have CagA – negative (Table 1, Figure 1).The mean level of SOD increased significantly in patients infected with *H.pylori* CagA positive strains p<0.001 when compared with healthy subjects and patients infected with *H.pylori* CagA negative (Table 2,Figure 2).The difference in SOD level between with *H.pylori* that have CagA positive or CagA negative is statistically significant p<0.01.

Table (1): Expression of CagA mRNA in *H.pylori*– positive patients with chronic gastritis by (ISH).

	CagA status	No.	(%)
***H.pylori* positive**	positive	37	74
	negative	13	26
	Total	50	100

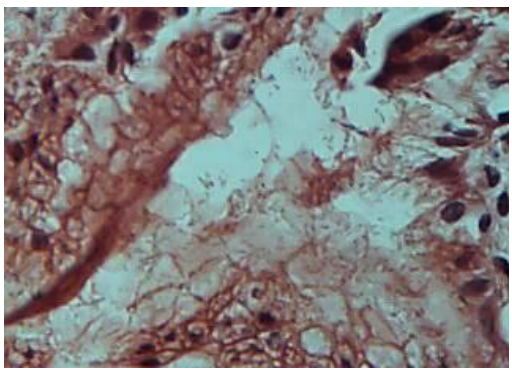

Figure (1): Gastric antral biopsy specimen from stomach infected with *H.pylori* that appear curved or round (Giemza stain) (100X).

Table(2): The serum level of SOD in patients infected with *H.pylori* and CagA status.

Group	Mean ± S.E SOD	S.D.	*P* Value	F Test
Control (Hp-)	177.70 ±2.91	9.20		18.56
Hp+ CagA-	* 284.00 ± 14.22	44.95	<0.05	
Hp+ CagA+	** 477.00 ±54.71	73.84	<0.001	

No significant difference *p*> 0.05
* Significant at the 0.05 levels
** Significant at the 0.001 level
Hp: *H.pylori*

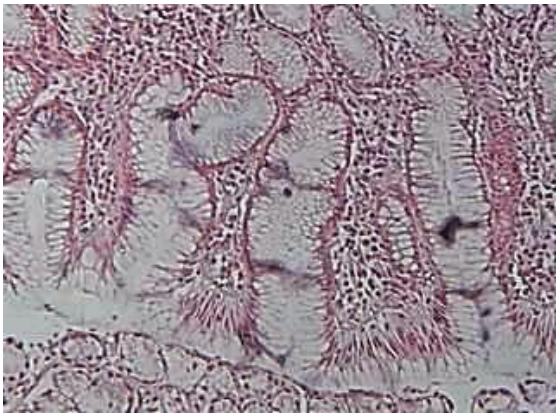

Figure (2): Detection of CagA, in patients with gastroduodenal disease by in situ hybridization. staining of CagA mRNA by BCIP/NBT (blue-black) counterstained with nuclear fast red. Tissue from patients with antral gastritis shows positive CagA by hybridization signals.

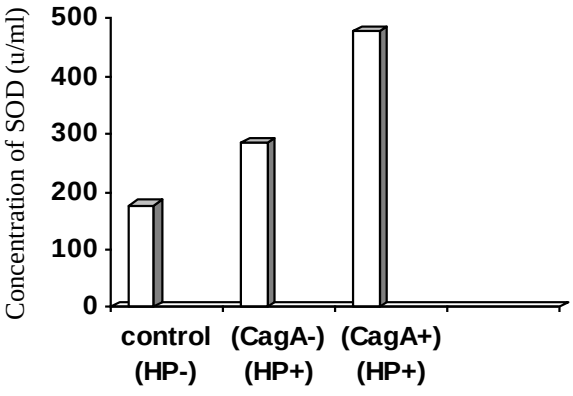

Figure (3): The serum level of SOD in patients infected with *H.pylori* and CagA status.

Discussion

Cytotoxine associated antigen – positive strain was significantly higher ($p<0.001$) in chronic gastritis patients than in control group. It can be seen that 74% of patients who have *H.pylori* infection have CagA positive strains. *H.pylori* strains were positive for the CagA 74.4% of Costa Rica patients [22]. Other study reported the prevalence of CagA was more than 80% among patients with chronic gastritis [23]. Increasing of CagA mRNA among those patients may explain the role of CagA positive *H.pylori* in the development of gastritis [24]. The mechanisms by which CagA modify the activity of epithelial cells is explaining by serving as scaffolding protein able to interact and modify the function of a variety of molecules involved in cell to cell interaction, cell motility, and proliferation [23, 25].The differences between the results, possibly by using different methods to assess the expression of CagA positive *H.pylori* in patients with gastritis, such as ELISA methods, PCR that the correct design of primers is very important , the different sets of CagA primers give different results, and this will attributed to divergence in the primer target sequences [26, 27].*H.pylori* infection of the gastric mucosa is associated with abundant inflammatory response; this bacterium is capable of stimulating oxidative bursts from noutrophils [28].Gastric tissue from *H.pylori* infected persons contains more ROS than normal tissue and there is a direct correlation between bacterial and the amount of ROS in the gastric mucosa [29, 30].This study supports the direct correlation between ROS and gastric mucosal damages in patients infected with *H.pylori* which can increase the susceptibility of gastric epithelial cells to ROS – associated cell injury. The increase of SOD and activity in patients with *H.pylori* – CagA positive strains is probably responsible for the increased survival of these cells. The generation of intracellular ROS on the presence of CagA positive *H.pylori* strains is possible explanation for the increases in activity of ROS – scavenging enzymes. That *H.pylori* induces the production of intracellular ROS; this increase in ROS in gastric cells was enhanced by increasing the concentration of *H.pylori* and inhibited by use of antioxidant[22].There are more ROS in the gastric mucosa of patients infected with *H.pylori* CagA positive strains, is significantly difference from patients with *H.pylori* CagA negative strains. As a response to increase formation of ROS the antioxidant enzyme are increase in these cells, suggest that gastric epithelial cells have a higher concentration of these enzymes (SOD and catalase) so they will be able to avoid lethal injury in patients infected with CagA positive strains than in patients infected with CagA negative strains [13].Gastric cells infected with CagA-positive *H.pylori* strains have higher catalase level, catalase enzyme convert hydrogen peroxide to H2O and oxygen molecule. Accumulations of hydrogen peroxide reduce the activity of SOD; these conversions of hydrogen peroxide protect the cells from sudden exposure to superoxide, giving them a survival advantage [13, 14].The presence of ROS along with the reduction of antioxidants, such as vitamin C in the gastric mucosa of people infected with *H.pylori* potentially increases the risk of oxidant – related cellular injury and DNA damage, change in the levels of cellular ROS-scavenging enzymes induced by *H.pylori* may further increase this risk of developing gastric cancer from ROS in patients infected with *H.pylori* [31].

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Cephalothin as a Carrier of 6-Mercaptopurine for Targeting Cancer Tissues

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Abstract

A lower extracellular pH is one of the few well-documented physiological differences between tumour and normal tissues. On the other hand, elevated glutathione (GSH) level has been detected in many tumours compared with healthy surrounding tissues. The compound II: 3-(9H-purin-6-yl-thio) carbonothionyl methyl-8-oxo-7-(2-thiophen-2-yl) acetamido-5-thia-1-azabicyclo-4-octo-ene-carboxylic acid was a cephalothin derivative contain 6-mercaptopurine (6-MP). Compound II react with general base catalysis in slightly acidic pH or with sulfhydryl nucleophiles to release the chemotherapeutic drug 6-MP. The generation of compound II was accomplished following multistep reaction procedures. The structure of compound II and its intermediate was confirmed by their melting point, infrared spectroscopy, CHN and NMR analysis. The hydrolysis of compound II in aqueous buffer solution of pH 6 and in the presence of GSH at pH 7.4 was studied. The partition coefficient (PC) of compound II was also determined. Compound II has acceptable rate of hydrolysis at slightly acidic medium pH 6 (t1/2 = 56.34 min.) and 80% of compound II had been converted to 6-MP within 30 min in the presence of GSH. And the compound has acceptable stability at pH 7.4(t0.5=639.65 min.) and the rate of hydrolysis was effected by change the buffer concentration. This compound can selectively deliver 6-MP into the tumour tissues which have acidic pH or elevated GSH level. Compound II had an improved PC value of 12.23 compared to 1.22 for free drug 6-MP confirming higher lipophilicity.

Key words: 6-mercaptopurine, cancer, prodrug, targeting

الخلاصة

ان انخفاض الاس الهيدروجيني (pH) خارج الخلية هو احد العلامات الفسلجية الفارقة الموثوقه بين الانسجة السرطانية والانسجة الطبيعية. ومن جهة اخرى فقد لوحظ ارتفاع في مستوى الغلوتاثيون (GSH) في عدد من الاورام مقارنة بالانسجة الطبيعية المحيطة. ان المركب II:

3-(9H-purin-6-yl-thio) carbonothionyl methyl-8-oxo-7-(2-thiophen-2-yl) acetamido-5-thia-1-azabicyclo-4-octo-ene-carboxylic acid

وهو مشتق للسيفالوثين (Cephalothin) يحتوي على مركب الـ 6-مركبتوبيورين (6-MP). ان المركب II يتفاعل مع القاعدة العامة في المحيط الحامضي الضعيف او مع سلفهايدريل (sulfhydryl) الباحث عن النواة لتحرير الـ 6-مركبتوبيورين. تم تحضير المركب II باتباع طريقة التفاعل متعدد الخطوات وقد تم اثبات الصيغ الكيمياوية للمركب النهائي والوسطي والتاكد منها بواسطة قياس درجات الانصهار والتحليل الطيفي للاشعة تحت الحمراء والتحليل الدقيق للعناصر. وتم دراسة تحلل المركب II في المحلول الدارئ ذي الاس الهيدروجيني (pH 6) وكذلك تم دراسة معامل التأين للمركب II (partition coefficient). اوضحت النتائج المستحصلة من هذه الدراسة ان المركب II يمتلك سرعة تحلل مقبولة في المحلول الدارئ ذي الاس الهيدروجيني (pH 6) (بعمر نصف = 56.34 دقيقة) وثباتية مقبولة في المحلول الدارئ ذي الاس الهيدروجيني (pH-74) ، كما تبين ان سرعة تحلل المركب تتاثر بتغير تركيز المحلول الدارئ. لذلك من المتوقع ان المركب II يستطيع ايصال الـ 6-ميركبتوبيورين (6-MP) الى الخلايا السرطانية ذات الاس الهيدروجيني المقارب للـ (6). وقد اتضح ايضا ان الغلوتاثيون يزيد من سرعة تحلل المركب II، حيث ان 80% من المركب قد تحول الى الـ 6-مركبتوبيورين خلال 30 دقيقة. كما تم ملاحظة ان معامل التأين قد تحسن مما يدل على تحسن نفاذية المركب داخل الجسم، حيث ان معامل التأين للمركب II هو 12.23 في حين ان معامل التأين للـ 6-مركبتو بيورين هو 1.22.

Introduction

The use of antineoplastic agent 6-MP accompany by several disadvantages including sever adverse effects [(1)], poor absorption, low bioavailability [(2)], limitation of uses [(3)], thiopurine associated leukemia [(4,5)] and drug resistance[(6,7,8)]. Cephalosporins are β-lactam antibiotics; their degradation depends on the side chain at C-7 and the substitution on C-3 [(9)]. The presence of good leaving group at C-3 facilitate spontaneous expulsion of the 3-substituent by any general nucleophile, β-lactamase or pH change [(10,11)], scheme (1).

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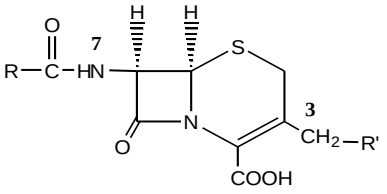
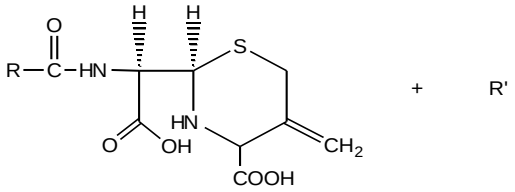


Cephalosporine-C

Beta-Lactamase,
OH^-
H_3O^+

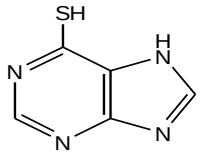


Anhydrodesacetyl cephalosporinic acid

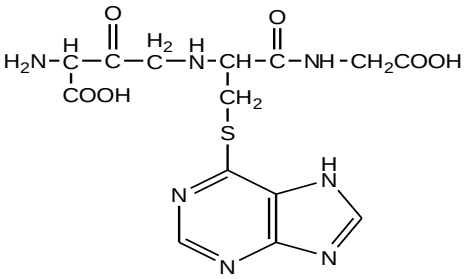
R'= good leaving group

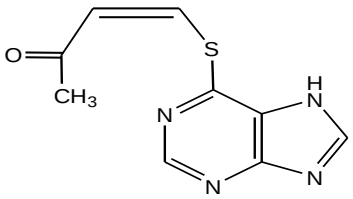
Scheme (1): Possible degradation of cephalosporin C [11].

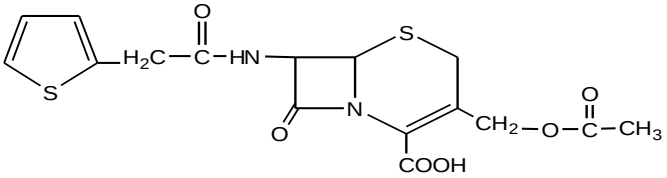
Lowering extracelular pH (pHe) is one of the few well-documented physiological differences between solid tumour and normal tissues. The main pHe in tumour tissues is 0.6-0.8 unit lower than normal tissues, with an absolute as low as 5.8 [12,13]. Changes in GSH content and level of glutathione S-transferase have been detected in tumours [14]. Moreover, increased levels of GSH have been linked with drug resistance [15] and poor patient prognosis[16]. Thus, an excellent opportunity exists for the design of prodrugs that specifically target tumours with low pHe or with abnormal GSH level. Doxorubicin armed antibody was a prodrug designed to target tumours with low pHe [17]. The 6-MP prodrug S-(9H-purin-6-yl) glutathione (figure 1a) which can be further metabolized *in vivo* to yield 6-MP[18]. Cis 6-(2-acetyl vinyl) thiopurine (AVTP). (figure 1a), a potential 6-MP prodrug targeting tumours with up-regulated GSH level. Structurally AVTP is butanone conjugate of 6-MP and a Michael acceptor that undergoes addition-elimination reaction with nucleophiles to yield 6-MP, the AVTP metabolism to 6-MP was GSH dependent [19].



6-Mercaptopurine







AVTP

Figure (1a): Chemical structure of 6-mercaptopurine, S-(9H-purin-6-yl) glutathione, Cis-AVTP and cephalothin.

Materials and Methods

Chemicals

6-mercaptopurine and HCl were purchased from Fluka (Germany); Cephalothin sodium was purchased from Laboratories TORLAN, S.A. (Spain); GSH was purchased from Sigma (USA); and Carbon disulfide was purchased from Riedel-Dehen (Germany). All chemicals were reagent grade and obtained from standard commercial sources. Elemental micro analysis were performed using Carlo Erba elemental analyzer 1106 (Italy); Melting points were measured on Thomas Hoover Electronic melting point apparatus; and are uncorrected; Infra red spectra were recorded as KBr disks on Back IR spectrophotometer (College of Pharmacy, University of Baghdad); and H-NMR spectrum was carried out on Mercury MHZ-NMR spectrometer (sppm) at MDIT center in the University of Toronto.

Synthesis of potassium 7H-purinylcarbonotrithionate (compound I)

To a stirred solution of potassium hydroxide (0.313gm, 5.58 mmol) in absolute ethanol (10ml) at 15-20 ^{o}C , a 6-MP (0.96gm, 5.58 mmol) was added over (0.5 min). After 0.5 hr carbon disulfide (0.425gm, 5.58mmol) was then added and the reaction mixture was stirred for 3 hr at 25 ^{o}C. Then the precipitate of the desired compound was obtained by the addition of diethyl ether. The precipitate was filtered and re-crystallized from ethanol to give white crystalline powder of compound I, percent yield (63%), melting point (240-243 ^{o}C) and infrared yield absorption band, (cm^{-1}): 3450 of NH stretching vibration (str. Vib.) of purine, 1610 and 1556 of C=N str. Vib., 1598 of NH bending vibration (bend. Vib.) of purine, 1497 and 1423 of C=C str. Vib., 1410 of CH bend. Vib. of purine 1205 of C=S str. Vib. of trithiocarbonate and 870 of aut plane CH bend. Vib. of purine.

Synthesis of 3-(9H-purin-yl thio) carbonothionyl-methyl-8-oxo-7(2-thiophen-2-yl) acetamido-5-thia-1-azabicyclo-4-octa-2-ene-carboxylic acid (compound II)

A mixture of cephalothin sodium (0.73gm, 3.4mmol) and of compound I (0.5gm, 3.4mmol) in (30ml) of 0.1M phosphate buffer (pH 7) was heated for 3hr at 60-70^{o}C. The mixture was cooled to 5 ^{o}C and acidified with HCl (1N) to reach pH 4.0, a precipitate was obtained and collected by filtration, dried and recrystallized from ethanol to give a yellow crystalline powder of compound II. Percent yield (60%), melting point (196-199 ^{o}C), elemental microanalysis, calculated/found: (C 42.539/42.996, H 2.855/3.011, N 14.882/15.189, O 11.339/11.637, S 28.390/28.427, the infrared absorption band (cm^{-1}): 3450 of NH str. Vib., 3000-2500 group of small band due to OH str. vib. of OH of COOH, 2682 of CH_2-S str. vib. of cephalothin, 1773 of C=O str. vib. of β-Lactam, 1610 a mide str. vib., 1617 and 1570 of C=N str. vib. 61598 of NH bend-vib. of purine, 1500 and 1430 of C+C str. vib., 1408 of CH bend. vib. of purine, 1228 of C=S str. vib. of trithiocarbonate ester, 875 out plane CH bend vib. of purine, 690 str. vib. of thiophen: and HNMR in $CDCl_3$ 3.06, 3.16 (q, 2H of methylene α to C=C and α to S-C-), 3.44 (s, 2H of methylene α to C-R and α to (C=O)-N), 4.07 (s, 2H of methylene α to –C=C and α to –S-C-R), 5.1 (d, 1H of propiolactam α to –S R, β- to –N-C=O), 5.45 (d, 1H of propiolactam α to N-C=O, β- to –S-R), 6.83 (d, 1H of 2-thiophen), 6.93 (t, 1H of 2-thiophen), 7.4 (d, 1H of 2-thiophen), 8.32 (s, 1H of sec. amide), 8.57 (s, 1H of purine), 13.65 (s, 1H, NH of purine) as shown in (Figure 1b).

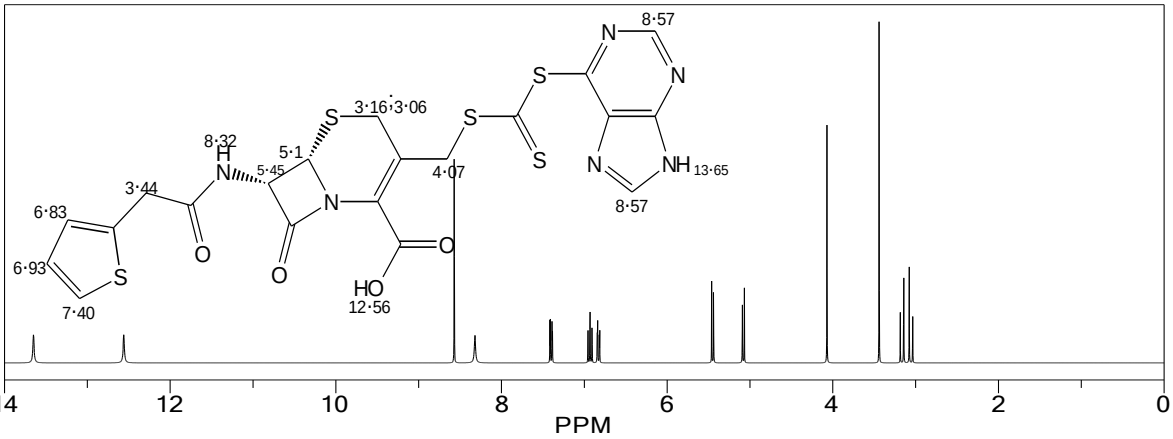


Figure (1b) : H NMR spectrum of compound II .

Hydrolysis of compound II at pH 6 and pH 7-4

The hydrolysis of compound II was carried out for the equivalent of (0.01mg/ml) in aqueous phosphate buffer solution of pH 6 and pH 7.4 at 37 ^{o}C. The total buffer concentration was 0.1M and the ionic strength (μ) of 1 was maintained by adding calculated amount of NaCl. Different sample were taken for analysis at specific time interval (16, 30, 60, 120, 240 min) and the rate of hydrolysis was followed spectrophotometrically by recording 6-MP absorbance increase accompanying the hydrolysis at 324nm and 316 for pH6 and pH7-4 respectively. The observed pseudo-first order rate constant was determined from the slope of the linear plot of log concentration of 6-MP *vs* time.

The effect of the buffer concentration at the rate of hydrolysis

The same procedure mentioned above was fallowed for studing the hydrolysis of compound II in different buffer concentration (0.2, 0.5 and 0.8) and the ionic strength (μ=1) to determine the effect of buffer concentration at the rate of hydrolysis.

Hydrolysis of compound II in the presence of glutathione

Compound II (0.96gm, 0.17mmol) was incubated with glutathione (0.52gm, 0.17mmol) in phosphate buffer (0.1M and μ=1) at pH 7.4 in shaking water bath 37^{o}C. A 1.0 ml sample was removed and added to 4ml of phosphate buffer. The concentration of 6-MP was determined spectrophotometrically at 314nm.

Partition coefficient estimation

Partition coefficient of compound II at pH 7-4 was estimated by using shake flask method. The amount of compound II in both phase were measured spectrophotometrically at 298 nm, and the partition coefficient value was calculated by the following equation [20].

$$PC = \frac{Conc.\,in\,organic\,layer}{Conc.\,in\,inorganic\,layer}$$

Results and Discussion

Target compound II was obtained following procedure outlined in scheme (2), compound I was obtained from reaction of 6-MP and carbon disulfide in the presence of potassium hydroxide. The reaction was a nucleophilic addition reaction in which the thiolate anion was added to the carbon atom of the disulfide [21]. Compound II have been synthesized using the method previously established for the synthesis of cephalosporin derivative [22]. The reaction followed a nucleophilic substitution in which the thiolate moiety attached the methylene moiety at position 3 of cephalothin leading the formation of trithiocarbonate ester with liberation of acetic acid. Under the experimental conditions used the hydrolysis of the compound II followed pseudofirst order kinetic, since the plot of log conc. of 6-MP *vs* time resulted in straight line from it slope, the observed rate constant of hydrolysis (K_{obs}) was calculated from figure (2a) and (2b) which representive graph for hydrolysis of compound II. The K_{obs} were 0.0123 min^{-1} and 1.083x10^{-3} at pH 6 and pH 7.4 respectively and the half-life of hydrolysis of compound II were 56.34 min and 639.65 min at pH6 and pH 7.4 respectively. The half life was calculated using the equation:

$$t_{0.5} = \frac{0.693}{K_{obs}}$$

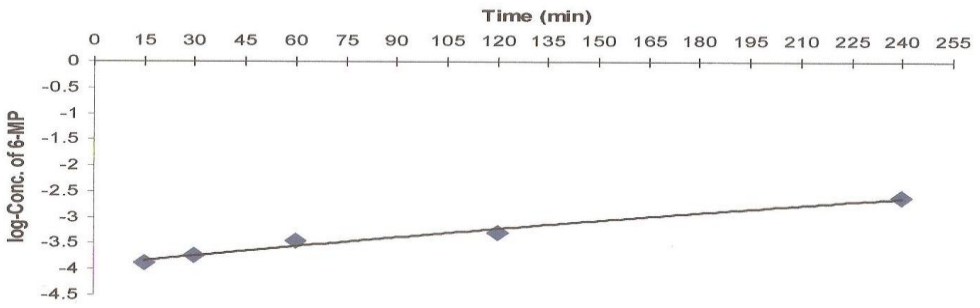


Figure (2a): First order plot for the hydrolysis of compound II in 0.1 M phosphate buffer of pH 6.0 at 37 ^{o}C (μ =1).

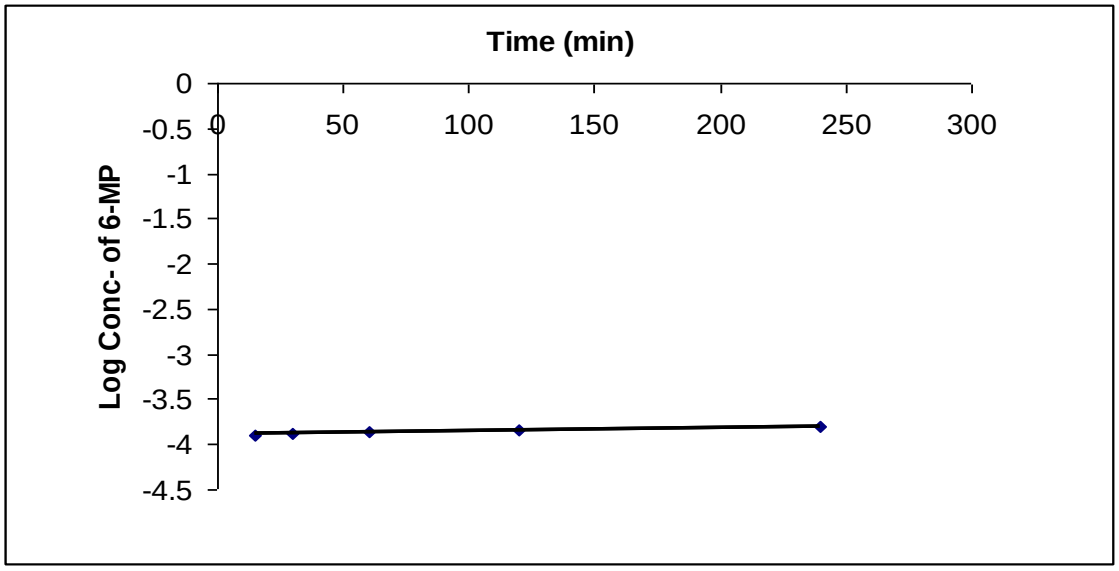
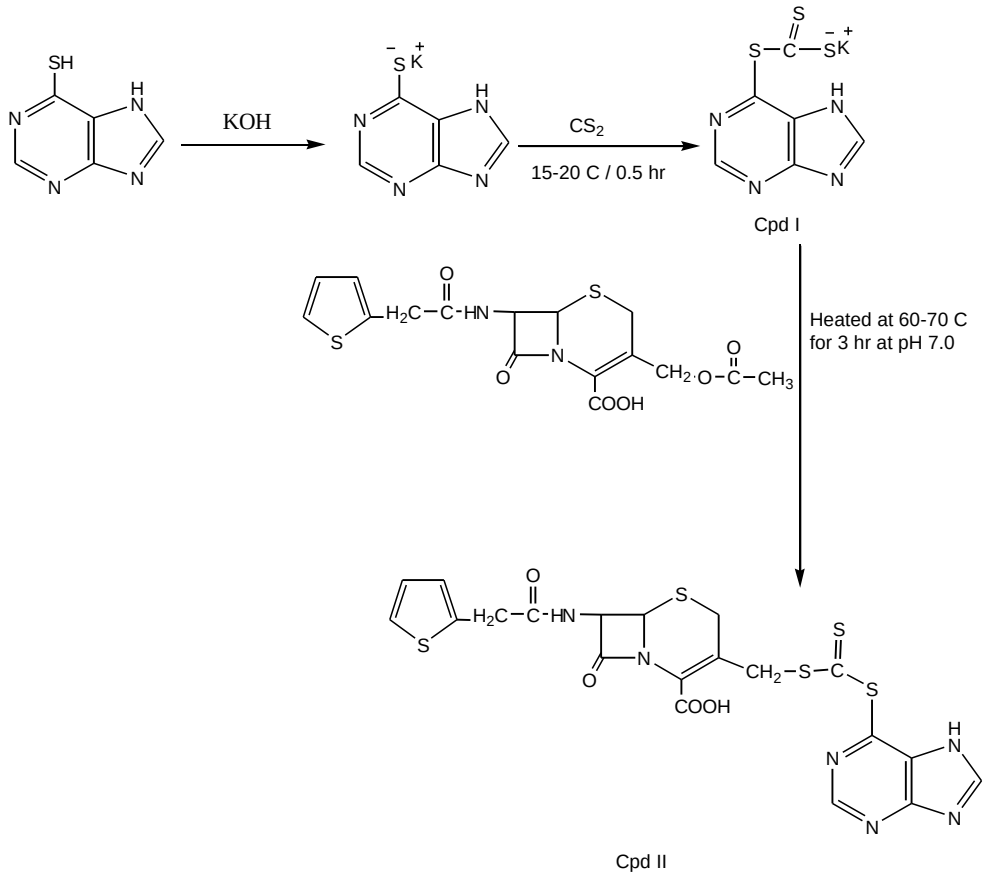
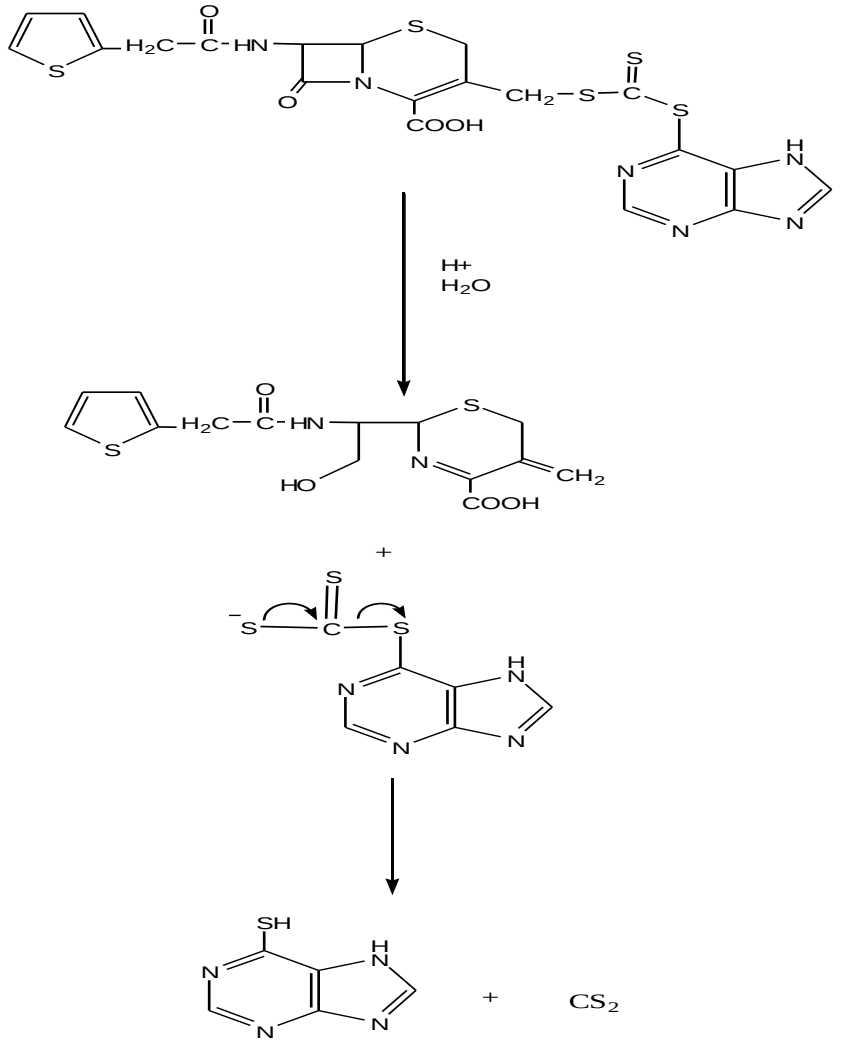


Figure (2b): First order plot for the hydrolysis of compound II in 0.1 M phosphate buffer of pH 7.4 at 37 ^{o}C (μ =1).

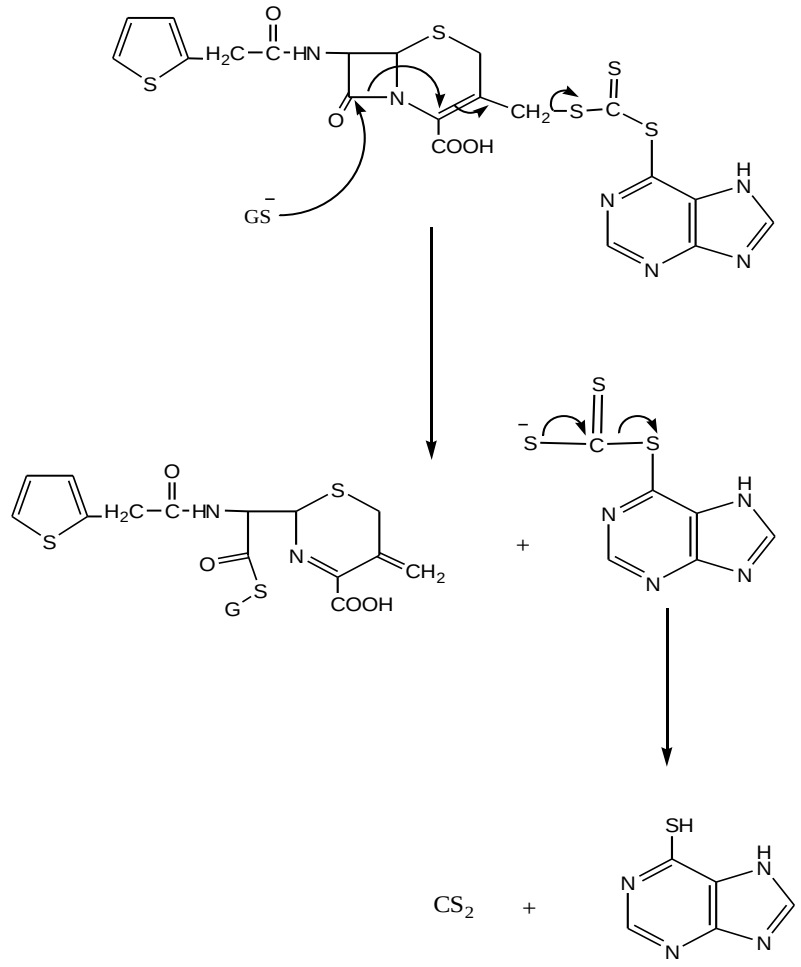


Scheme (2): Synthesis of compound II.

The release of 6-MP from compound II depend on the opening of β-lactam ring which in turn depend on the pH of the media and the ease with which the substitution at position 3 of cephalosporin will leave the molecule [10,11]. The tithiocarbonate is better leaving group than acetoxy group due to it soft base [23], and this will cause the hydrolysis of compound II at pH 6 is faster than hydrolysis of cephalothin at the same pH (Scheme 3). The rate of hydrolysis of compound II at pH 6 in the presence of different buffer concentration was calculated from figure (4) which represent the hydrolysis of compound II at different buffer concentration. The K_{obs}. Were 0.0133mmin^{-1}. 0.0154 min^{-1} and 0.0166 min^{-1} at buffer concentration 0.2, 0.5 and 0.8M respectively. The half-lives were 52.13min, 45.00min. and 41.1 min. for buffer concentration 0.2, 0.5 and 0.8M respectively. Under experimental condition used the hydrolysis of compound II followed second order kinetic, since plot of log conc. of 6-MP *vs* time result in curve line. The formation of 6-MP from compound II was linear for at least 30 min. (figure 3). 80% of compound II had been converted to 6-MP within 30 min. Compound II reacted rapidly with thiolate nuceophile of GSH to yield the 6-MP. The thiolate was attack the β-lactam ring of cephalosporin resulted in the opening of this ring with the release of trithiocarbonate derivative from position 3 [24] (scheme 4). The partition coefficient has become the most common physicochemical property [25]. The partition coefficient of compound II equal to 12.23; so this compound had an improved partition coefficient value compared to 1.2 for 6-Mp, confirming higher lipophilicity and improvement of the drug bioavailability.



Scheme (3): The liberation of 6-mercaptopurine from Compound II at pH 6.0[10].



Scheme (4): Liberation of 6-mercaptopurine from compound II in the presence of glutathione.

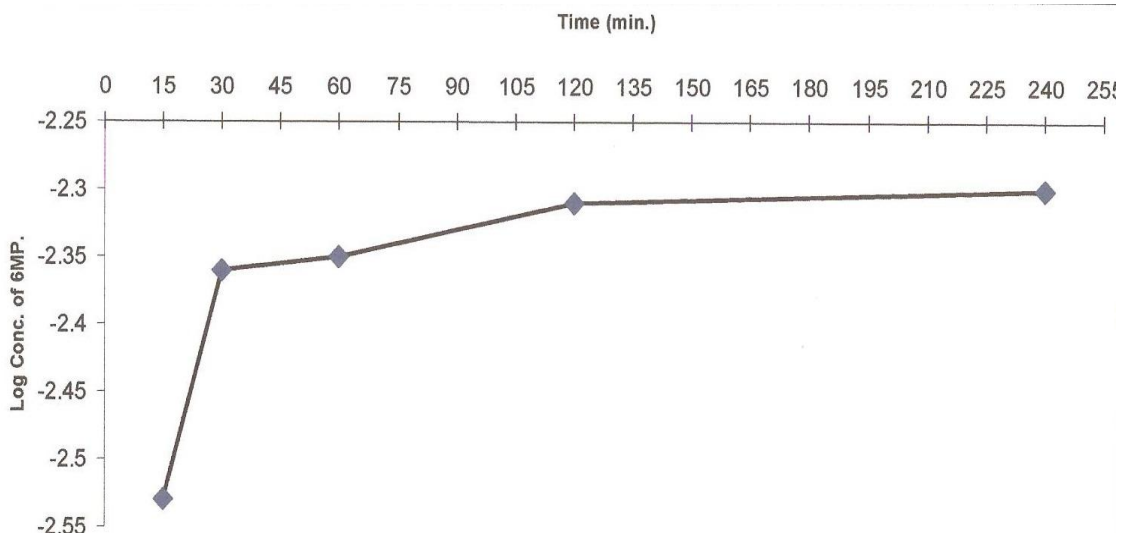


Figure (3): Plot of the hydrolysis of compound II in the presence of glutathione (0.17 mmole) in phosphate buffer of pH 7.4 at 37 ^{o}C (μ =1).

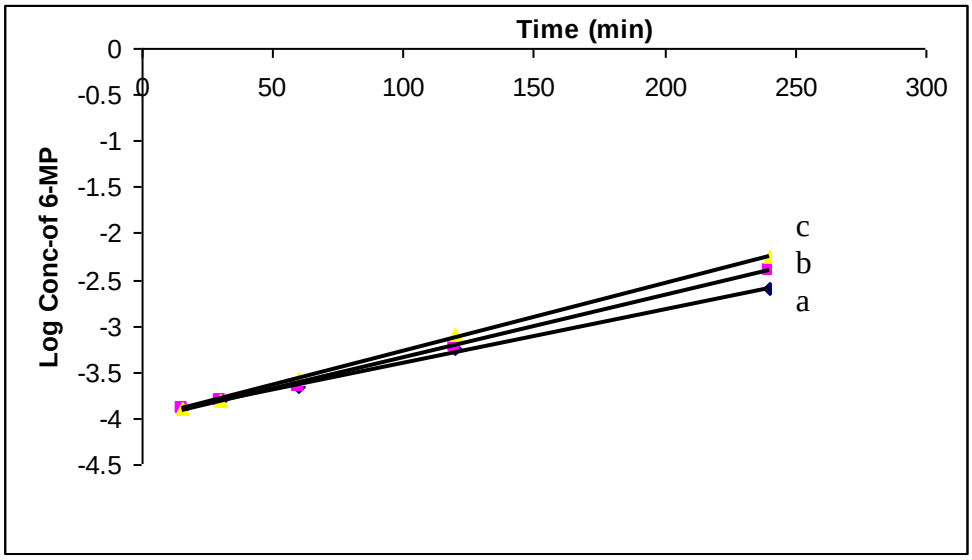


Figure (4): The effect of buffer concentration on the rate of hydrolysis of compound II at Ph 6 at 37 ^{0}C (μ =1).

* buffer concentration:
a= 0.2 M , b= 0.5 M , c= 0.8 M

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Single Dose Antibiotic Prophylaxis in Outpatient Oral Surgery Comparative Study

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Abstract

It is clear that correct application of antibiotic prophylaxis can reduce the incidence of infection resulting from the bacterial inoculation in a variety of clinical situations; it cannot prevent all infections any more than it can eliminate all established infections. Optimum antibiotic prophylaxis depends on: rational selection of the drug(s), adequate concentrations of the drug in the tissues that are at risk, and attention to timing of administration. Moreover, the risk of infection in some situations does not outweigh the risks which attend the administration of even the safest antibiotic drug. The aim of this study was to compare between 2 prophylactic protocols in out patients undergoing oral surgical procedures. Thirty patients, selected from the attendants of oral surgery clinic in Al-Karamah Dental Center, were subjected to different oral surgical procedures under local anesthesia. These patients were given single dose antibiotic prophylaxis in 2 groups; 1st group were 15 patients given 1 million i.u. of procaine penicillin I.M. 30 minutes before oral surgery, 2nd group were 15 patients given 600mg clindamycin orally 1 hours before oral surgery. The maximum time for all procedures was 2 hours. There was no difference between procaine penicillin (1 million i.u.), and clindamycin (600mg), regimens concerning post operative infection in out patient's oral surgical procedures.

Key words: Antibiotic prophylaxis, outpatient oral surgery

الخلاصة

من الواضح أن التطبيق الصحيح لإعطاء المضـــادات الحيوية لإغراض وقائية يمكن أن يقـلل نسبة حدوث االاختـلاطات البكتيرية الناتجة من النمو البكتيري بسبب عـدد من الحالات السريريـة المتنوعة. إن التطبيـق ألصحيح لا يمنع كل الاختلاطات البكتيرية ولكنه يستطيع التخلص من الاختلاطات الموجودة فعلا. إن النتيجة االأفضل للتعامل الوقائي للمضاد الحيوي يعتمد على الاختيار المنطقي للعقار المعطى والتركيز الكافي للعقـار (المضاد الحيوي) في النسيج المهدد بالاختلاط البكتيري والانتباه للتوقيت عند إعطاء المضاد الحيوي . من الجدير بالذكر مخاطر بعض الاختلاطات البكتيرية لاتستدعي إعطاء المضاد وهو أمرله سلبياته حتى مع أكثر المضادات أمانا.إن غرض هذه الدراسة هو المقارنـة بين بروتوكولين علاجيين وقائيين للمرضى الذين يخضعون لتداخلات جراحية في العيادات الجراحية لطب الأسنان (تحت التخدير الموضعي). تم اختيار ثلاثون مريضا من قبـل المراجعين الذين يزورون قسم الجراحة في مركز الكرامة التخصصـــي لطب الأسنان حيث تم إخضاعهـم لتداخلات جراحية متنوعة تحت التخدير الموضعي. هؤلاء المرضى ، تم إعطاؤهم جرعة مفردة من عقـار البروكاين بنسلين وهي مليون وحدة عالمية وتم إعطاؤها عضليا (للمجموعة الأولى المكونة من 15مريضا) وعقار الكلندمايسين 600 ملغم عن طريق الفم (للمجموعة الثانية وتضم 15 مريضا) لـم يتجاوز الوقت اللازم لإجراء التداخلات الجراحية ساعتان.كانت النتيجة انه لم يكن هناك أي اختلاف بين المجموعتين من ناحية عدم حدوث الاختلاطـات البكتيريـة بعد التداخل الجراحي الفموي بأنواعه.

Introduction

The use of antimicrobial agents to prevent infection is effective in many circumstances, and it is limited to specific, well-accepted indication to avoid excess cost, toxicity, and antimicrobial resistance.[1] Preoperative topical, oral, and intravenous antimicrobial prophylaxis has been important in decreasing the incidence of surgical site infection.[2,3] The time taken for an antibiotic to reach an effective concentration in any particular tissue reflects its pharmacokinetic profile and the route of administration. [4] Administration of prophylaxis more than three hours after the start of the operation significantly reduces its effectiveness. For maximum effect, it should be given just before or after the start of the operation. [5] Preoperative antimicrobial surgical prophylaxis is recommended for operative procedures that have a high rate of postoperative wound infection, when foreign materials must be implanted, or when the wound infection rate is low but the development of a wound infection results in a disastrous events. [2,3,6]

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Infection of the incised skin or soft tissues is a common but potentially avoidable complication of any surgical procedure. Some bacterial contamination of a surgical site is inevitable, either from the patient's own bacterial flora or from the environment. [7] In procedures that require the insertion of implants or prosthetic devices, the term surgical site infection is used to encompass the surgical wound and the implant. Surgical site infection also encompasses infections involving the body cavity (e.g. a. subphrenic abscess), bones, joints, meninges and other tissues involved in the operation. [8] Prophylactic administration of antibiotics inhibits growth of contaminating bacteria and their adherence to prosthetic implants, thus reducing the risk of infection. [9] The goals of prophylactic administration of antibiotics to surgical patients are to: reduce the incidence of surgical site infection, use antibiotics in a manner that is supported by evidence of effectiveness, minimize the effect of antibiotics on the patient's normal bacterial flora, minimize adverse effects and cause minimal change to the patient's host defenses.[2] It is important to emphasize that surgical antibiotic prophylaxis is an adjunct to, not a substitute for, good surgical technique. Antibiotic prophylaxis should be regarded as one component of an effective policy for the control of hospital-acquired infection. [10,11] The American college of surgeons classified wound surgery into 4 categories: clean, clean-contaminated, contaminated and dirty wound, according to this classification trans-oral wound is considered Clean contaminated, That is, Class II, these wounds should receive protection if (a) the patient has depressed host defenses. (b) A prosthetic device is being inserted. (c) The sequel of an infection is serious; and (d) some aspect of the procedure, such as increased duration or decreased local blood supply, makes infection more likely. [8,11] Prophylactic antimicrobial agents should be administered not more than 30 to 60 minutes before surgery.[8-9] Exceptions to this rule are cesarean procedures, colonic and urologic procedures. Therapeutic concentrations of antimicrobial agents in tissue should be present throughout the period that the wound is open. The duration of antimicrobial prophylaxis for the majority of procedures is controversial; however, experts recommend at most one or two postoperative doses. [2,3] The antibiotics chosen for prophylaxis can be those used for active treatment of infection. However, the chosen antibiotics must reflect local, disease-specific information about the common pathogens and their antimicrobial susceptibility.[12] Procaine penicillin is one of the semi-synthetic penicillin obtained by alterations in the prosthetic group differ from the naturally occurring product (penicillin G) in three dimensions: their resistance to acid makes oral administration possible, they may be resistant to the action of penicillinase and their spectrum of antimicrobial activity is usually broadened for many streptococcal infections. [14] It is bactericidal, act by interfering with bacterial cell wall synthesis.[10] Clindamycin is a bacteriostatic act by interfering with protein synthesis of bacteria. It is active against Gram positive cocci, including streptococci and penicillin-resistant staphylococci, and also against many anaerobes, especially *B. f*ragilis[15].

Subjects and Methods

After a thorough history taking, clinical, and radiographic examination, thirty patients attending Al-karamah Dental Center were selected to participate in this study. These patients are mostly from the residents of the neighborhood, which is a relatively a low socioeconomic level. None of patients had medical history or active infectious process. All patients in this study are not allergic to penicillins. These patients were subjected to oral surgical procedures under local anesthesia maximally 2 hours the surgical procedures involved bone and soft tissue and these includes: removal of impacted lower 3rd molar, Apicectomy for upper central and lateral incisors. Patients were classified into two groups according to the antimicrobial agent:

1. 1st group were 15 patients given single I.M. doses of 1 million i.u. procaine penicillin 30 minutes before oral surgery.
2. 2nd group were 15 patients given 600mg clindamycin orally 1 hour before surgery.

Number of female patients included in our study was 17, while the number of male patients was 13. Patients were classified into 3 groups. Group one (10-19) nine patients, group two (20-29) thirteen patients and group 3 (30-39) eight patients. Surgical procedures included in this study were: removal of impacted lower RT 3rd molar (11 cases), removal of impacted lower LT 3rd molar (8 cases), removal of impacted of upper RT 3rd molar (1 case), apicectomy for upper RT central incisor (5 cases) apicectomy for upper LT central incisor (4 cases) apicectomy for upper RT lateral incisor (1 case).Meticulous handling of the tissues, avoidance of unnecessary surgical trauma and copious irrigation of the wound before closure to remove foreign bodies and debris, leaving

no potential foci for bacterial infections were of crucial importance in our measures. Patients were examined 48 hours post-operatively to investigate the presence of any local and general signs of post operative infection these signs are: increased pain or tenderness, post operative swelling at the site of surgery, enlarged, tender regional lymph node and fever. The same investigated parameters were also examined 7th day after surgery, for suture removal.

Results

Characterization of patients according to age, gender and type of oral operation is given in figures 1, 2, and 3. No postoperative infections were recorded in the two groups, and no postoperative complications in the two groups.

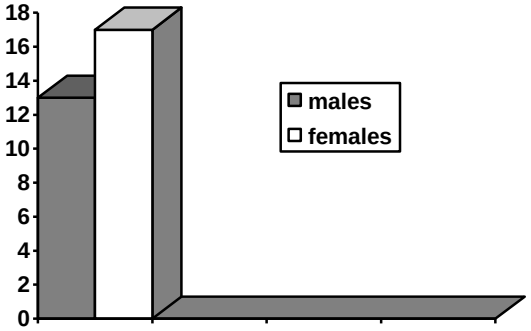


Figure (1) No. of patients according to gender

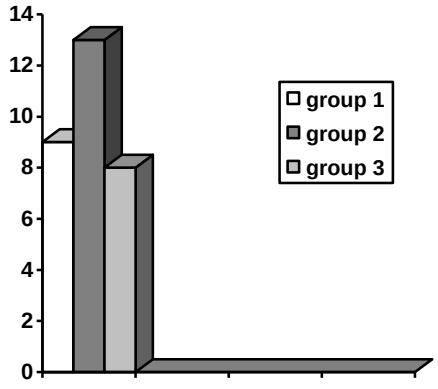
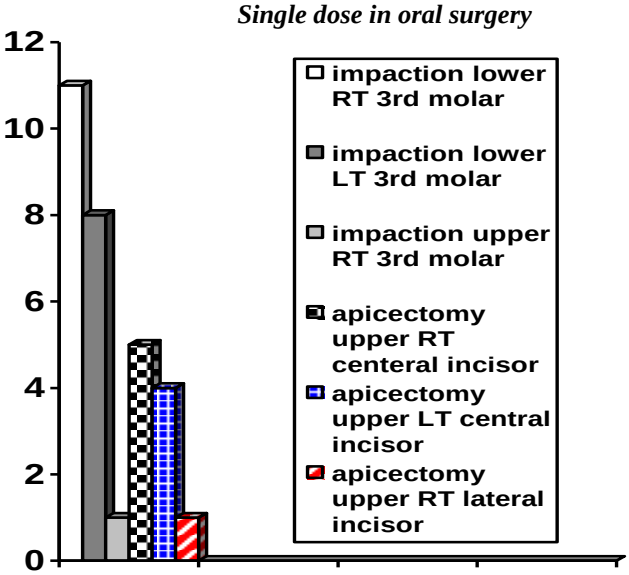


Figure (2) : No. of patients according to age group



Figure(3) No. of patients according to surgical procedures

Discussion

Although some studies found that antibiotic prophylaxis in some oral surgical procedures is controversial [12,16,17].Its generally agreed that when antibiotic prophylaxis is decided, the antibiotic must be present in the systemic circulation at a high level at the time of surgery and is usually given as one dose [17,18,19]. In spite of the fact that preoperative antibiotic prophylaxis is an established practice [17, 20], there is no consistent protocol for the method or duration of drug administration in oral surgical procedures, [21] and this is true for Iraqi dental surgical centers. Although it is agreed that procedures entailing entry into the oropharynx or esophagus, need antibiotic coverage of aerobic cocci is indicated. Prophylaxis has been shown to reduce the incidence of severe wound infection by approximately 50 percent. [22]. Our choice for procaine penicillin depends on two factors

1. most of oral infections caused by penicillin sensitive bacteria [23]
2. The use of penicillin is an established clinical practice in advanced surgical centers [22,23], on the other hand some of the studies select Clindamycin for antimicrobial prophylaxis in oral surgery, clindamycin is occasionally chosen for orthopedic surgical prophylaxis, where it has an excellent activity against *Staphylococcus* spp. and *Bacteroides fragilis*, but have no activity against enteric microorganism.[22,24]. Also it has good reputation for tissue penetration, with almost the same effectiveness of penicillin against anaerobes. [13]

The minimum inhibitory concentration (MIC) of clindamycin is achieved within the first 2-3 dose intervals. Thus, stable drug concentration is then maintained for greater than 6 hours

after the last dose. [13] In our selected sample; female patients were more than the males, this may be explained by the fact that females are more interested in oral hygiene. We have noticed that the number of patients in the age group (20-29) is higher than other age groups; this could be attributed to the fact that the problems of impacted 3rd molar or its complications are usually experienced in this age group. No post operative infections were recorded in our sample, for all patient groups (no difference between parenteral and oral route of administration). We conclude that there is no difference in surgical prophylaxis between procaine penicillin (1 million i.u.), and clindamycin 600mg concerning post operative infection in out patient's oral surgical procedures, and this may be explained by the fact that both antibiotics used in this study covered both pathogens that are mostly involved in oral infections. This conclusion shown in figure (4) which represents surgical removal of impacted lower 3rd molar (group 2)and figure (5) which represents apicectomy for upper central incisor intraoperatively (group 1), figure (6) postoperatively for the same case, while figures 7,8 and 9 represent apicectomy for lower central incisor, preoperative, intraoperative and postoperative respectively (group 2).

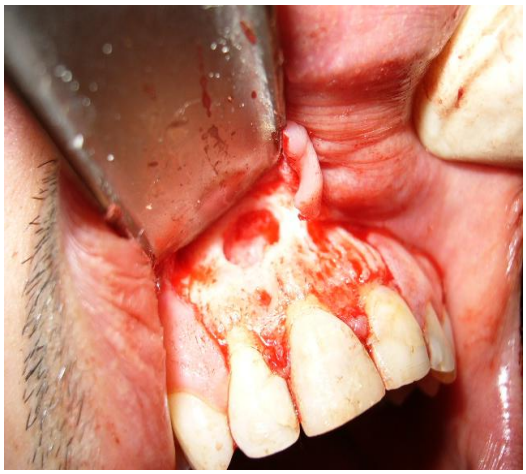


Figure (4): Apicectomy with periapical dental cyst enucleation for upper central incisor (Intra operative picture) The patient has been given clindamycine 600 mg 1 hr. preoperatively (group 2)

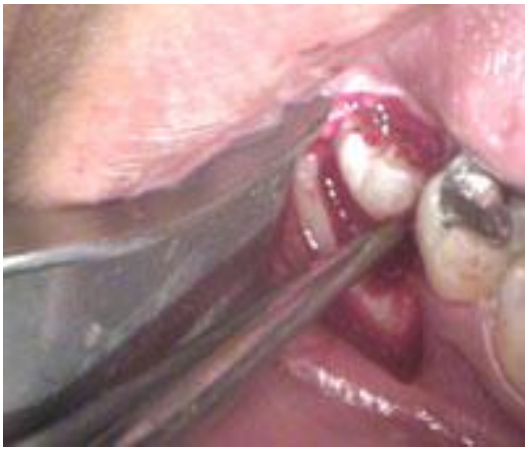


Figure (5): Surgical removal of impacted lower 3rd molar (intra operative picture) The patient has been given 1 million i.u. Procaine penicillin 30 minute pre operatively (group 1)

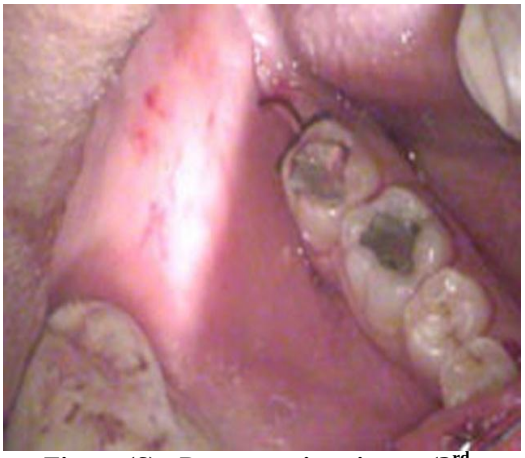
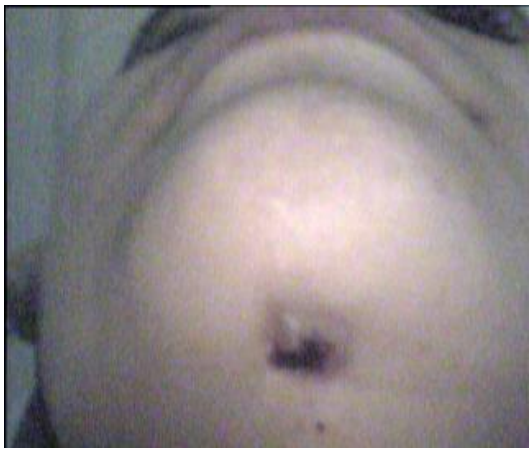


Figure (6) : Postoperative picture (3rd postoperative day)For the site of operation (postoperative oedema subsided, no signs of infection noticed)



Figure(7): 21 years old female with extra oral sinus due to infected cyst associated with necrotic lower central incisor (pre operative picture), (group 2)

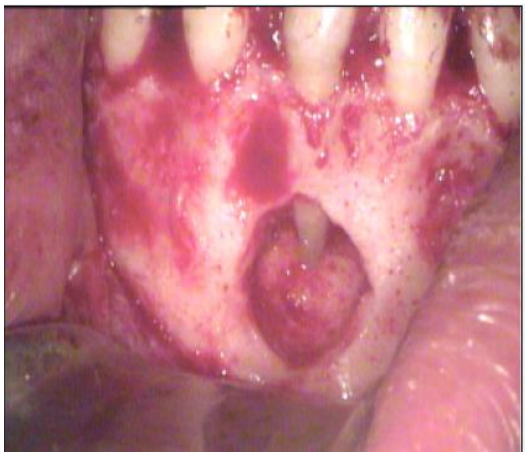


Figure (8): inta operative picture after the removal of the infected cyst. This patient has been given 600 mg Clindamycin 1 hr. pre operatively

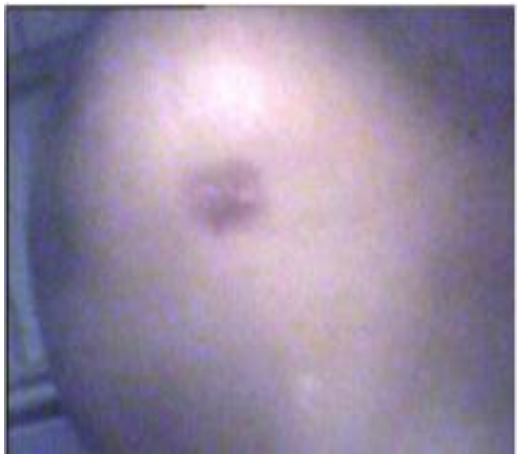


Figure (9): Extra oral picture after one month of the operation shows the process of healing of the extra oral sinus

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Therapeutic Effects of Melatonin in Lead-Induced Toxicity in Rats

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Abstract

Exposure to lead results in significant accumulation in most of vital organs, and free radical damage has been proposed as a cause of lead-induced tissue damage, where oxidative stress is a likely molecular mechanism. This study was designed to evaluate therapeutic effects of melatonin in lead-induced organ toxicity in rats. The therapeutic effects of melatonin on lead induced toxicity in rats were evaluated using 36 rats, which were allocated into 3 groups and treated as follows: Group I, includes 12 rats injected subcutaneously with 0.2 ml physiological saline for 30 days, followed by treatment with a daily dose of 20mg/kg melatonin, administrated I.P for the successive 30 days; groups II and III, each includes 12 rats , injected with lead acetate 100 mg/kg/day s.c for 30 days, followed by treatment with intraperotoneal injection of physiological saline (0.2 ml) or melatonin 20mg/kg/day for the next 30 days. At the end of treatment period, the rats were sacrificed by an overdose (100mg/kg) of thiopental (twenty-four hour after the last injection). Craniotomy and laparotomy were performed to obtain the brains, livers and kidneys for the assessment of tissue damage. The changes in total body weight, weight of major organs (brain, liver and kidney), oxidative stress parameters, hemoglobin content, liver and renal functions, and histological appearance of the studied organs were evaluated and compared with that of negative and positive controls. Treatment with melatonin reverses the damage induced by lead in many organs and tissues through the reduction of MDA levels in RBCs, brain, liver and kidney; increases GSH levels in all studied organs; in addition to the improvement in the indices of the functions of the organs studied. These findings demonstrated that melatonin is capable of reversing damage of rat tissues caused by successive doses of lead acetate, and animals had restored their organ functions due to treatment with melatonin.

***Key words*: Melatonin, Lead poisoning, Oxidative stress**

الخلاصة

ان التعرض للرصاص يمكن ان يتسبب بتركزه في معظم الاعضاء الحيوية كما ان الاضرار الناجمة عن الجذور الحرة قد تكون هي المسببة للاضرار بهذه الاعضاء , حيث ان الاجهاد التأكسدي هو الاكثر احتمالية ليكون الميكانيكية المسؤولة .تم تصميم هذه الدراسة لتقييم الفعالية العلاجية للميلاتونين في الفئران المصابة بالتسمم بالرصاص. ان التأثيرات العلاجية للميلاتونين على الفئران المصابة بتسمم الرصاص ليتم تقييمها باستخدام 36 فأرا , قسمت الى ثلاث مجاميع تلقت المعالجات التالية :المجموعة الاولى تتضمن 12 فأرا .حقنت تحت الجلد ب 2,. مل من المحلول الملحي لمدة ثلاثين يوما , وبعدها بجرعة 20 ملغم/كغم من الميلاتونين اعطيت في البريتون لثلاثين يوما . و المجموعتين الثانية والثالثة والتي ضمت 12 فأرا لكل منهما تم حقنها بخلات الرصاص بجرعة 100 ملغم / كغم / يوم تحت الجلد لثلاثين يوما اتبعت بجرع من المحلول الملحي (2،0 مل) او الميلاتونين 20 ملغم /كغم / يوم عن طريق حقن البريتون للثلاثين يوما التالية .في نهاية فترة المعالجة تم قتل الحيوانات باستخدام جرعة عالية من ثايوبنتال بعد مرور 24 ساعة من اخر معالجة . تم اجراء التشريح لاستحصال الادمغة و الاكباد والكلى لفحص تضررها . ان التغييرات في معدل اوزان اجسام واعضاء الحيوانات (الادمغة و الاكباد والكلى) و مؤشرات الاجهاد التأكسدي ومحتوى الخضاب فحوصات وضائفكل من الكبد والكلى والتغيرات النسيجية في الاعضاء المدروسة تم تقييمها بعد مقارنتها ب كل من مجاميع المقارنة السالبة والموجبة . ان المعالجة بالميلاتونين يمكن ان يعاكس الضرر الناتج بالرصاص في العديد من الاعضاء والانسجة من خلال تقليل اكسدة الدهون (مستوى المالوندالديهايد) في كل من خلايا الدم الحمر والدماغ والكبد والكلى ، اضافة الى زيادة مستوى الكلوتاثايون المدروسة مع تحسن ملحوظ في مؤشرات وظائف الاعضاء المدروسة .ان هذه النتائج توضح ان الميلاتونين له القابلية على عكس الاضرار الناجمة في انسجة الفئران من التعرض المتعاقب لجرع من خلات الرصاص ، وان الحيوانات قد استعادت وظائف الاعضاء فيها نتيجة المعالجة بالميلاتونين .

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Introduction

Lead Poisoning is one of the oldest occupational hazards in the world. Despite its recognized hazards, lead continues to have a wide spread commercial applications, including the production of storage batteries, pipes and metal alloys such as brass, solders, paints, glass and ceramics [(1)]. Once lead enters the body it binds sulfhydryl (SH) moiety of proteins with consequent impairment of their functions; by disrupting protein structure, it interferes with many enzyme systems in the body, thereby affecting the functions of most organs [(2)]. Lead also interferes with regulatory mechanisms that control the metabolism of many essential cations, particularly calcium, iron, zinc, sodium and potassium; it also alters the integrity of the cellular and mitochondrial membranes, thereby, increasing cellular fragility and facilitate degenerative processes [(3)]. Clinical manifestations of lead toxicity include symptoms referable to the central and peripheral nervous systems, hematopoietic, renal and gastrointestinal systems [(4)]. Lead poisoning is a potential factor in brain damage, mental impairment with severe behavioral problems, as well as anemia, kidney insufficiency, neuromuscular weakness and coma [(5)]. At the molecular level, it disturbs heme biosynthesis leading to accumulation of a variety of heme precursors including δ-aminolevulinic acid (ALA) [(6)]. Lead has effects on the hormonal regulation of calcium absorption, and lead toxicity is exacerbated in the presence of low dietary calcium [(7)]. It also displaces calcium in the mineral bone matrix, which may affect bone quality [(7)]. The effects on heme synthesis are the best studied toxic effects of lead; it inhibits the key enzymes, δ-ALAD and ferrochelatase (heme synthetase) [(8)]. As a result heme synthesis is retarded, and because heme moiety is important for the functions of cytochrome systems and cellular respiration, so lead shows an impact on the entire organism; it inhibits Na^+-K^+-ATPase pump attached to erythrocytes membrane leading to their lyses [(9)]. Many compounds with antioxidant properties have been evaluated for their protective effects against lead-induced toxicity in animal and human models [(10)]; moreover, melatonin has been used successfully to protect the nervous system against lead toxicity in rats [(11)]. The present study was designed to evaluate the therapeutic effects of melatonin in rats intoxicated with successive doses of lead.

Materials and Methods

Thirty six male rats (*Rattus norvegicus*) are used in the present study, weighing 200-250 g, housed in the animal house of the College of Pharmacy, University of Baghdad. The animals were maintained at controlled temperature (25 ± 2ºC) from November 2006 to April 2007, allowed free access to water, and fed standard rat chow *add libitum*. The therapeutic effects of melatonin on lead-induced toxicity in rats were evaluated using 36 rats, which were allocated into 3 groups and treated as follows: Group I, includes 12 rats injected subcutaneously with 0.2 ml physiological saline for 30 days, followed by treatment with a daily dose of 20mg/kg melatonin, administrated I.P for the successive 30 days; group II, includes 12 rats, injected with lead acetate 100 mg/kg/day s.c for 30 days, followed by treatment with intraperotoneal injection of physiological saline (0.2 ml) for the next 30 days; group III, includes 12 rats injected with 100mg/kg lead acetate s.c daily for 30 days, followed by treatment with intraperotoneal injection of melatonin 20mg/kg/day for the latter 30 days. At the end of treatment period, the rats were sacrificed by an overdose (100mg/kg) of thiopental (twenty-four hour after the last injection). Craniotomy and laparotomy were performed to obtain the brains, livers and kidneys for the assessment of tissue damage. After animals were sacrificed, blood samples were obtained by heart puncture and immediately placed into two tubes; an EDTA tube to get whole blood for the estimation of lead by atomic absorption in the Poisoning Consultation Center [(PCC), Medical City/ Baghdad], Hb, PCV, MDA and GSH in RBCs. The second fraction was transferred into plane tube to obtain the serum for analysis of other parameters (ALT, AST, ALP, Urea, and creatinine). In the plane tube, blood allowed to clot and serum was separated after centrifugation for (15-20) minutes at 3000 rpm and the resulted serum was kept frozen at (-18ºC) unless immediately analyzed was. Brains, livers, and kidneys were excised from each animal immediately, placed in chilled saline phosphate buffer solution, blotted with filter paper and accurately weighed. A 10% (W/V) tissue homogenate was prepared in phosphate buffer at 4ºC, using metal head tissue homogenizer which was adjusted at set 3 for one minute. All samples were kept frozen at (-18 ºC) unless analyzed immediately. Specimens from the brain, liver and kidneys were prepared for histopathological examination according to the method of Bauer [(12)], using paraffin sections technique.The significance of differences between mean values was calculated using unpaired Student's ***t***-test and analysis of

variance (ANOVA). *P* values less than 0.05 were considered significant for all data presented in the results.

Results

Administration of 100mg/kg lead acetate s.c for one month and treatment with saline for another month resulted in significant reduction in body weight after two months (25%). Therapeutic treatment with 20 mg/kg melatonin I.P for one month after intoxicated of rats with lead acetate resulted also in significant reduction in total body weight (6%), this level seem to be less than that reported when lead acetate was administered with saline (Table 1) . Malondialdehyde (MDA) levels in the RBCs, brain, liver and kidney tissues were significantly elevated after exposure of animals to 100mg/kg lead acetate (479%, 109%, 178% and 101% respectively, $p<0.05$) compared with 20 mg/kg melatonin treated animals. Therapeutic treatment with 20 mg/kg melatonin resulted in significant decrease in MDA levels in studied tissues (55%, 33%, 54% and 23% respectively, $p<0.05$) compared with animals challenged with 100 mg/kg lead acetate and saline only (Table 2).

Table 1. Effects of therapeutic use of 20 mg/kg melatonin on the total body weight and the weights of brain, liver and kidney in rats previously intoxicated with 100 mg/kg lead acetate.

Treatment groups	Weight (g)		Organ /body weight		
	Pre-treatment	Post-treatment	Brain/body	Liver/body	Kidney/body
Saline +Melatonin (20mg/kg) (n=12)	353.3 ± 1.88	385.8 ± 7.0 ^{a*}	0.004 ± 0.0002 ^{a}	0.027 ± 0.0005^{a}	0.003 ± 0.0001 ^{a}
Lead acetate (100mg/kg) + Saline (n=7)	349.8 ± 1.84^{a}	263.5 ± 9.55 ^{b*}	0.0057 ± 0.0002^{b}	0.05 ± 0.001^{b}	0.005 ± 0.0002^{b}
Lead acetate (100mg/kg) + Melatonin(20mgk) (n=10)	351.0 ± 3.14^{a}	330.0 ± 7.15 ^{c*}	0.004 ± 0.0001^{c}	0.033 ± 0.001^{c}	0.003 ± 0.0002^{c}

Data are expressed as mean ± SEM; n= number of animals;*Significantly different compared to pre-treatement value($P>0.05$) values with non-identical superscripts (a, b, c) within the same variable considered significantly different ($P<0.05$).

Table 2. Effects of therapeutic use of 10 or 20 mg/kg melatonin on the malondialdehyde (MDA) in erythrocytes, brain, liver and kidney in rats previously intoxicated with 100 mg/kg lead acetate.

Treatment groups	Malondialdehyde (MDA)			
	RBC (nmol/g Hb)	Brain (nmol/g tissue)	Liver (nmol/g tissue)	Kidney (nmol/g tissue)
Saline +Melatonin (20mg/kg) (n=12)	5.4 ± 0.12^{a}	48.9 ± 1.62^{a}	52.7 ± 1.31^{a}	24.4 ± 1.21^{a}
Lead acetate (100mg/kg) + Saline (n=7)	31.2 ± 2.48^{b}	101.9 ± 4.71^{b}	144.8 ± 5.56^{b}	49.1 ± 2.17^{b}
Lead acetate (100mg/kg) + Melatonin (20mgkg) (n=10)	13.9 ± 0.83^{c}	68.2 ± 1.89^{c}	66.5 ± 2.13^{c}	37.8 ± 1.87^{c}

Data are expressed as mean ± SEM; n= number of animals; values with non-identical superscripts (a, b, c) within the same variable considered significantly different ($P<0.05$).

Table 3. Effects of therapeutic use of 20 mg/kg melatonin on the glutathione (GSH) levels in erythrocytes, brain, liver and kidney in rats previously intoxicated with 100 mg/kg lead acetate.

Treatment groups	Glutathione (GSH)			
	RBC (µmol/g Hb)	Brain (µmol/g tissue)	Liver (µmol/g tissue)	Kidney (µmol/g tissue)
Saline +Melatonin (20mg/kg) (n=12)	13.9 ± 0.13[a]	11.8 ± 0.12[a]	8.9 ± 0.13[a]	7.8 ± 0.23[a]
Lead acetate (100mg/kg) + Saline (n=7)	3.2 ± 0.19[b]	4.4 ± 0.18[b]	3.3 ± 0.12[b]	4.1 ± 0.12[b]
Lead acetate (100mg/kg) + Melatonin (20mgkg) (n=10)	6.1 ± 0.14[c]	5.9 ± 0.11[c]	7.0 ± 0.09[c]	5.8 ± 0.10[c]

Data are expressed as mean ± SEM; n= number of animals; values with non-identical superscripts (a, b, c) within the same variable considered significantly different (P<0.05).

Daily treatment of rats with 100mg/kg lead acetate significantly reduces GSH levels in RBCs, brain, liver and kidney (77%, 63%, 64%, and 48% respectively, $p<0.05$) compared with 20 mg/kg melatonin treated animals. Meanwhile therapeutic treatment with 20 mg/kg melatonin, administered one month after lead acetate results in significant elevation of GSH in the studied tissues (88%, 34%, 115% and 41% respectively, $p<0.05$) compared with lead acetate and saline treated animals (table 3). Administration of 100 mg/kg lead acetate to the rats result in significant decrease in Hb levels and PCV %(12% and 9% respectively, $p<0.05$), when compared with melatonin 20 mg/kg treated group (table 4). Exposure of animals to s.c injections of lead acetate (100 mg/kg) for one month and saline for another month produces significant elevation in the serum levels of hepatic enzymes activity (AST, ALT, ALP)(162%, 232%, and 102% respectively, $p<0.05$) compared with 20 mg/kg melatonin treated animals. Therapeutic administration of melatonin in a dose of 20 mg/kg (39%, 53% and 42%) significantly reduces enzymes activities both with respect to lead acetate and saline treated animal group and between each other (table 5). However, therapeutic treatment of animals with melatonin, one month after lead acetate challenge, significantly reduces serum levels of urea and creatinine in which the reduction were (28% and 25% respectively, $p<0.05$), the reduction in serum level of their parameters was significantly different when compared with lead acetate and saline treated animals and between each others(table 6). Lead acetate, when administered subcutaneously, in a consecutive 100 mg/kg doses for one month and saline for another month produces significant elevation in blood lead levels (513%), and lead levels in brain, liver and kidney of these animals were also significantly elevated (3810%, 4736% and 2849% respectively, $p<0.05$) compared with 20 mg/kg only melatonin treated animals. Melatonin reduces lead levels significanty in all studied compartments (blood 28%, brain 46%, liver 40% and kidney 42%) compared with lead acetate and saline treated animals (table 7).

Table 4. Effects of therapeutic use of 20 mg/kg melatonin on the hematological parameters of rats previously intoxicated with 100 mg/kg lead acetate for one month.

Treatment Groups	Hb (mg/dl)	PCV %
Normal saline + Melatonin (20 mg/kg) (n=12)	14.6 ± 0.20[a]	44.3 ± 0.81[a]
Lead acetate (100 mg/kg) + Saline (n=7)	12.2 ± 0.29 [b]	37.3 ± 0.87[b]
Lead acetate (100 mg/kg) + Melatonin (20 mg/kg) (n=10)	13.6 ± 0.15 [c]	40.7 ± 0.72[c]

Data are expressed as mean ± SEM; n= number of animals; values with non-identical superscripts (a, b, c) within the same variable considered significantly different ($P<0.05$).

Table 5. Effects of therapeutic use of 10 or 20 mg/kg melatonin on the liver enzymes (AST, ALT, and ALP) of rats previously intoxicated with 100 mg/kg lead acetate for one month.

Treatment groups	Liver enzymes level (U/L)		
	AST	ALT	ALP
Normal saline + Melatonin (20mg/kg) (n=12)	55.0 ± 1.53 [a]	36.0 ± 1.30 [a]	95.6 ± 2.27 [a]
Lead acetate (100mg/kg) + Saline (n=7)	144.2 ± 3.87 [b]	119.8 ± 3.23 [b]	192.9 ± 3.44 [b]
Lead acetate (100mg/kg) + Melatonin (20mgkg) (n=10)	87.7 ± 2.71 [c]	56.8 ± 2.14 [c]	112.5 ± 3.33 [c]

Data are expressed as mean ± SEM; n= number of animals; values with non-identical superscripts (a, b, c) within the same variable considered significantly different (P<0.05).

Table 6. Effects of therapeutic use with 10 or 20 mg/kg melatonin on serum urea and creatinine of rats previously intoxicated with 100 mg/kg lead acetate for one month.

Treatment groups	Serum urea (mmol/L)	Serum creatinine (µmol/L)
Normal saline + Melatonin (20mg/kg) (n=12)	5.4 ± 0.13 [a]	72.8 ± 2.61 [a]
Lead acetate (100mg/kg) + Saline (n=7)	11.9 ± 0.62 [b]	190.7 ± 11.39 [b]
Lead acetate (100mg/kg) + Melatonin (20mgkg) (n=10)	8.5 ± 0.24 [c]	142.2 ± 4.73 [c]

Data are expressed as mean ± SEM; n= number of animals; values with non-identical superscripts (a, b, c) within the same variable considered significantly different (P<0.05).

Table 7. Effects of therapeutic use of 10 or 20 mg/kg melatonin on lead levels in blood, brain, liver and kidney of rats previously intoxicated with 100 mg/kg lead acetate for one month.

Treatment groups	Lead level			
	Blood (µg/dl)	Brain (µg/gm)	Liver (µg/gm)	Kidney (µg/gm)
Saline + Melatonin (20mg/kg) (n=12)	12.98 ± 0.29 [a]	0.9 ± 0.05 [a]	2.18 ± 0.1 [a]	8.23 ± 0.26 [a]
Lead acetate (100mg/kg) + Saline (n=7)	79.54 ± 3.51 [b]	35.19 ± 1.33 [b]	105.43 ± 2.98 [b]	242.69 ± 2.28 [b]
Lead acetate (100mg/kg) + Melatonin (20mgkg) (n=10)	57.48 ± 2.15 [c]	18.92 ± 0.83 [c]	63.38 ± 1.88 [c]	141.57 ± 2.1 [c]

Data are expressed as mean ± SEM; n= number of animals; values with non-identical superscripts (a, b, c) within the same variable considered significantly different (P<0.05).

Sections prepared from livers of rats, previously intoxicated with lead acetate 100 mg/kg, treated with saline for one month, showed a wide area of normal appearance with presence of small area of degeneration and necrosis with inflammatory cells infiltration (figure 1). Meanwhile, treatment of rats with 20 mg/kg melatonin previously intoxicated with 100 mg/kg lead acetate for one month, the liver sections showed normal structure appearance with few discrete degenerative changes (figure 2).

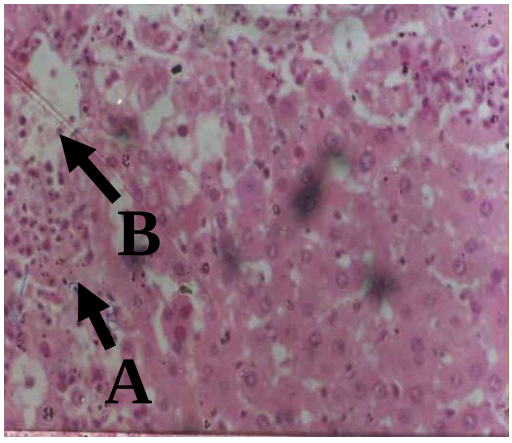

Figure (1). Section of liver tissue showing a wide area of normal appearance with presence of small area of degeneration and necrosis (arrow A) with inflammatory cells infiltration (arrow B) in rats treated with saline previously intoxicated with 100mg/kg lead acetate for one month. Magnification: 200X (hematoxylin and eosin stain).

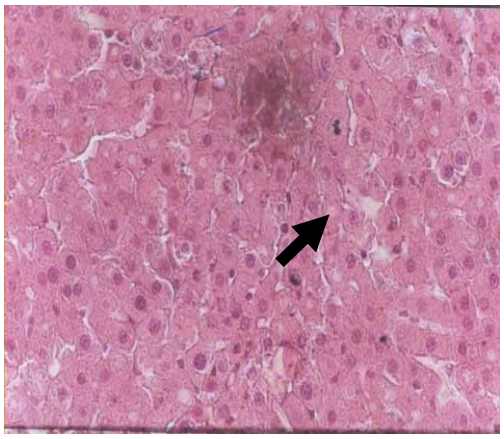

Figure (2). Section of liver tissue showing normal histology with appearance of few discrete degenerative changes (arrow) in rats treated with 20 mg/kg melatonin previously intoxicated with 100mg/kg lead acetate for one month. Magnification: 200X (hematoxylin and eosin stain).

Sections prepared from kidneys of rats treated with saline and previously intoxicated with 100 mg/kg lead acetate for one month, showed mild degenerative changes and necrosis in the kidney tubules (figure 3). Meanwhile, administration of 20 mg/kg melatonin to group of rats previously intoxicated with lead acetate 100 mg/kg, the kidney sections showed normal histology but still there is slight dilatation of the renal tubules (figure 4).

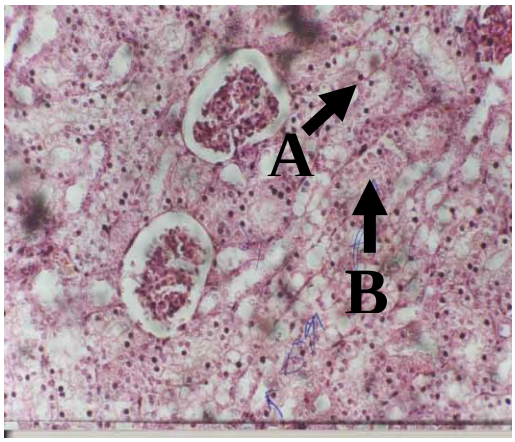

Figure (3). Section of kidney tissue showing mild degenerative changes (arrow A) and necrosis (arrow B) in the kidney tubules in rats treated with saline previously intoxicated with 100mg/kg lead acetate for one month. Magnification: 200X (hematoxylin and eosin stain).

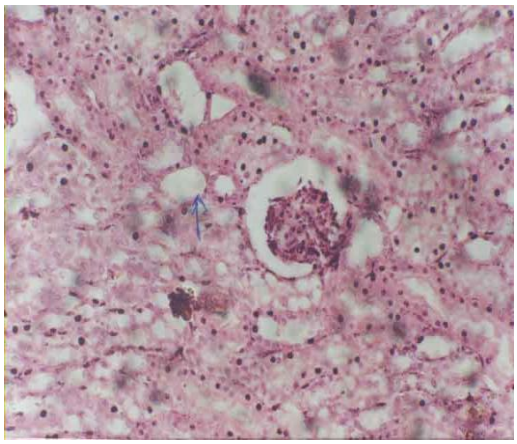

Figure (4). Section of kidney tissue showing normal histology but still there is slight dilatation of renal tubules (arrow) in rats treated with 20 mg/kg melatonin previously intoxicated with 100mg/kg lead acetate for one month. Magnification: 200X (hematoxylin and eosin stain).

Discussion

Daily administration of 100 mg/kg lead acetate to rats, reduce their total body weights compared with control animals with subsequent elevation of organ/body weight ratios, and treatment with melatonin restores body weights and the impaired organ/total body weight ratio. Lead poisoning is very well known to affect numerous organ systems, and is associated with a number of morphological, biochemical and physiological changes that include kidney dysfunction, impaired glucose metabolism, CNS disturbances, impairment of liver function and hematological disorders [(13)]. Among their effects, the impaired glucose metabolism is considered as a major pathway that may be followed by changes in total body or organ weights; in this respect intoxication with lead reduces the rate of glucose metabolism, with consequent reduction of the required energy for many anabolic process, and the profound decrease in serum glucose level which is reported in rabbits intoxicated with lead, might also be a cause for tissue wasting due to inappropriate availability of energy. The findings of the present study are found compatible with those reported by others [(14)], where loss of total body weight is found parallel with the increase in blood lead levels; furthermore, the increase in oxidative stress exhibited contributing factor, where lipid peroxidation might predispose to perturbation in the content of lipids in many organs and tissues. Exposure to lead acetate significantly elevates MDA levels in erythrocytes, brain, liver and kidney; while therapeutic use of melatonin results in significant reduction in the MDA levels in all compartments compared with control groups; their results are found compatible with those reported previously [(15)]. In this respect also, lead depletes the natural antioxidant molecule, the glutathione in the erythrocytes, brain, liver and kidney, and the use of melatonin therapeutically improves the levels of this antioxidant thiol in their compartments; their results are in agreement with those reported by others [(16)]. Lead-induced enhancement of lipid peroxidation is a major mechanism for some of the toxic effects of lead in different organ and tissues have certainly been suggested earlier [(17)]. Lead crosses the blood brain barrier and causes immediate effects by altering the metabolism and physiology of the brain and other organs like liver and kidney. One likely molecular mechanism involved in lead toxicity is the disruption of the pro-oxidant/ antioxidant balance [(18)] which leads to tissue injury via oxidative damage to critical biomolecules such as lipids, proteins, and DNA. After absorption of lead into the blood, 99% of lead is bound to erythrocytes and the remaining 1% stay in plasma to be carried to other tissues. Decreased hematocrit and hemoglobin levels might arise from reduction in serum copper as well as reduced iron metabolism and consumption induced by lead [(19)]. Development of anemia in lead toxicity may be attributed to the decreased red blood cell survival because of the increased membrane fragility, reduced RBC count, decreased hemoglobin production, or summation of all these factors [(20)]. The activities of serum enzymes AST, ALT, and ALP showed significant elevation in rats exposed to lead, administration of melatonin reduces these activities but remain significantly elevated when compared with control groups, these findings are compatible with other previous studies [(21)]. Increasing the activities of AST, ALT and ALP in serum was most likely a consequence of the hepatotoxic effect of lead, the accumulated lead in the liver directly damaging the hepatocytes, primarily by destroying the permeability of the cell membrane, which results in the increased release of cytosolic enzymes AST, ALT and ALP into the circulation. The results of the present study demonstrated significant increase in both urea and creatinine levels in the serum of rats exposed to 100mg/kg lead acetate daily for one month, indicating renal damage. Lead poisoning causes renal dysfunction and such type of toxicity might be due its ability to cause oxidative damage to the renal tissue, which includes enhanced lipid peroxidation, DNA damage and the oxidation of protein sulfhydryl groups[(22)].Lead is a pervasive environmental pollutant known to induce a broad range of physiological, biochemical and behavioral dysfunction in human and laboratory animals. Based on the present results, it seems that lead levels in blood and tissues became significantly elevated when compared with control and melatonin treated groups, and in agreement with other previous studies [(23)]. The results of the present study enables the conclusion that melatonin, attenuates and reverses the tissue damage induced in experimental animals by lead acetate, and the therapeutic use of this pleiotropic hormone support the idea of the oxidative stress-induced damage due to lead toxicity.

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The *In Situ* Expression of IL-6 and IL-1β in breast cancer patients

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Abstract:

Breast cancer is the second most common cancer in women world. Multiple Cytokines appear to have a dominant role in human breast cancer formation. Estimation of the *in situ* expression of IL-6 and IL-1β in breast cancer patients. A sixty patients with breast cancer BC were divided into two clinical subgroups, (30) with malignant breast cancer MBC and (30) with benign breast tumor as a control group according to histological examination. In situ hybridization technique used for detection of IL-6 and IL-1β mRNA sequence in two groups. The results showed that percentages of mRNA expression of IL-6 and IL-1β were in (≥ 11-50%) for malignant breast cancer. This research also investigated that (73.3%) of benign breast tumor were expression less than (<10%) for IL-6 and IL-1 β mRNA. The ISH expression of the mean percentages of IL-6 and IL-1 β were higher levels in malignant breast cancer patients (48.13 and 56.07 ,respectively) than benign tumor (2.73 and 1.40 ,respectively), with highly significantly differences (P<0.01) of ISH expression for IL-6 and IL-1 β mRNA among two studied groups., the expression of IL-6 and IL-1 β mRNA are significantly elevated in the tissue of breast cancer patients compared with benign tumor and was found a significant correlation between the expression of IL- 6 and IL-1β mRNA in the tissue of breast cancer patients, thus the results of the present study might be explain the pathological role of these two cytokine in breast cancer.

Key words: IL-6 mRNA, IL-1β mRNA, Breast cancer, ISH.

الخلاصة

يعتبر سرطان الثدي ثاني الامراض السرطانية انتشارا عند النساء في العالم . وتلعب العديد من السايتوكينات دورا اساسيا في تكوين سرطان الثدي . الهدف من البحث هو التحري عن التعبير في الموضع لكل من انترلوكين - 6 و 1 بيتا لدى مرضى سرطان الثدي . شملت الدراسة (60) مريضة مصابة بسرطان الثدي تم تقسيمهم الى مجموعتين اعتمادا على الفحص النسيجي ، المجموعة الاولى (30) مريضة مصابة بسرطان الثدي الخبيث ، والمجموعة الثانية (30) مريضة مصابة بورم حميد . تم استخدام تقنية التعبير في الموضع للتحري عن الحامض النووي الرايبوسومي المراسل لكل من انترلوكين -6 و 1 بيتا في المقاطع النسيجية لكلتا المجموعتين . اظهرت الدراسة ان نسبة التعبير للانترلوكين-6 و1 بيتا هي (≤ 11-50%) عند مجموعة مريضات سرطان الثدي الخبيث بينما كانت نسبة التعبير هي (< 10%) لـــــ 73.3 % من مريضات الورم الحميد لكلا من انترلوكين -6 وانترلوكين -1 بيتا . وبينت تقنية التعبير في الموضع معدل نسب مئوية عالية لأنترلوكين -6 و1 بيتا لمجموعة الورم الخبيث (48.13 و 56.07)على التوالي مقارنة بمجموعة الورم الحميد والتي شكلت النسب (2.73 و 1.40) على التوالي وهذه الاختلافات ادت الى ظهور فروقات معنوية عالية عند مستوى معنوية(< 0.01) .كذلك بينت الدراسة وجود علاقة موجبة معنوية بين التعبير لأنترلوكين - 6 وانترلوكين -1 بيتا في المقاطع النسيجية لمريضات سرطان الثدي .وهكذا فأن نتائج الدراسة الحالية قد توضح الدورالامراضي لهذين النوعين من السايتوكينات في تكون سرطان الثدي وتطوره .

Introduction

Breast cancer is the most frequently diagnosed cancer and the second leading cause of death after lung cancer in women [(1)]. There is strong evidence that the tumor growth can be actively controlled by host immune system.[(2)] . Cytokines are known to have both stimulatory and inhibitory effects on breast cancer growth depending on their relative concentrations and the presence of other modulating factors in the tumor microenvironment. Certain cytokines appear to prevent an effective immune response being mounted; permitting cancer growth, whereas others promote the immune system's anti-tumor capability [(3)] .Interleukin-6 (IL-6) is a cytokine with multiple biologic activities on a variety of cells. It is produced by macrophages, T cells, B cells, endothelial cells and tumor cells. IL-6 is able to promote tumor growth by upregulating antiapoptotic and angiogenic proteins in tumor cells.

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It is associated with worse survival in patients with metastatic breast cancer and is correlated with the extent of disease [4]. In human breast cancer, an important role of IL-1β and IL-1RA mRNA expression was noted in various studies [5], Interleukin-1β is a highly inflammatory and prototypical multifunctional cytokine that affects nearly all cell types, often in concert with other cytokines or small mediator molecules. IL-1β elicits important proinflammatory and immunological responses, such as fever, hypotension, increasing circulating NO, recruiting neutrophils,and costimulating T cell activation by increasing IL-2R expression and inducing IL-2 production [6]. The basis of the various biologic properties of IL-1β depends on its regulatory effects on the expression of various genes and/or receptors. IL-1β induces the gene expression of the IL-1 family, other inflammatory cytokines, colony stimulating factors, and mesenchymal growth factors [7].This study aimed at estimation of the in situ expression of IL-6 and IL-1β in malignant breast cancer patients comparing to the benign breast cancer and find out the correlation between these two marker in malignant and benign patients.

Materials and Methods

Sixty Iraqi patients with breast cancer who were admitted to AL -Yarmook and Baghdad Teaching Hospital. Patients with breast cancer (BC) were divided into two clinical subgroups according to histological examination: (30) with malignant breast cancer and (30) with benign breast tumor as a control group. Fresh samples were obtained during routine examination of surgically removed tissue, each specimen was fixed in 10% formalin then processed paraffin wax embedded section and cut into 5µm thickness, put on Fisherbrand positively charged slides for our research. ***In situ hybridization***: For in situ hybridization technique (ISH), DNA Probe Hybridization/Detection System in situ kit (Maxim Biotech, Inc., USA) was used.The probes were biotin-labeled DNA probes for human IL-6 (360 bp), and human IL-1β (556 bp), (Maxim Biotech, Inc., USA). In situ hybridization (ISH) is a technique used the high specificity of complementary nucleic acid binding to detect specific DNA or RNA sequence in the cell [8] For detection of this markers, the biotinylated DNA probe hybridize the target sequence (IL-6 and IL-1β mRNA sequence) then a streptavidin-AP (streptavidin - alkaline phosphatase) conjugate is applied followed by addition of the substrate promo-chloro-indolyl-phosphate /nitro- blutetrazolium (BCIP/NBT)which yields an intense blue-black signal appears at the specific site of the hybridized probe [9] . This directly streptavidin-AP conjugate like the biotinylated probe provides a rapid and highly sensitive detection method.Evaluation of ISH signal was done with the assistance of a histopathologist .The expression of both IL-6 and IL-1β mRNA was measured by the same scoring system, counting of the number of the positive cells in the tissue that has given a blue-black (BCIP/NBT) nuclear staining under the light microscope. The score was the average from 10 distinct high-power fields observed under ×100 magnification. The percentage of positively stained cell was calculated for each case by taking the mean of the percentages of the positively stained cell in the 10 fields. A score of 0 was given when no staining was detected, 1 if there was weak to moderate staining in less than 10% of cells, 2 if moderate to strong staining was present in 11 to 50% of cells, and 3 if strong staining in more than 50% of cells was detected [10].

Statistical Analysis

The suitable statistical methods were used in order to analyze and assess the results. Descriptive statistics results presented as percentages of frequencies ,mean, SD, SEM, minimum & maximum levels .Inferential statistics used to accept or reject the statistical hypotheses, includes: Chi-square (χ^2),T-test.Pearson Correlation (r). P - value < 0.05 and P < 0.01 were considered statistically significant. [11].

Results

The expression of IL-6 and IL-1β were detected by ISH technique. Tables **1** and **2** show the percentage of frequency scoring for IL- 6 and IL-1β mRNA expression among study groups, respectively. Chi-square test was conducted to examine the association between IL-6 and IL-1β mRNA expression in the tissue in the two groups of investigated women ,it was found that highly significant association ($p<0.01$) between them among the four scoring levels. The results showed that percentages of mRNA expression of IL-6 and IL-1β were in (≥ 11-50%) for malignant breast cancer. This research also investigated that (73.3%) of benign breast tumor were expression less than (<10%) for IL-6 and IL-1 β mRNA. On the other hand, the mean percentages of these two cytokines was significantly higher($p<0.001$) in malignant breast cancer compared with benign tumor as demonstrated in (Table 3) . The expression of IL-6 and IL-1β was heterogeneous blue-black nuclear staining in the tissue, as shown in Figure (1). In addition, this study demonstrated highly significant positive correlation ($P<0.01$)

between IL-6 and IL-1β in two studied groups, as shown in (Table 4) and Figure (2).

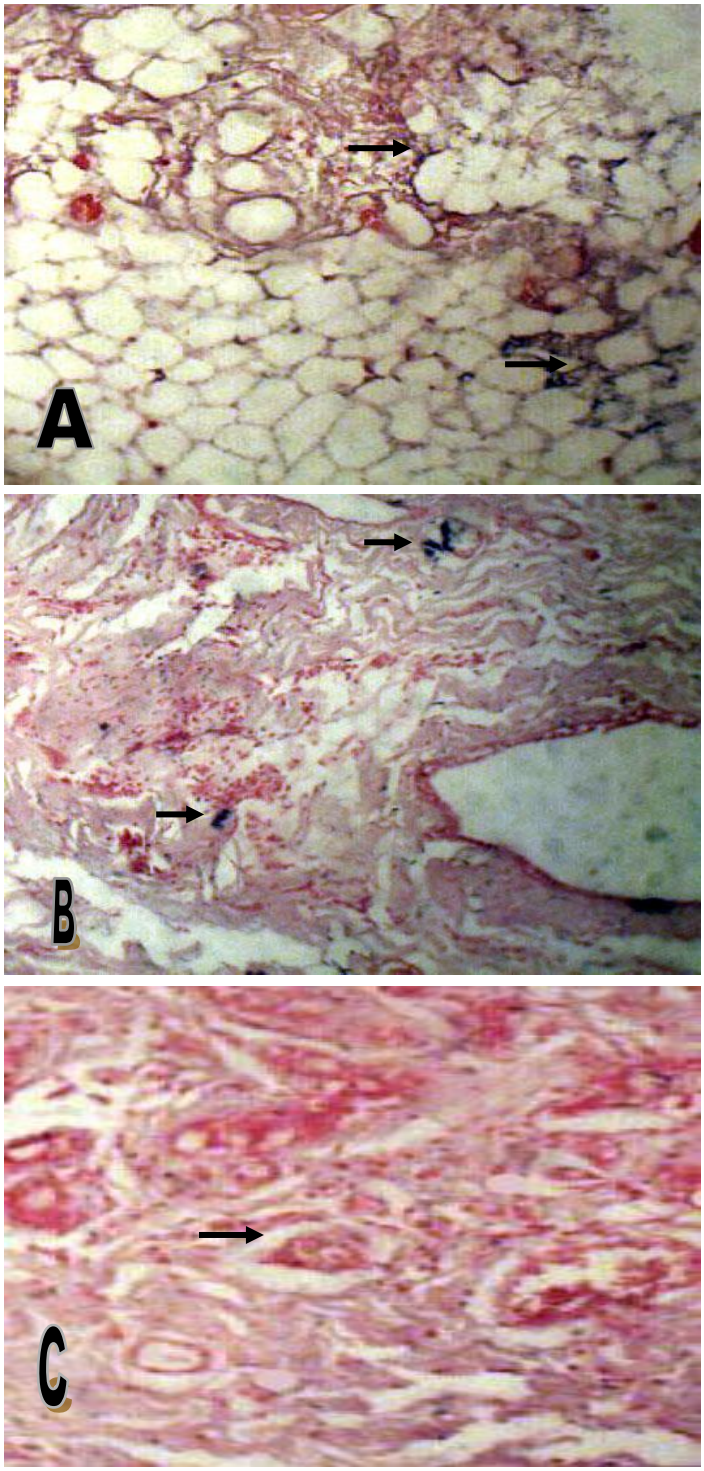

Figure (1): **Detection of IL-6and IL-1β in studied groups by in situ hybridization (ISH)**. Staining of IL-6 and IL-1β mRNA by BCIP/NBT (blue-black)counterstained with nuclear fast red. **(A)** Tissue from breast cancer patients shows positive IL-6 hybridization signals (X400)**. (B)** Tissue from breast cancer patients shows positive IL-1 β hybridization signals (X400)**. (C)** Negative control tissue.

Table (1): Distribution of ISH%IL-6 among studied groups (Malignant breast cancer & Benign breast tumor patients).

ISH%IL-6 groups		Studied groups		Total	Comparison of significant	
		Malignant BC*	Benign BT**		P-value	Sig.
0%	N	0	8	8	0.00	Highly Sig. (P<0.01)
	%	0	26.7	13.3		
< 10 %	N	0	22	22		
	%	0	73.3	36.7		
11-50 %	N	16	0	16		
	%	53.3	0	26.7		
>50%	N	14	0	14		
	%	46.7	0	23.3		
Total	N	30	30	60		
	%	100	100	100		

* = breast cancer ** = breast tumor

Table (2): Distribution of ISH%IL-1β among studied groups (Malignant breast cancer & Benign breast tumor patients).

ISH%IL-1β groups		Studied groups		Total	Comparison of significant	
		Malignant BC*	Benign BT**		P-value	Sig.
0%	N	0	8	8	0.00	Highly Sig. (P<0.01)
	%	0	26.7	13.3		
< 10 %	N	0	22	22		
	%	0	73.3	36.7		
11-50 %	N	11	0	11		
	%	36.7	0	18.3		
>50%	N	19	0	19		
	%	63.3	0	31.7		
Total	N	30	30	60		
	%	100	100	100		

* = breast cancer ** = breast tumor

Table (3): Mean of ISH%IL-6 & IL-1β levels among studied groups (Malignant breast cancer & Benign breast tumor patients)

Studied groups / Interleukins	Malignant BC* N=30	Benign BT** N=30	(T-test)	
			P-value	Sig.
ISH%IL-6				
Mean	48.13 21.03	2.73	0.00	Highly Sig. (P<0.01)
SD	3.84	2.49		
SEM	15 ─ 85	0.45		
Mini.─ Maxi.		0 ─ 8		
ISH%IL-1β				
Mean	56.07	1.40	0.00	Highly Sig. (P<0.01)
SD	17.87	1.13		
SEM	3.26	0.21		
Mini.─ Maxi.	20 ─ 86	0 ─ 3		

* = breast cancer ** = breast tumor

Table (4): Correlation between ISH%IL-6 level & ISH%IL-1β level among total breast cancer patients, Benign BC patients & Malignant BT patients.

Pearson Correlation	Total		Malignant B C*		Benign BT**	
	ISH%IL-6 level	ISH%IL-1β level	ISH%IL-6 level	ISH%IL-1β level	ISH%IL-6 level	ISH%IL-1β level
r	0.893		0.576		0.516	
P-value	0.00		0.001		0.004	
Sig.	Highly Sig. (P<0.01)		Highly Sig. (P<0.01)		Highly Sig. (P<0.01)	

* = breast cancer ** = breast tumor

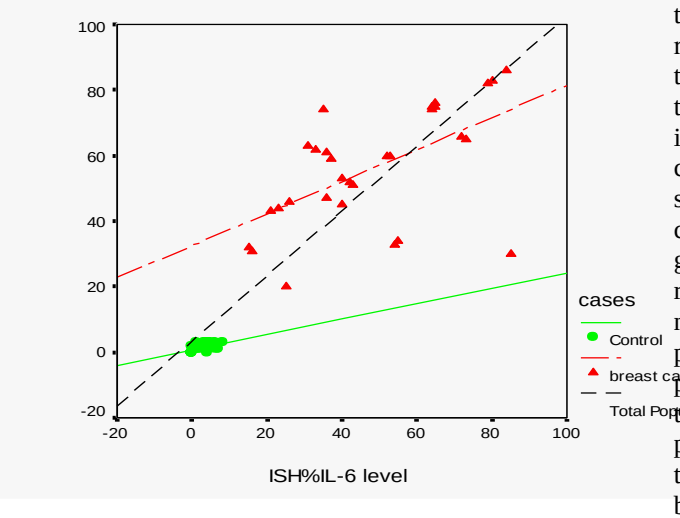

Figure (2): Correlation between ISH%IL-6 level and ISH%IL-1β level among total breast cancer patients, Benign BT patients& Malignant BC patients.

Discussion

Cytokines in general are thought to be involved in numerous physiologic and pathologic conditions. Among cytokines, IL-1β and IL-6 probably seem to play the most important role in breast carcinogenesis [12; 13] .In the present study, IL-6 and IL-1β mRNA expression was examined by in situ hybridization technique in tissue of malignant breast cancer compared with benign tumor . The IL-6 and IL-1β were expressed in a higher percentage in breast cancer tissue compared to benign tumor and we found the positive expression of IL-6 mRNA and IL-1β mRNA among malignant breast cancer were 46.7% and 63.3% were more than fifty percent (>50%),respectively . This results suggests that IL-6 and IL-1β are over expressed in breast carcinoma compared to benign tumor and might play a pathological role in malignant breast cancer. Evidence supporting this suggestion includes the fact that in human breast cancer, the elevated expression of IL-6 and IL-1β were observed in breast carcinoma tissues [5; 14; 15; 16] and in serum [17; 18] .Several studies suggest that the IL-1 system is vital in the local control of tumor growth, important in regulating ''protumorigenic'' activities within the tumor microenvironment, and contributes to angiogenesis, tumor proliferation, and tumor invasion [19; 20; 21]. Furthermore, IL-1ß and IL-6 cause tumor regression and increase median survival time in a variety of cancer patients. In contrast, elevated circulating concentrations of growth factors such as IGF-I are a surrogate risk for cancers of the breast [22; 23; 24]. It is noteworthy that IL-1 β is a prototypical proinflammatory cytokine that exerts a plethora of biological activities, including tumor regression [25]. The tumor-suppressing property of IL-1 β has been attributed mostly to its ability to prime antitumor immunity [26], but the mechanism for its direct cytostatic actions in suppressing cell cycle progression is largely unknown. The antiproliferative action of IL-1 β on human breast cancer cells is exhibited not by killing the cells but rather by preventing the ability of the late G1 progression factor, insulin-like growth factor (IGF)-I, to promote progression from late G1 into the S phase of the cell cycle [27] .This cross-talk between proinflammatory cytokine and growth factor receptors is similar in principle to that between the B cell receptor and the 2-adrenergic receptor for the neurotransmitter norepinephrine [28] and that between the IGF-I receptor and integrin-associated protein for thrombospondin-1 [29] .Moreover, the role of the IL-1 system in human breast cancer is conflicting. IL-1 has been shown to inhibit growth of breast cancer cells and to promote cellular differentiation in vitro, but it is equally known to stimulate the expression of several proteolytic enzymes in human cancer [30;31] .The consecutive degradation of extracellular matrix is a key element of local invasion and metastasis [32, 33] .In addition, they are many confounding studies about the role of IL-6 and IL-1β in tumor cell growth, but its exact role remains varied and unclear [19; 34]. It appears that the effect of IL-6 on tumor cell growth may depend on the tumor cell type, IL-6 plays a

new role in cancer biology; it promotes multidrug resistance [34] and it has been shown to be involved in intercellular signaling between mesenchyme and breast cancer epithelium.. These display an oncogenic role for IL-6; however, lacking is an understanding of the mechanisms governing IL-6 production in tumors and the biological role of this cytokine in tumorigenesis [19, 35]. The human IL-6 shows antiadhesive effects, and modulates the estrogen receptor and progesterone receptor content of these cells [35] .The elevated expression of IL-6 has been detected in multiple epithelial tumors [36] .An interesting finding , in the current study that in situ expression of IL-6 was significantly correlated ($p< 0.01$) with in situ expression of IL-1β ($r= 0.576$; $p< 0.01$) in malignant breast cancer . This results indicating that IL-6 and IL-1 β are strongly interact with each other and act synergistically, subsequently increasing their effect. This finding in agreement with that of Robison and colleagues who reported that a significant correlation between IL-6 and IL-1 immunoreactivity [13] , thus both ILs, i.e., IL-1β and IL-6 have been shown to be strongly interact and to act additively in breast carcinogenesis, subsequently inhibiting tumor growth [37] . In conclusion, in this study both IL-6 and IL-1 β mRNA showed the significant increased expression in the tissue of malignant breast cancer patients compared with benign tumor , this might be implicated in the development of malignant breast cancer. Although a significant positive correlation between these two markers in all studied group, this might reflect a close relation between these two parameters.

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Cytotoxic Assay of *Nigella sativa* Leaf Callus Extract (Thymol) on Hep-2 Cell Line Using ELISA Assay

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Abstract

Extract from cell culture of medicinal plant like *Nigella sativa* have been assessed for its cytotoxic properties. Thymol is likely responsible for the theraputic effects of *Nigella sativa* leaf callus extract. In this short study the inhibitory effect of *Nigella sativa* leaf callus extract (Thymol) has been studied on Human Lorgnx Epidrmoid Carcinoma (Hep-2) cell line during different exposure period of time (24, 48 and 72 hrs.) using different concentration of the extract (1000, 500, 400, 300, 200 and 100 µg/ml). The optical density of the Hep-2 cells has been readed on 492 nm wave length. Thymol – induced cytotoxicity was (500 µg/ml) which inhibit cell growing compared to the control and this ratio increased at the 48 hrs of exopsure and stopped at 72 hrs.

Key wards: Nigella sativa, callus extract, cell line, ELISA assay.

الخلاصة

تم عمل مستخلص من النسيج الزرعي للنبات الطبي الحبة السوداء لمعرفة خصائصه السمية للخلايا. الثايمول يعتبر هو المسؤول عن التأثيرات العلاجية لنبات الحبة السوداء من مستخلص أوراق الكالس. تم في هذه الدراسة القصيرة دراسة تأثير مستخلص أوراق كالس الحبة السوداء (الثايمول) على نمو الخلايا السرطانية Hep-2 ضمن أوقات تعريض مختلفة (24, 48 و 72 ساعة) باستخدام تراكيز مختلفة من المستخلص (1000, 500, 400, 300, 200 و100 µg/ml). تم قياس الO.D للخلايا على الطول الموجي 492 nm. وجد ان الثايمول يكون ساما للخلايا السرطانية عند التركيز 500µg/ml والذي ثبط نمو الخلايا بالمقارنة مع معاملة السيطرة وزادت هذه النسبة في المدة 48 ساعة من التعريض وتوقف عند اليوم الثالث من التعريض.

Introduction

The main inspiration of black seed comes from the famous saying (Hadith) of our Prophet Mohammed; (God peace be upon him), that "Habbat Al-soda is remedy for all disease except death." [(1)].It is an annual herbaceous plant believed to be endogenous to the Mediteranean region but has been cultivated in other parts of the world including India and Pakistan [(2)]. Black seed oil contains about 0.5-1.5 % volatile oil including nigellone and thymoquinone used as anti-histanimic, antioxidant, antiinfective and bronchodilating effects[(3)].Thymol, is one of the active compounds in *N. sativa* extract, plays important role in the inhibition of cancer cells, and can attach with the mutagenic substance, because thymol is one of the antioxidant phenolic compounds [(4)].Plant tissue culture techniques inters in several applications like plant micropropagation, genetic study, plant improvement, study of plant cell physiology, the production of secondary metabolites in addition to production of viruse free plant diseases (bacterial and fungal) [(5)]. So, to increase the production of this compound (thymol) all the year round without depending on the mother plant, plant tissue culture techniques formed callus and then increased the production of thymol. The anticancer activity of *N. sativa* was first revealed by [(6)] who observed enhancement of natural killer (NK) cell activity ranging from 200-300% in advanced cancer patients receiving multimodality immunotherapy programme in which *N. sativa* was one of these components. Thymoquinone and dithymoquinone, active principles of *N. sativa*, had cytotoxic effect against parental and multi-drug resistant human tumour cell lines which were over 10-fold more resistant to doxorubicin and etoposide [(7)] . Radiation protection activity of *N. sativa* in mice against induction of chromosomal aberrations by gamma ray was also reported[(8)]

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The using of plant tissue culture techniques make the easy of pharmaceutical compounds production instead of depending on the mother plant and become possible to produce these compounds at high amount and at high rate of pure may be over than these isolated from the complete mother plant, and its production may be quickly and independent on the season, also limit the surface area that is used in the medicinal plant culturing [9].The objective of the present study was to assess the cytotoxic properties of this extract from cell culure of *N. sativa* leaf callus using against Hep-2 cell line using ELISA (enzyme linked immunosorbant assay) assay.

Material and Methods:

Collection of plant material:

Seeds of *Nigella sativa* gotten from Dr. Aws Al-Ani (Directorate of Agriculture Research and Food Technology/ Ministry of Science and Technology/ Baghdad/ Iraq) to be used.

Callus culture condition:

The establishment and maintenance of callus were carried out using the procedures described before[10].

Extraction prepration:

For preliminary screening, the seeds were cultured and callus induced and material from callus culture were lyophilzed and extracted by a method described elsewhere [11]. In short, one g of callus was mixed with 30 ml of NaOH solution 5% and then diethyl ether was then added in a ratio of (2:1) (v /v) and mixed well as described elsewhere [12]. The extract was then filtered and concentrated in vacum at 45º C and then kept in the dark at 4º C until tested.

Cytotoxicity test using ELISA assay:

For this test, the extract were weighed (0.05 g) and dissolved in phosphate buffer saline (PBS) and dimethylsulphoxide (DMSO) to prepare extract solution at 1000 µg/ml. The following dilutions of extract were then prepared: 500, 400, 300, 200 and 100 µg/ml.Hep-2 cell line, obtained from Iraqi Center For Cancer and Medical Genetics Researches at the passage level 326 were used in this study. The origin and description of this cell line was mentioned by [13]. It was a human laryngeal carcinoma excised from 57 years old man, then transplanted in immune suppressed rat by cortisone. After growth of the tumour in the rat, it was then excised and transplanted as an *in vitro* tissue culture. It was kept at -169˚C (in liquid nitrogen). In preparation to any *in vitro* assay, the frozen cell line was withdrawn and maintained in RPMI-$_{1640}$ containing 10% bovine calf serum. When the *in vitro* cells culture forms a monolayer. These cells were treated with trypsin/ versine mixture in order to pursue subculture process.The percentage of inhibition was calculated according to the following equation: [14]

Inhibition % = [(OC – OT) /OC] x 100

Where :
OC: optical density of control wells
OT : optical density of test wells

From the above calculation, a graph was plotted for the precentage of growth inhibition against each extract concentration. Activity against Hep-2 cell line was determined by the inhibition assay using an ELISA assay. In short, cell cultures in the microtitration plate were exposed to a range of plant extract concentrations during the log phase of growth and the effect determined after recovery time. The following protocol as descriped in [15] was performed the extracts of *Nigella sativa* leaf callus :

a) After trypsinization, cell suspension seed in a micro titration plates at 50000 cells/ml RPMI-$_{1640}$ growth medium with serum 5% was used for seeding.
b) Plates then incubated for 24 hours.
c) By using maintenance medium, two-fold serial dilution were prepared starting from 1000 µg/ml ending with 100 µg/ml.
d) After incubation for 24 hrs, cells exposed to different extract dilutions. Only 200 µl of each concentration added for each well (6-replicates for each tested concentration). 200 µl of maintenance medium added to each well of control group.The times of exposure were (24, 48 and 72 hrs). The plates sealed with self adhesive film then returned to the incubator at 37º C.˚
e) After the end of the exposure period, the medium and the cells decanted off and replaced by 200 µl of 0.01% crystal violet dye. After 20 min. the stain was washed gently with tap water for three times. The plate was left until become dry.
The optical density of each well was read by using a micro-ELISA reader at 492 nm transmitting wave length [15 , 16].

Statisitical analyses:

A one-way analysis of variance was performed to test whether group variance was sifgnificant or not, the comparison between groups were used analyses of variance Least Significant Differences Test (L.S.D.)[16].

Results

Cytotoxic effect of Nigella sativa leaf callus extract (thymol) on Hep-2 cell line:

The result of the cytotoxic effect of the extrcat readed using ELISA micro reader with wave length 492 nm indicate the presence of a relation ship between color density of the stain and the number of the viable cells. The result showed presence of significant at level ($p \leq 0.05$) differences between the concentrations compared with control started with the concentration (1000, 500, 400, 300, 200 and 100 µg/ml) with inhibition value ranged (67.2%, 75.4%, 74%, 75%, 72.2% and 74%) respectively at 48 hr of exposure periods, while there is no significant differences at level ($p \leq 0.05$) between the concentration itself as shown in figure (1).

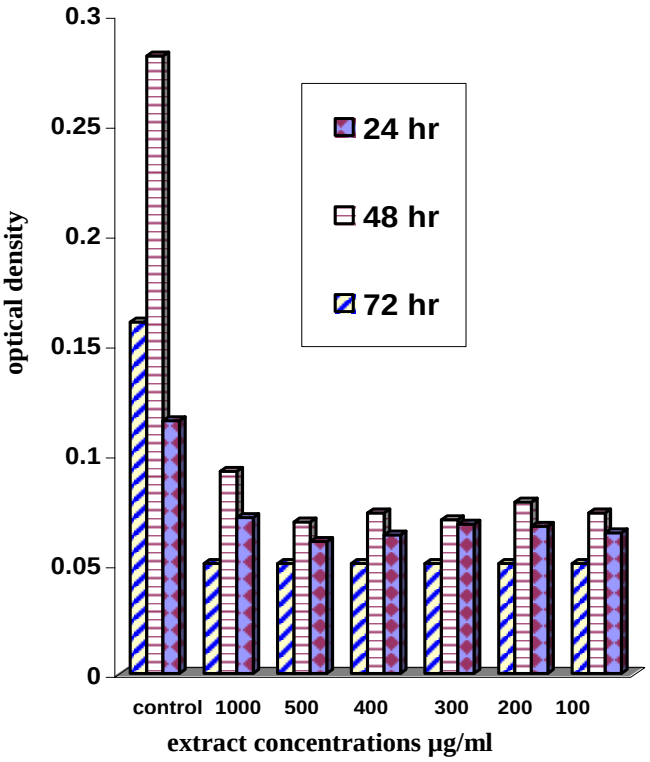

Figure (1): Effect of extract concentrations on the growth of Hep-2 cell line during different exposure preiod.

The results also showed the best exposure period was 48 hr than the other periods (24 and 72 hr), the inhbition to cell line begins at 48 hr, there is no significant differences at level ($p \geq 0.05$) in inhibition when compared with the period 72 hr. This means exposure the extract to cell line at 48 hr with lowest concentration showed significant differences. After that result we choose the inhibitory concentration which inhibit the growth of Hep-2 cell line depending on the changes that appears on the optical density and the changes in the color that appears on the plate itself from the stain reaction with the cells[17]. So, the concentration 500 µg/ml has inhibitory effect compared to other concentration that also showed a minimum inhibition on the growth of Hep-2 cell line at 48 hr exposure period as shown in table (1), and the inhibition ratio was shown in table (2).

Table 1: A comparison optical density of growth inhibition of Hep-2 cell line, by using different concentration of callus extracts of *Nigella sativa* during three periods of exposure.

Type of extract	Conc. (µg/ml)	Optical density (OD)		
		24hrs	48hrs	72hrs
Callus extract (thymol)	1000	0.071	0.092*	0.05
	500	0.060	0.069*	0.05
	400	0.063	0.073*	0.05
	300	0.068	0.070*	0.05
	200	0.067	0.078*	0.05
	100	0.064	0.073*	0.05
	control	0.115	0.281	0.16

(*):means the presence of siginificant differences at level ($p \leq 0.05$) between the concentrations.

Table 2: A comparison of growth inhibition percentage of Hep-2 cell line, by using different concentration of callus extracts of *Nigella sativa* during three periods of exposure.

Type of extract	Conc. (µg/ml)	Inhibition%		
		24hrs	48hrs	72hrs
Callus extract (thymol)	1000	38.2	67.2	68.7
	500	47.8	75.4	68.7
	400	45.2	74.0	68.7
	300	40.8	75.0	68.7
	200	41.7	72.2	68.7
	100	44.3	74.0	68.7

Discussion

Although the quinone thymol has demonstrated significant *in vitro* and *in vivo* antineoplastic activities against different tumor cell lines[7,18]. In this study , thymol demonstrated different cytotoxicity *in vitro* toward Hep-2 cell line according to its concentration. This study appeared that the concentration 500 µg/ml affact on the inhibition ratio when compared with the lowest concentrations which show a minimum inhibition compared with the control. This

inhibition increased when reacrhed 48 hr of exopsure and stopped at the 72 hr of exposure. The using of these concentrations (1000, 500, 400, 300, 200 and 100 µg/ml) in this study affect on the growth of Hep-2 cell line with slight differences between them , the OD of Hep-2 cell line ratio was the lowest on the concentration 500 µg/ml compared with other concentrations. So, the extract make an inhibition on the growth of Hep-2 cell line compared the control depending on the concentration that is used and the length of incubation period. The result of this study suggest that thymol inhibited proliferation of tumor cell line by a mechanism that involves cytotoxicity, in fact, it is known that thymoquinone (a quinone from *Nigella sativa*) inhibited the proliferation of COS 31 (canine osteosarcoma) at concentration 100µM by inducing apoptosis and cell cycle arrest at G1. Non-cancerous cells are relatively resistance to thymoquinone [(19)] . *Nigella sativa* and other plants were tested on human hepatoma Hep G2 cell line, the effect were determined on 24 hr of incubation. The greatest inhibitory effects were observed on *Nigella sativa* plant extract even at low concentration [(20)].

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Preparation and *In Vitro* Permeation of Chlopheniramine Maleate (CPM) from Gel through Rat Skin

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Abstract

Chlopheniramine maleate (CPM) , is one of the H- receptor antagonist , widely used in allergic diseases ,like skin rash and pruritis .CPM 3%w/w was successfully loaded in 2%w/w sodium alginate (SA) as a gel base , and to be considered as a selected formula .It was found that the diffusion of CPM through the skin of albino rat was increased as the concentration of CPM increased from 2 %w/w sodium alginate , More over , the addition of Triethanolamine 5 % w/w, to sodium alginate 2 % w/w , loaded by CPM 3 % w/w , enhanced the amount of CPM diffuse through the skin of albino rat . Mean while the addition of PEG 1000 2% w/w , and urea 5 % w/w, separately to sodium alginate 2 % w/w , loaded by CPM 3 % w/w , hindered significantly $P<0.05$ the amount of the drug diffused through the skin of the rat .The selected formula of sodium alginate 2% w/w as a base loaded by CPM 3% w/w was physically acceptable , with shelf life approximately 3.3 years .

Key wards: chlopheniramine maleate , gel , skin permeation

الخلاصة

الكلورفنيرامين ماليبت هو احد المثبطات لمستقبلات هه 1 والمستعملة بشكل واسع في الطفح الجلدي والحكة الخارجية .لقد نجحت المحاولات بتركيب الكلورفنيرامين ماليبت 3%(وزن \وزن) في 2% (وزن\وزن) من الجينات الصوديوم كقاعدة هلام , واعتبرت كتركيبة مختارة . لقد وجد ان تخلل الكلورفنيرامين ماليبت من خلال جلد الجرذ الابرش يزداد بزيادة تركيز العقار في 2%(وزن\وزن) من الجينات الصوديوم ,علاوة على ذلك فان اضافة الترايبثانولامين 5% (وزن\وزن) الى2% (وزن\وزن) من قاعدة الجينات الصوديوم محملة ب 3%(وزن\وزن) من الكلورفنيرامين ماليبت , ادى الى زيادة كمية العقار النافذة خلال جلد الجرذ الابرش. وعلى صعيد اخر, فأن أضافة 2%(وزن\وزن) من مادة بولى أثلين كلايكول(1000) و 5%(وزن\وزن) من اليوريا بشكل منفصل الى 2% (وزن\وزن) من الجينات الصوديوم ,محملة ب 3% (وزن\وزن) من الكلورفنيرامين ماليبت, ادى الى الاعاقة بشكل مهم $P<0.05$ كمية العقار النافذة خلال جلد الجرذ للابرش . أن التركيبة المختارة كانت تحتوي على 2% (وزن\وزن) من الجينات الصوديوم كقاعدة هلام محملة ب 3% (وزن\وزن) من الكلورفنيرامين ماليبت , اضافة الى صفات فيزياوية جيدة وفترة نفاذية للعقار بحدود3,3 سنة .

Introduction

Gels are semi solids consisting of dispersions made up of either small inorganic particles or large organic molecules enclosing and interpenetrated by a liquid [(1)] .The delivery of the drug into and through the skin is recognized an effective means of therapy for local dermatological and systemic disease ,In recent years, the development of transdermal permeation has been attracting an attention due to several advantages , Such as better control of blood levels , reducing systemic toxicity and avoid firs pass metabolism [(2)] . Sodium alginate , a naturally occurring poly saccharide has been widely used as a disintegrant and gelling agent in pharmaceutical preparations [(3)] . It has several unique properties that have been enabled it to be used as a gel matrix for delivery of many drug [(4)] . Chlorpheniramine maleate as a potent H1-receptor antagonist can be indicate for many types of allergy such as rhinitis and pruritis , it can prevent but does not reverse histamine mediated response [(5)] . This study aimed to both suggest new alternative dosage form for enhancing topical penetration of CPM , and to evaluate the potential and transdermal absorption .

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Experimental

Materials and Equipments :

Chlorpheniramin maleate CPM , supplied by Sammraa drug industry SDI , Sodium alginate , triethanolamine TEA , from (Hopkins and William LTD , England) , Sodium carboxymethylcellulose NaCMC , diethyl ether , glycerin, from (BDH chemical limited , Pool , England) , Formaldehyde 37% (v/v) , urea , from (Fluka AG , Switzerland) , Polyethylene glycol PEG1000 , methyl and propyl hydroxyl benzoate , from (Merk-Shuchardt , Germany), UV- spectrophotometer ,carrywin UV ,Varian , Australia USP dissolution apparatus ,magnetic stirrer ,ultra sonic cleaner , VWR cpley , England , Water bath shaker ,hot air oven , memmert , Germany

Preparation of sodium carboxymethylcellulose (NaCMC) 5%w/w gel base :

Simply , the method employed for base was fusion method . it was carried out by incorporation

CPM equivalent to 1%w/w in the following base content :

NaCMC powder	5gm.
Glycerol	15gm.
Methylhydroxybenzoate	0.1gm.
Purified water to	100gm.

The base was prepared by mixing NaCMC with glycerol in a glass mortar , while methylhyd-roxybenzoate (methyl paraben) was dissolved in 40ml. of distilled water using heat to about 70˚C with vigorous stirring by stirrer for 15 minutes , and cooled, then the later mixture was mixed with polymer-glycerol mixture and stirring until clear gel-base was gained . then the CPM was incorporated to the base with 5 minutes continous traturation and stirring to obtain homogenous clear drug-gel solution [6].

Preparation of sodium alginate (SA) 2% w/w gel base:

Sodium Alginate	2gm.
Glycerol	15gm.
CaCl2	0.2gm.
Propylhydroxybenzoate	0.2gm.
Distilled water to	100gm.

The same principle of procedure was done as in preparation of Na-CMC gel base. The polymer of sodium alginate was mixed with glycirin in a glass mortar and the mixture was poured in small amounts to the vehicle with stirring , while calcium chloride was dissolve in small amount of water and added to the vehicle with stirring then complete the volume with distilled water with 5 minutes continuous stirring , until translucent – white clear gel was formed (6).Different concentration of polymers corresponding to 1% , 2% and 4% (w/w) of Sodium alginate and only 4% (w/w) of NaCMC were used with physical mixture through studying their effect on the release process.

In vitro release of CPM from gel base:

A small glass container with 3cm. in diameter of its opening mouth was modified in order to be filled with one gram of each formula , which was containing equivalent weight of 1%w/w of CPM .The mouth of container was covered with the filter paper which secured in place with rubber band . the dialysis cell was inverted in 500ml. of phosphate buffer pH 7.4 contained in a beaker of the dissolution apparatus . The system maintained at 37˚C, the samples were withdrawn after 1 ,2 ,3 , 4 and 5 hours , and replaced with an equal volume of fresh buffer solution [7] . the sample were analyzed for their CPM content using uv-spectrophotometer at λmax 261 nm.

Preparation of the diffusion membrane :

The albino rat (4-6 week old male) , was scarified by ether inhalation , then the skin was shaved lightly with an electrical clipper , taking care to prevent any damage to the skin , a rectangular section of abdominal skin several centimeters in each dimension was excised using a sharp blades . The defating procedure [9], of the skin was carried out by weeping the skin with a cotton tip soaked in diethyl ether to remove the subcutaneous fat and scraping the dermal side to remove the muscles and blood vessels , the adhering fat was again removed by another cotton tip soaked in diethyl ether , and kept in phosphate buffer pH 7.4 for 2 hours in a water bath maintained at 37˚C, to allow water soluble uv absorbing materials to leach out. The buffer was changed three times during this period with fresh amounts [10] .then the prepared skin for diffusion was stored in phosphate buffer for 24 hours in the refrigerator at 2˚C before use.

In vitro diffusion of CPM through rat skin membrane[8] :

One gram of each formula containing CPM was introduce in a small container and the epidermal surface of the rat skin was stretched over the mouth of the container with diameter 3cm. and legated with rubber band, the diffusion cell then inverted and

immersed in 500ml of phosphate buffer at pH 7.4 contained in a beaker of dissolution apparatus . The system was maintained at 37˚C and the buffer solution was stirred at 100 r.p.m. during 5 hours of the study .Samples of 5 mls.were pippeted from the collection medium after 1 ,2 ,3 ,4 , and 5 hours replaced with an equal volume of freshly prepared phosphate buffer pH 7.4 at 37˚C . The samples were analyzed using uv-spectrophotometer at λ max 261 nm.

Effect of different enhancers and their concentrations on the diffusion :

In order to evaluate best release profile of CPM from selected formula , different enhancers, urea 5%w/w, polyethylene glycol (PEG1000) 2% w/w, and triethanolamine (TEA) 1% , 2.5 ,and 5% w/w were used on diffusion of CPM through rat skin

Skin irritation test [11]:

Skin male albino rats weighing approximately 500gm. were used to study the irritation test of the selected formula , on the rat skin . The dorsal side of the rat was carefully shaved and two circular areas of 2.5 cm. in diameter in each animal were done .then 0.8%v/v aqueous solution of formalin as standard irritant to one circular area , and 5% w/w TEA gel formula contain 3% w/w CPM to the other circular area for three rats, and 2% w/w sodium alginate gel containing 3% w/w CPM to other circular areas of other three rats .The fresh gel samples and formalin solution were applied for 7 days , Finally the application sites were graded to the visual scoring scale always by the same investigator .

Stability study :

The estimation of the shelf life of a selected formula 3% w/w CPM kept in a collapsible tubes at room temperature and oven maintained separately at 40,50 , and 60˚C , samples were taken every seven days for 4 weeks . each sample of the gel equivalent to 250mg. CPM in 50 ml. of phosphate buffer at pH 7.4 . These samples were quantitatively transferred to volumetric flasks and appropriate dilutions were made with the same buffer , then the resulting solution were filtered with 0.45µ filter paper , The absorbance of each collected sample was calculated for CPM content at λ max 261 nm [12] .

Effect of the temperature on the pH of the gel :

The pH of the gel was measured every week for one month , by taking one gram of the gel from each stored sample at 40 , 50 , and 60˚C, and shaken up with 10mls. of distilled water . the pH of the final solution was measured and recorded .

Statistical analysis

The significance between mean values was analyzed by student t- test , P-value of less than 0.05 was considered significant for all analyzed data shown in the results of this study .

Results and Discussions

Effect of gel bases on the release of CPM :

Table 1. and figure 1 , show the amount of drug release from gel bases , the results indicated that the drug released is significantly increased $P< 0.05$ as a function of polymer type used in an order of 4%w/w SA > 4% NaCMC , this result may be referred to the hygroscopic effect of cellulose derivatives that affect water entrapment in the cross linking gel of 4%w/w NaCMC more than that of 4% w/w SA .since this amount of water may hinder another water molecules diffuse inside gel structure and then more drug releasing occurred . This result is in consistent with results obtained by Cetin T. et . al [13] .

Table (1) . Effect of different bases on the release rate constant (K) of CPM 1%w/w in phosphate buffer pH7.4 at 37˚C .

Type of Bases	Amount of CPM released (mg.)/5hr.*	CPM released %	K ($mg.min^{-1/2}$)
4%w/w SA	9.06 ±0.09	90.6	0.427
4%w/w NaCMC	6.77 ±0.11	67.7	0.401

Each value represents the mean SD
(n=3 readings in each group *)

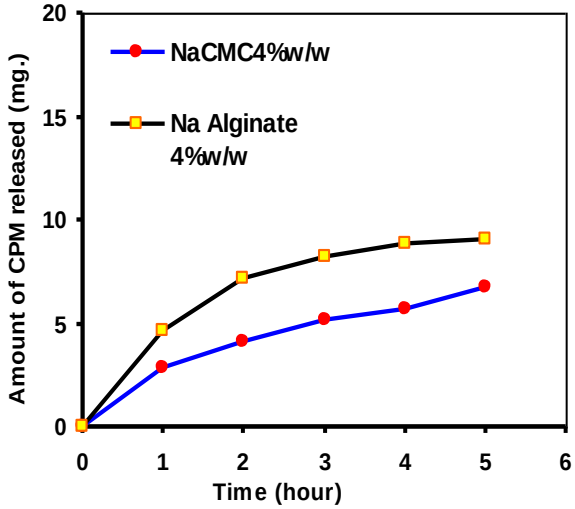

Figure (1) . The effect of different bases on the release of CPM 1%w/w at pH 7.4 and 37˚C

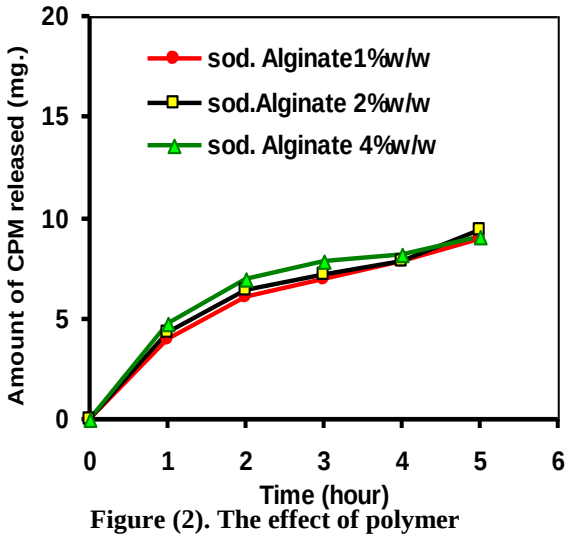

Figure (2). The effect of polymer concentration on the release of CPM 1% w/w through rat skin at pH 7.4 and 37˚C

Effect of polymer concentrations on the release of 1%w/w CPM gel :

Table 2. and figures 2 and 3 , demonstrate the effect of SA concentrations on the release profiles of the CPM through rat skin , it was seen that the drug released from SA at different concentration and diffused through the filter paper was not affected by the concentration of the polymer , since no significant increase in the drug release , this behavior gives an impression that the drug release from SA followed zero- order kinetics in these concentrations , since water up take by polymer is not affected by the concentrations of the polymer it self , Meanwhile the plot of the amount of the drug released versus square root of time demonstrates that there is a linear relationship of the drug release followed Higuchi principle in the diffusion process from semisolids in percutanous absorption . these results were in agreement with the results obtained from the permeation of carvedilol transdermal patches [11] .

Table (2) . Effect of Sodium Alginate (SA) concentrations on the rate constant (K) of CPM 1%w/w phosphate buffer pH 7.4 at 37˚C .

Sodium alginate Concentratin	Amount of CPM (mg./5hr.)*	CPM released %	Rate constant (K) ($mg.min^{-1/2}$)
1%w/w	8.934±0.17	89.3	0.4983
2%w/w	9.435 ±0.14	94.3	0.5713
4%w/w	9.060 ±0.04	90.6	0.4273

Each value represents the mean SD (n=3 readings in each group *)

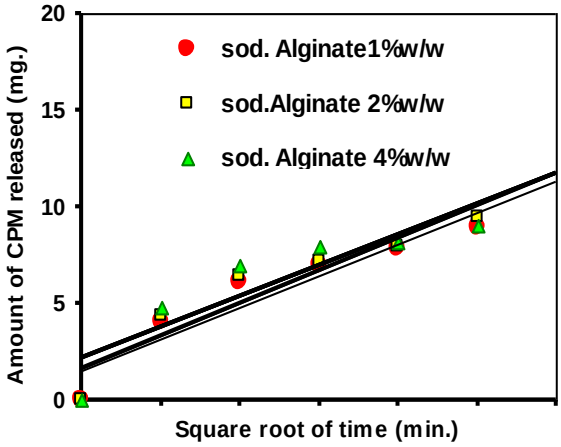

Figure (3) . The kinetic analysis of CPM 1% w/w release from different polymer concentration at pH 7.14 and 37˚C

Effect of CPM concentrations on the diffusion process :

Table 3. and figure 4 , illustrate the effect of different CPM concentration 1% w/w and 3% w/w on the amount of CPM diffused through the rat skin , using 2% w/w SA as a gel base , the results showed that the amount of CPM diffused during the period of application (5hours) , increased as a function of increasing drug concentration . This behavior confirmed that the drug diffusion followed first- order mechanism , and the penetration rate is proportional to the concentration , since this diffusion depend on many factors , among them , the partition coefficient (K) and the concentration of the drug [14] . In this experiment the rate limiting step of the drug

diffusion through the rat skin can't be estimated , because there are two types of partitioning , one of the partition of the drug for the skin (Ds) , and the other for vehicle (Dv) ,or gel base . so these two magnitudes of the two diffusion coefficient Ds and Dv , determines whether the release from vehicle or skin is the rate limiting step, and by this approach, the concentration of incorporated drug in the gel base may solve this problem , regardless the diffusion or partition coefficient [11 ,14].

Table 3. Effect of CPM concentration on the diffusion rate constant (K) using 2%w/w S0dium Alginate (SA) gel .

CPM concentration %	CPM amount diffused mg./5hr. *	Rate constant (mg.min $^{-1}$½)
1%w/w	7.54±0.09	0.582
3%w/w	11.765±0.21	0.8217

Each value represents the mean SD (n=3 readings in each group) *

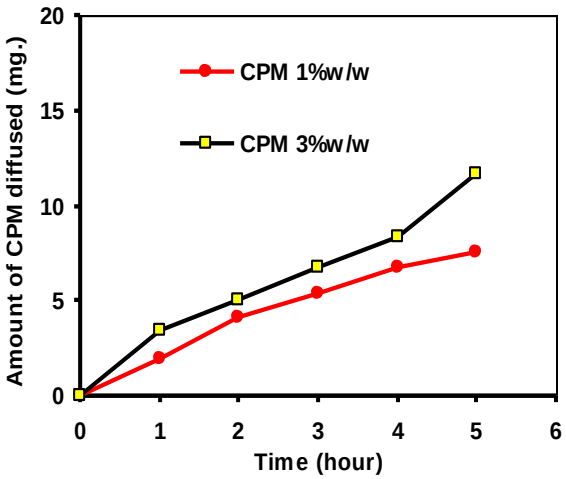

Figure (4) . The effect of CPM concentration on the diffusion process of through rat skin from sodium alginate 2%w/w gel at pH 7.4 and 37°C

Effect of different enhancers and their concentrations on the diffusion of CPM 3%w/w through rat skin:

The enhancers which they are used in this study are water soluble types , they include urea 5% w/w , PEG 1000 2 % w/w , and TEA 2.5% w/w Table 4 . and figure 5 , illustrate the effect of incorporation the above enhancers separately on the diffusion of 3% w/w CPM loaded in 2% w/w SA gels through rat skin . It was seen that both urea 5% w/w and PEG 1000 2% w/w significantly decrease $P < 0.05$ the amount of the drug diffused through rat skin. Since incorporation of urea in hydro gel may activate the hydrolysis process of urea to form ammonia and carbon dioxide , which lead to elevate the pH of the medium of environment , which in turn dissociate the CPM into maleate anion and chlorpheniramine cation , these ionized species may hinder the diffusion process of the drug [15] . Moreover , the alkyl amine group , of the drug may complex ethylene oxide (CH2-Ö-CH) group of PEG1000 that decrease the amount of the drug available for permeation, this result is in a consistent with that result obtained , when lidocaine was formulated as a topical gel [16] . On the other hand , incorporation of 2.5%w/w of TEA which counter irritant to the skin as an enhancer , showed a slight increase of CPM permeation through the rat skin , this effect may be attributed to both effects of TEA as a basic tertiary amine enhancer once that is compatible with alkyl amine anti histamine (CPM) , and second may be referred to the effect of emulsification of TEA with malic acid to form water soluble salt that easily allow the drug penetration through the rat skin [17].

Table (4). Effect of different enhancers on the diffusion rate constant (K) of CPM 3%w/w through the rat skin

Enhancer type	CPM amount diffused mg./5hr.	Rate constant (mg.min $^{-1}$½)
No addition	11.765±0.11	0.8217
Urea 5%w/w	5.648±0.24	0.3659
PEG1000 2%w/w	6.820±0.12	0.4807
TEA 2.5%w/w	13.138±0.16	1.09

Estimation of irritation property of selected formula :

The selected formula which was introduce to specify the irritation test consist of 3%w/w CPM loaded in 2% w/w SA and fortified by 5% w/w TEA as an enhancer. After 7 days of gel application on the dorsal shaved skin of albino rat , it was seen that a recognized redness area on the skin developed during this period , while the application of

the same formula free from 5% w/w TEA showed no appearance of this irritation . This observation may be related to the irritation effect of TEA itself at this concentration , since most of the quaternary ammonium surfactant are strongly catoionic irritant enhancers [18] .

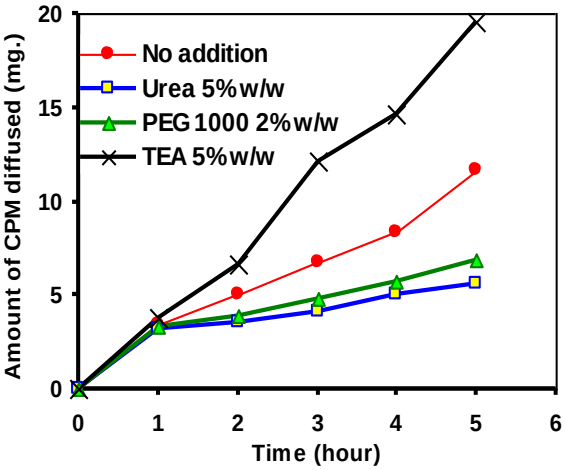

Figure (5) . The effect of different enhancers on the diffusion process of CPM 3% w/w through rat skin at pH 7.4 and 37˚C

Stability study

Determination of the shelf life

The results of this study showed CPM followed first order kinetic degradation , when the selected formula kept in a collapsible tubes maintained separately at , 40 , 50 , and 60˚C , the contents of these tubes for CPM amount were determined every seven days for 4 weeks , and the rate of degradation at 25 ˚C (K25˚C) was found to be 0.872¯ x 10 .day⁻¹ , when Arrhenius plot was constructed as a logarithm of degradation rates constants for above exaggerated temperatures against reciprocal of absolute temperatures of CPM storage as shown in figures 6 and 7 To estimate the shelf life , the following expression was used to estimate 90% of the drug content remain at that time .

t 90% = 0.105/0.872¯day = 1204 days

So the calculated shelf life for a selected formula was found to be about 3.3 years .

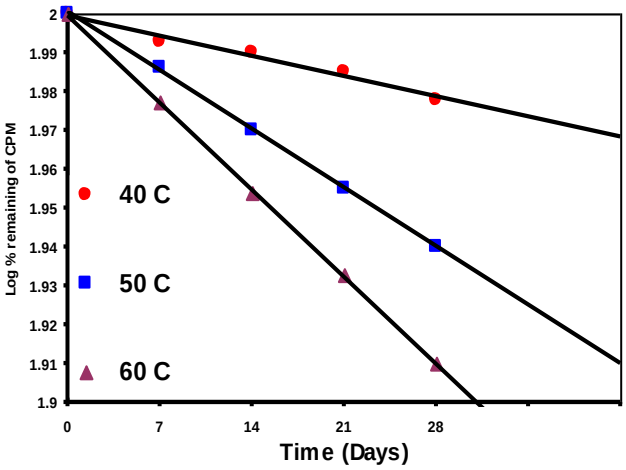

Figure (6) . Accelerated degradation of CPM in a selected formula at different exaggerated temperatures

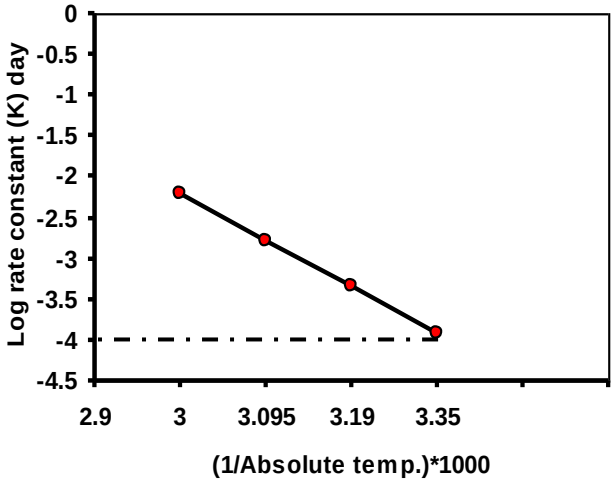

Figure 7 . Arrhenius plot for estimation of shelf life of CPM of a selected formula

Effect of temperatures and storage time on the pH , color and odor of 3%w/w CPM gel

The result after 30 days storage time at different storage exaggerated temperatures of CPM gel , revealed that slight increase in the pH of CPM gel from 4.25 to 4.6 , which may be attributed to the ionization of CPM that releases chlorpheniramine base , which belongs to the basic properties of this types of antihistamines [19] . More over no change in the original translucent white color or appearance of unpleasant odor was observed . These results indicated no probability of physical instability, or growth of micro- organisms in the selected formula .

Conclusions

Concerning of the results obtained , one can conclude , the followings :

1. Maximum CPM release was achieved, when 4% w/w of SA was introduce as a gel base.
2. The diffusion of CPM through the skin of the rat ,was increased as a function of increasing CPM concentrations , loaded in 2%w/w SA gel base .
3. Addition of 5%w/w TEA to 2%w/w SA loaded by 3%w/w CPM , enhances the amount of the drug diffused through the skin of the rat .
4. Addition of 2%w/w PEG1000 , and 5%w/w urea , separately to 2%w/w SA loaded by 3%w/w CPM , decreases significantly $P< 0.05$ the amount of the drug diffused through the skin of the rat .
5. There was a marked irritation spots recognized, when TEA 5%w/w used as an enhancer in the selected formula , compared with no effect when this enhancer is avoided .
6. The selected formula of SA 2%w/w as a base loaded by 3%w/w of CPM was acceptable with calculated shelf life about 3.3 years .

This formula may need a further clinical study on volunteers to ensure its therapeutic , and economic value .

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Effects of Interleukin-2 (IL-2) and Interleukin-6 (IL-6) in Recurrent Spontaneous Abortion (RSA).

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Abstract

Recurrent Spontaneous Abortion (RSA) is the most painful experience for couples expecting a child. This study aimed to determine the relevance of IL-2 and IL-6 in recurrent spontaneous abortion (RSA). Serum samples were collected from 90 women attend Al Kadhmiya teaching hospital in Baghdad. 60 women (first group) had recurrent abortion the women were negative for rubella virus, herpes simplex virus and toxplasma gondii. And they were negative from bacterial infection eg. Niesseria gonorrhea and Chlamydia trachomatis. The histopathological tests for fetus abnormalities were negative in this group, and 30 women (second group) with successful pregnancy (normal delivery). All samples were analyzed for IL-2 and IL-6 by commercially available Enzyme-Linked Immunosorbent Assay (ELISA) kits. The data showed highly significant increase in the serum level of IL-2 in group 1 compared with group 2 (P<0.001). However, IL-6 showed highly significant increase level in group 2 compared with group 1. In addition, there was no significant correlation between these two markers in studied groups. The data of this study strengthen the possibility that high level of IL-2 and low level of IL-6 may explain the role of type-1 cytokines in the pathogenicity of recurrent spontaneous abortion.

Key words: - interleukin 2, interleukin 6 and recurrent spontaneous abortion (RSA)

الخلاصة

يعد الإسقاط الذاتي المتكرر من التجارب الأليمة في حياة الزوجين الراغبين في إنجاب الأطفال. أجريت هذه الدراسة للتحري عن دور الانترلوكين 2 و6 في إحداث الإسقاط الذاتي المتكرر حيث جمعت تسعون عينة مصل من مجموعتين من نساء ادخلن إلى مستشفى الكاظمية التعليمي المجموعة الأولى ستون عينة كانت من نساء عانوا من الإجهاض الذاتي المتكرر واللاتي سبق واثبت خلوهن من الإصابة بفيروس الحصبة الألمانية (الحميراء) Rubella virus, وفيروس الحلأ التناسلي Herpes simplex virus وداء المقوسات Toxoplasma gondii وكذلك عدم إصابتهن ببكتريا المسببة السيلان Niesseria gonorrhea , والكلاميديا Chlamydia trachomatis وكذلك أثبتت الدراسة النسيجية من عدم وجود تشوهات في أجنة هذه المجموعة. المجموعة الثانية كانت من ثلاثون عينة كانت من نساء وضعن حملهن بصورة طبيعية. جميع العينات فحصت بطريقة الاليزا (ELIZA) للتحري عن الانترلوكين 2 و 6 , أظهرت النتائج فروق معنوية عالية في مستويات الانترلوكين 2 في مصول المجموعة الأولى مقارنة مع المجموعة الثانية ($p< 0.001$) , وكذلك كانت معدلات الانترلوكين 6 في مصول المجموعة الثانية مرتفعة مقارنة مع المجموعة الأولى. بالإضافة إلى ذلك لم تظهر النتائج ارتباط معنوي بين هذين الواصمين في المجاميع قيد الدراسة . نتائج هذه الدراسة دعمت احتمالية دور(السايتوكاين 1) في أمراضية الإسقاط التلقائي المتكرر في حالات ارتفاع الانترلوكين 2 وانخفاض الانترلوكين 6.

Introduction

Recurrent spontaneous abortion (RSA) is one of the important complications in pregnancy. Half of recurrent miscarriages loss is multifactorial, can be explained by genetic, hormonal,anatomical, metabolic abnormalities infections or autoimmune mechanisms and an be divided into embryological driven causes (mainly due to abnormal embryonic karyotypes) and maternally driven causes which affect the endometrium and/or placental development [(1)]. Known causes of maternal defects include coagulation disorders, autoimmune defects, endocrine disorders and endometrial defects [(2)]. Mammalian pregnancy is thought to be a state of immunological tolerance. The mechanisms underlying this phenomenon are still poorly understood, Successful mammalian pregnancy depends upon tolerance of a genetically incompatible fetus by the maternal immune system. When tolerance is not achieved pregnancies fail [(3)] .

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Immunological rejection of the fetus due to recognition of paternal antigens by the maternal immune system, resulting in abnormal immune cells and cytokine production, is postulated to be one cause of unexplained pregnancy loss [4]. Cytokines have traditionally been divided into families dependent upon the immune cell of origin and the immunological effects that they bring about. CD4+ T-helper cells are the major immune cells involved in cytokine production, and these can be divided into functional subsets based on their cytokine production, T-helper 1 (Th1) cells produce interferon gamma (IFNg), IL-2 and tumor necrosis factor beta (TNFb) are the main effectors of cell mediated immune response [5] T-helper 2 (Th2) cells produce IL-4, IL-5, IL-6 and IL-10, which are the main effectors of antibody-mediated humoral responses [6]. Local mechanisms may play an important role in evading immune attack because maternal alloreactive lymphocytes are not systemically depleted. The specialized fetal tissue in contact with maternal uterine tissue might contribute to tolerance by several mechanisms, such as depleting tryptophan,[7] by inactivating natural killer cells through HLA-G expression,[8] or by provoking apoptosis of activated maternal lymphocytes [9]. Incomplete tolerance might therefore result in disturbed pregnancy such as spontaneous abortion and pre-eclampsia. Further, Th1/Th2 cytokine balance has been seen as a very important mechanism determining the survival of the fetus in the maternal uterus. Recent evidence suggests that maternal tolerance is established at the feto-maternal interface, by factors deriving from the decidualized endometrium and from the trophoblast itself, is maintained throughout gestation in physiological pregnancy [10]. Cytokines released at the feto-maternal interface have been proposed to play an important role in regulating embryo survival controlling not only the maternal immune response but also angiogenesis and vascular remodeling [10; 11]. Th-1 cytokines are considered to be detrimental to pregnancy, via direct embryo toxic activity, or via damage to the placental trophoblast, or possibly by activating cells that are deleterious to the conceptus, whereas Th-2 cytokines may directly or indirectly contribute to the success of pregnancy by down regulating potential Th-1 reactivity [12; 13]. Protect the fetus and placenta from being rejected and to aid in the maintenance of normal pregnancy. In humans an important role for the T-helper 2 immune response has also been reported during normal pregnancy **[14; 15]**.

Methods : Studied group

This study included ninety (90) women from the Obstetrics and Gynecology Department of Al- Kadhmiya teaching hospital in Baghdad. Patients' ages ranged between (18-36) years with a mean of (27.5 − 30.1) year. The patients were divided into two groups:

Group 1: sixty (60) women were admitted to the hospital for recurrent spontaneous abortion (3-6 numbers of abortions) for evacuation.

Group 2: thirty (30) women with successful pregnancy (normal delivery) as control group.

Sample collection

From each women included in the study blood samples were collected to obtain the serum.

Procedure

* Enzyme Linked Immunosorbent Assay (ELISA) for the detection of IL-2, IL-6 in **serum:** IL-2, IL-6: ELISA Test Kits provided by (Mabtech Australia Pty Ltd). Product cod: (3460-IA-6) IL-6, (3430-IA-6) IL-2. Estimation of IL-2, IL-6 level in serum or plasma by ELISA method. This method has two immunological steps. In the first step, the cytokine is captured by monoclonal antibody bound to the wells of a micro titer plate. In the second step a monoclonal antibody linked to a biotinylated monoclonal antibody is add together with streptavidine-peroxidase conjugate. The solid phase antibody-antigen complex and in turn, binds the conjugate. After incubation, the wells are washed and the antigen complex bound to the well detected by addition of a chromogenic substrate. The intensity of the color developed is directly related to the specific monoclonal antibodies concentration of the sample [16].

Statistical analysis

The Student test (t-test) analysis program was used to calculate the values, Mean, and standard error were all used in the analysis and the relationship between the indicators was measured qualitatively by using the correlation coefficient

(r).Values of $P<0.05$ were considered as statistically significant [17].

Results

The expression of IL-2 and IL-6 was detected by ELISA technique. Table (1) shows the mean value of concentration (pg/ml) of IL-2 in sera of studied group which show highly significant ($p<0.01$) increased expression of IL-2 in aborted women compared with control. Table (2) shows the mean value of concentration (pg/ml) of IL-6 in sera of studied group which show highly significant ($p<0.01$) increased expression of IL-6 in aborted women compared with control. In addition, the study failed to found a significant correlation ($P>0.05$) between IL-2 and IL-6 in aborted women and control groups, as shown in Table(3) .

Table (1): The mean value of concentration (pg/ml) of IL-2 in sera of studied group.

Studied group	N	Mean± Std. Error	Comparison of significant	
			P-value	Sig.
Control	20	97.45± 0.90	0.00	Highly Sig. (P<0.01)
Aborted women	20	259.32± 82.6		
Total	50			

Table (2): The mean value of concentration (pg/ml) of IL-6 among studied group.

Studied group	N	Mean± Std. Error	Comparison of significant	
			P-value	Sig.
Control	20	375.30±73.3	0.00	Highly Sig. (P<0.01)
Aborted women	20	82.41±0.86		
Total	50			

Table (3): Pearson correlation (r) between IL-2 and IL-6 levels in serum of studied group

Pearson Correlation		IL-2 IL-6
Abortion	r	0.532
	P-value	>0.05
	Sig.	NS
Control	r	0.421
	P-value	>0.05
	Sig.	NS

NS= Non Significant difference

Discussion

The level of IL-2 increased in women with abortion in comparison with that successful pregnancy as shown in table (1). Evidence supporting this result showed that the administration of one of the Th-1 cytokines like Interferon- γ (IFN- γ), Tumor necrosis factor- α (TNF-α) or interleukin-2 (IL-2) to normal pregnant mice causes abortion [18]. During a normal pregnancy, allogenic fetal tissues are exposed to the maternal immune system. Rejection reactions normally develop after allogenic recognition following the principle of transplantation immunology [19]. Although the immune system is functional in the uterus and the embryo expresses paternal major histocompatibility complex (MHC) molecules, the conceptus nevertheless escapes the deleterious effect of maternal rejection. It may be due to local tolerance or even perhaps immune suppression after interactions between fetal antigens and the maternal immune system[19]. T cells must change from a resting to an activated state during immune responses and lead to de-novo synthesis of interleukin IL-2 and expression of IL-2 receptors {IL-2Rs} [20]. Interaction of IL-2 and its receptors triggers cellular proliferation, culminating in the emergence of effectors T cells that are required for the full expression of immune responses. [21] Spontaneous abortions in humans have been shown to be associated with increased production of interleukin (IL)-2 and IFN-γ by peripheral blood mononuclear cells (PBMC) and with decreased production of IL-10, as compared to normal pregnancy [22]. Studies by Hill and colleagues have shown that

trophoblast antigens activate the PBMC of women with a history of unexplained recurrent spontaneous abortion (RSA) to produce the embryotoxic cytokines IFN-γ and TNF-ß[22,23]. Interleukin-2, tumor necrosis factor-α, and interferon-γ are deleterious and used to stimulate the apoptosis of human primary villous trophoblast cells. In addition this study reveals in table 2 that the expression of IL-6 proteins in circulation of women with successful pregnancy was significantly higher (p<0.001) than that of women with abortion. Further studies showed that there is a greater increase in IL-6 secretion during pregnant compared to not pregnant state that may be detected by ELISA of endometrial biopsy samples [24]. human trophoblasts express IL-6 receptor and produce IL-6, which induces the production of hCG in an autocrine manner, suggesting a role of IL-6 in early implantation and its continuation in early pregnancy[25]. IL-6 may play a role in physiological mechanisms involved in uterine contractions and the propagation of labour. Thus, increased concentrations of IL-6 may reflect a systemic reaction in the mother, leading to labour and delivery [26]. This study showed that IL-6 concentrations were lower in women with RSA than in those undergoing normal delivery. Considering that IL-6 is a Th2-type cytokine and that normal pregnancy appears to be a Th2-biased condition. These cytokines encourage vigorous antibody production, and are therefore commonly associated with strong antibody responses that are important in combating infections with extracellular organisms. IL-6 may also induce prostaglandin synthesis by intrauterine tissues; thus, it seems to play a physiological role in labour development. High levels of IL-6 have been detected in pregnant women at term and in preterm at labour [27]. Such human studies do not clearly indicate a cause-and-consequence between increased IL-6 concentrations and events associated with labour, studies in monkeys have shown that an increase in IL-6 concentrations precedes uterine contractions that may play a role in physiological mechanisms involved in uterine contractions and the propagation of labour [28]. The IL-6 found in the serum may originate from the trophoblast [29]. Thus the study by Makhseed and colleagues, demonstrated that IL-6 concentrations were lower in women with RSA than in those undergoing normal delivery, considering that IL-6 is a Th2-type cytokine and that normal pregnancy appears to be a Th2-biased condition [13]. Th1 and Th2 cells are mutually inhibitory to each other when Th1 reactivity is high, Th2 reactivity is usually low and vice versa [30]. The current study shows in table (3) no significant correlation (p>0.05) between IL-2 and IL-6 in women with recurrent spontaneous abortion (RSA) and successful pregnancy. This un relation might be associated with different role of these two cytokine during pregnancy thus we suggested further study focusing on the role of IL-2 and IL-6 in pregnancy and RSA in placental tissue. In conclusion, IL-2 might play a pathological role in pregnancy in contrast IL-6 might play a role in successful pregnancy.

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Rosiglitazone , Metformin or both for Treatment of Polycystic Ovary Syndrome

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Abstract

This study was designed to show the advantages of using the combination of metformin and rosiglitazone over using each drug alone in treatment of women with polycystic ovary syndrome (PCOS).Forty four women with PCOS were classified into 3 groups , group 1 received rosiglitazone (4mg/day) for 3 months , group II received metformin (1500 mg/day)for three months and groupIII received the combination (rosiglitazone 4mg/day + metformin 1500 mg/day) for the same period of treatment . The blood samples were drawn before treatment and after 3 months of treatment . The fasting serum glucose , insulin , progesterone , testosterone , leutinizing hormone were measured before and after treatment. The reduction of serum insulin , glucose ,homostasis model assessment of insuline resistance (HOMA-IR) , LH and testosterone levels were greater in the group received the combination of rosiglitazone with metformin than that those taken each one alone. Testosterone levels decreased significantly ($P<0.05$) from baseline level 1±0.04ng/ml to 0.073±0.32ng/ml after treatment with combination.The rate of ovulation is 29.4%,36.4% , 62.5% in rosiglitazone , metformin and combination of both, respectively.The combination of rosiglitazone with metformin has more beneficial effect on ovulation rate.

Key words: polycystic ovary syndrome, rosiglitazone, metformin, ovulation rate .

الخلاصة

هذه الدراسة صممت لعرض الفوائد من استعمال خليط من الميتفورمين والروزيكليتازون على استعمال كل دواء منفردا عند علاج النساء المصابات بمتلازمة المبيض متعدد الاكياس. اربع واربعون امرأة مصابة بمتلازمة المبيض متعدد الاكياس قسمن الى ثلاث مجموعات : المجموعة الاولى تناولت الروزيكليتازون بجرعة 4ملغ يوميا لمدة ثلاثة اشهر و المجموعة الثانية تناولت الميتفورمين بجرعة 1500 ملغ يوميا لمدة ثلاثة اشهر اما المجموعة الثالثة فقد تناولت خليط من الروزيكليتازون بجرعة 4ملغ يوميا والميتفورمين بجرعة 1500 ملغ يوميا لنفس مدة العلاج. نماذج الدم سحبت قبل العلاج وبعد ثلاثة اشهر من العلاج.ان التغيرفي مستويات الكلوكوز , الانسولين, البروجيستسرون , الهورمون اللوتيني والتستوستيرون كان ملحوظا اكثرفي خليط الروزيكليتازون و الميتفورمين مما في حالة اخذ كل دواء منفردا.كما ان مستوى التستوستيرون يقل معنويا عن مستوى الشروع بعد العلاج بالمركب . وكانت نسبة القابلية على انتاج البيض هي29,4, 36,4 , 62,5 في الروزيكليتازون والميتفورمين وكلتاهما معا على الترتيب ان خليط الروزيكليتازون و الميتفورمين له تأثير نافع على نسبة انتاج البيض.

Introduction

Polycystic ovary syndrome (PCOS) is the most common abnormality in women during reproductive age, it is a hetrogenous disorder of uncertain etiology [(1)]. It is characterized by chronic anovulation and hyperandrogenism [(2)] affecting approximately 5-10 % of reproductive age women. The association between hyperinsulinemic insulin resistance and PCOS is well recognizes and may play an important pathogenic role in development of PCOS [(3)]. Obese and lean women with PCOS manifest insulin resistance independent on body weight, although obesity is an additive factor which aggravates insulin resistance [(4)]. There is some data to suggest that insulin enhances the effect of LH on preovulatory ovarian follicle arrest [(5)]. It is possible that hyperinsulinemia due to insulin resistance drives the LH affect on ovarian theca cells to cause androgen excess which are intrinsically programmed to produce more androgens [(6)] . Excess androgens are known to interfere with the process of follicular maturation , thus inhibiting ovulation and producing more arrested follicles. It has been postulated that in PCOS ovaries there is an increased rsistance to all insulin functions , except for steroidogenic effect and the ultimate result is excess androgen production even with normal insulin level[(7)]. Metformin is abiguanide hypoglycemic agent that is approved for the management of type II diabetes [(8)]. Its main mechanism of action is the reduction of hepatic glucose production (hnhibition of gluconeogenesis). It also increases insulin mediated glucose utilization in peripheral tissue and decreases intestinal absorbtion of glu**cose**[(9)].

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Several authors [(10,11)] have demonstrated the additional benefits of using metformin such as

these related to menstrual cycle regulation and induction of ovulation, protection from pregnancy losses ,improvement of cardiovascular risk factors ,moreover metformin markedly increases both spontenous ovulation rate and clomiphene-induced ovulation rate for obese women with PCOS [12] . Many studies have shown improvements in ovulatory function , development of normal menses , and restoring of fertility [13]. In spite all of these benefits , many workers reported that metformin effect may be to some extent transient and some cellular adaptation may occur during more prolonged therapy [14]. Rosiglitazone was approved in (1999) by food and drug administration (FDA) as an oral antidiabetic agent for the management of type II dibetes as montherapy and in combination with oral hypoglycemic agents [15]. Rosiglitazone increases insulin sensitivity without stimulating insulin secretion, its mode of action is by binding and activation of the nuclear peroxisome proliferators activator gamma (PPAR-γ) which is found in key target tissues for insulin action as adipose tissue, skeletal muscle and liver. Activation of (PPAR-γ) regulates the transcription of insulin –responsive gens involved in control of glucose and fatty acid metabolism [9,11] . Therefore this study was designed to show whether combination of (rosiglitazone and metformin) has advantages over using each drug alone in the treatment of women with PCOS.

Methods and Materials

This study was conducted at Baghdad city in AL-Elwia maternity teaching hospital from October-2004 till June-2005.The study groups included 60 raqi women, (44) case with PCOS aged 17-40 years with a mean of age 27.3±5.07 years , and 16 normal control subjects aged 18-38 years with a mean of age 27.1±6 years. The patients included in this study were diagnosed with PCOS were non-diabetic, non-hypertensive and non-pregnant.The patients were under gynecologist supervision during period of treatment. The diagnosis of PCOS was made by gynecologist depending on ultrasound examination, clinical features and laboratory tests (hormonal assay). The patients involved in this study were on normal diet.They were divided randomly into 3 groups:

1. Groiup 1 included 17 patients (BMI 28.8±3.9 Kg/m2), age 29.7±6.4 years. The patients received rosiglitazone 4mg daily in two divided doses (2mg) in the morning and (2mg) in the evening after meals for 3 months.
2. -Group II included (11) patients (BMI 29.1±5.2Kg/m2), age 24.9± years). The patients received metformin 1500mg daily in three divided doses (500mg after each meal) for three months.
3. -Group III included (16) patients (BMI 34.2±6 Kg/m2) , age 26.5±4 years. The patients received a combination of the two drugs (rosiglitazone 4mg/day + metformin 1500mg/day) for three months.
4. -Control group included (16) normal women (BMI 30±4.8 Kg/m2), age 27.1±6 years.

Sample collection:

Eight millilitrs (8ml) of venous blood samples used in this study were drawn from PCOS patients.The first sample was collected before treatment as a baseline level, and after three months of treatment with insulin sensitizing agents.Fasting blood samples were used for the measurement of glucose , insulin, hormones (LH, testosterone and progesterone). Blood samples were left at room temperature for 30 minutes for clotting, centrifugated and then serum was separated and collected in small aliquots(0.5ml) and stored at (-20 C) until biochemical and hormonal analysis was performed. The serum was used for measurement of fasting blood sugar,insulin ,testosterone, LH and progesterone levels.

Biochemical and hormonal assay:

Fasting insulin levels were determined using a commercial kit obtained from Randox, using Radioimmunoassay (RIA) method [17,18]. Serum testosterone levels were determined using a commercial kit obtained from Immunotech, based also on (RIA) [19]. By using of a kit from Immunotech, the radioimmunoassay of progesterone is compitiotion assay [20]. Serum LH determined using kit from Immunotech, by the immunoradiometric assay (IRMA) which is sandwich type assay [21].Fasting serum glucose was measured by a commercial kit obtained from BIOmghreb, using the enzymatic method[22].

Diagnosis of infertility depends on that inability of any couple to conceive a child within a 12 months period of unprotected coitus (sexual intercourse) [23].

Body mass index (BM I) was calculated using the standard formula :

BMI=weight (kg)/hight (m2). Obses patients were defined as having BMH> 27 Kg/m2[24,25].

Homeostasis model assessment of insulin rsistance (HOMA_IR) was calculated using the following formula:

HOMA-IR= basal glucose (mmol/L).basal

insulin(μIU/ml) 22.5
Insulin resistance patients were defined as having HOMA>2.7 [26].
The drugs used in this study were: Rosiglitazone 4mg tablets purchased from (Sunpharma)- India- and metformin 500mg tablets purchased from (MB and C) -syria-

Statistical analysis

1-The results were expressed as man±SD mean.
2- Student T-test was used to examine the difference in the mean of parameters tested.
3- P-value of less than (0.05) was considered significant.

Results

The most patients in this study were with oligomenorrhea (68.1%) and (22.7%) of the patients were infertile. The hirsutism was obvious symptom in (63.6%) of the patients (table 1). The combination of metformin and rosiglitazone reduced the levels of serum insulin, glucose , HOMA-IR, LH and testosterone which are more than that produced by rosiglitazone or metformion alone (tables 2 , 3 and 4)(P<0.05).The testosterone was significantly decreased (P<0.05) only after treatment with combination compatred to the baseline levels. The ovulation rates were 29.4%, 36.4%, 62.5% in rosiglitazone, metformin and combination of both , respectively (table 5).

Table(1): demographic data of 44 patients with PCOS.

Character	Number of patients (%)
Obese	31(70.4%)
Lean	13(29.5%)
Amenorrhea	10(22.7%)
Oligomenorrhea	30(68.1%)
Regular cycle	4(9%)
Infertility	10(22.7%)
Hirsutism	28(63.6%)

Table (2): Effect of treatment of rosiglitazone(4mg/day) on levels of insulin, glucose, HOMA-IR,LH and testosterone in group1.

Variables	Control levels (n=16)	Baseline levels (before treatment) (n=17)	After treatment (n=17)(%)
Insulin (μU/ml)	8.4±1.7	15.6±5.5a	10.4±3.6(33.5)
Glucose (mg/100ml)	82±4.9	89.8±7	84±5.8(6.4)
HOMA-IR	1.7±0.43	3.4±1.4a	2.1±0.9(38.2)
LH (mμ/ml)	4.5±0.15	11.6±3.4a	10.6±3.1a(8.6)
Testosterone(ng/ml)	0.34±0.01	0.94±0.44a	0.71±0.43a(24.4)

Values are means±SD
n= No. of women.
%= percentage of change compared with base line levels.
a p<0.05 for comparison with control group.
HOMA= homeostasis model assessment of insulin resistance.
LH= Leutinizing hormone.

Table (3) : Effect of treatment with metformin(1500mg/day) on levels of insulin , glucose , HOMA-IR,LH and tstosterone in group II.

Variables	Control levels (n=16)	Baseline levels (before treatment) (n=11)	After treatment (n=11)(%)
Insulin (µU/ml)	8.4±1.7	14.5±4.3a	10.3±2.8(28.9)
Glucose (mg/100ml)	82±4.9	84.2±4.2	82±5.1(2.6)
HOMA-IR	1.7±0.43	2.9±0.9a	2±0.5b(13)
LH (mµ/ml)	4.50.15±	12.4±4.7a	11.5±3.9a(7.2)
Testosterone(ng/ml)	0.34±0.01	0.91±0.51a	0.71±0.37a(21.9)

Values are means ±SD.
N=No. of women.
%=percentage of change compared with baseline level.
a P<0.05 for comparison with control group.

Table (4): Effect of treatment with the combination of metformin(1500 mg /d) and rosiglitazone (4mg/d) on the levels of insulin , glucose , HOMA-IR,LH and testosterone in group II.

Variables	Control levels (n=16)	Baseline levels (before treatment) (n=16)	After treatment (n=16)(%)
Insulin (µU/ml)	8.4±1.7	21.2±8a	12.5±4.8(41)
Glucose (mg/100ml)	82±4.9	90.3±8.3a	84.1±6.5(6.8)
HOMA-IR	1.7±0.43	5±1.5a	2.6±0.8(48)
LH (mµ/ml)	4.50.15±	11.9±2.8a	10.2±2.3a(14.2)
Testosteroe (ng/ml)	0.34±0.01	1±0.46a	0.73±0.32a,b(27)

Values are means ±SD.
n= no. of women .
%=percentage of change compared with baseline level.
a P<0.05 for comparison with control group.
b P<0.05 for comparison with base line alone .

Table (5): Ovulation rate in PCOS patients for treatment with insulin sensitizing agents.

Groups Secondary outcome	Group 1 (n=17)	Group II (n=11)	Group III (n=16)	Total
Ovulation	5(29.4)	4(36.4%)	10(62.5)%111	19
No ovulation	12(70.6%)	7(63.6%)	6(37.5%)	25
Total	17	11	16	44

n=no. of women
Group 1: women treated with rosiglitazone 4mg/d alone.
Grouop II: women treated with metformin 1500mg/d alone.
Group III : women treated with combination of roziglitazone 4mg/d and metformin 1500mg/d.

Discussion

In this study , the administration of insulin sensitizing agents rosiglitazone and metformin alone or in combination for three months showed non-significant reduction (P>0.05) in serum glucose levels , serum insulin levels nor HOMA _IR index. The efficacy and percentage of improvement was seen to be more obvious in combination group than with either drug alone (table 2,3 and 4) . Roziglitazone showed more improvement than metformin . However, in present study , rosiglitazone and metformin treatment improved insulin resistance because there was an improvement in fasting insulin and fasting glucose levels, similar results were reported by other studies [27,28]. All groups of patients who received rosiglitazone and metformin alone or in combination showed a slight (non-significant) decline in LH levels when compared with baseline levels .Lack of change in LH levels also reported by many researchers[28,29]. The effect of rosiglitazone and metformin combination for three months was associated with significant decline in testosterone levels (p<0.05) (table 4). The study also shows a greater decrease in insulin and HOMA-IR index leading to more decrease in testosterone level. These results are in agreement with studies showed a reduction in serum androgen levels after the reduction of insulin levels by insulin sensitizing agents, and these effect were independent in body weight [28,30,29]. In general, the favorable effect of insulin sensitizing agents on hyperandrogenemia in PCOS may be due to reduced pituitary secretion of LH, reduced ovarian androgen secretion, and increased levels of sex hormone binding globulin (SHBG)[31].The adminstrastion of rosiglitazone or metformin alone or both of them for three months demonstrated an improvement in ovulation rate assessed by measurement of mid-luteal phase progesterone level in group III more than group II and I (table 5) . This may be due to the synergestic effect of two drugs which lead to decrease the testosterone significantly (P<0.05). The percentage of ovulation was (62.5%0, (36.4%) and (29.4%) in groups III , II , I respectively. Several studies investigated effect of metformin on menstrual cyclicity, and a significant improvement in the frequency of menstrual cycles has been reported , with an incrase in the percentage of ovulatory cycles as assessed by mid-luteal phase progesterone [32,27]. K.J Meenanakumaari et al (2004) found a significant negative correlation between insulin and progesterone , and between progesterone and LH concentration in PCOS women treated with metformin and suggested that insulin resistance / hyperinsulinemia may be responsible for low progesterone levels during the luteal phase in PCOS. The luteal progesterone may be enhanced in PCOS by decreasing insulin levels with metformin [33]. Richardo Azziz et al (2001) studied the effect of rosiglitazone on menstrual cyclicity and ovulation in PCOS women. Azziz reported an increase in the mean rate of ovulation in dose-related fasion and he expected an improvement in the menstrual cycle after the improvement in ovulation [34]. Nicholas A. Cataldo et al (2006) showed a favorable effect of rosiglitazone on menstrual pattern or ovulation independent of rosiglitazone dose, furthermore they have found that ovulation occur in association with only modest change in insulin rsistance and insulinemia and claimed either that a small metabolic improvement is sufficient to promote preovulatory follicular maturation or that rosiglitazone exerts its effect indepently of insulin [35]. Similar results were reported by Didem Dereli et al (2005)[36]. In conclusion it is preferable to use a combination of rosiglitazone and metformin in infertile PCOS women as it has more potent effect in the improvement of ovulatiobn rate. The combination is also more beneficial to alleviate the hyperandrogenemioa in women with PCOS.

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