Effect of plasma isolated Orexin-A on the regulation of metabolites in male rats

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Abstract

This research includes the isolation and purification of Orexin-A from the plasma of healthy human via various biochemical techniques, it was proposed the therapeutic role of orexin on hyperlipidemia and lipid peroxidation and it has been suggested to study the effect of isolated orexin A on the metabolism of lipids and glucose in normal and hyperlipidemic rats, a high level of orexin-A had been found only in second peak (B) isolated by gel filtration chromatography (using Sephadex G-50) and showed (34.5) fold of purification, also, the effect of isolated orexin-A on some clinical parameters had been studied in normal and hyperlipidemic male rats. The rats were injected intraperitoneally with orexin-A at a dose of 1μmol/kg of body weight/day for one month. The results, obtained before treatment and after two and four weeks of treatment, had been showing a significant decrease in the concentration of total cholesterol, triglycerides, low and very low-density lipoprotein cholesterol, glucose, malondialdehyde and hyperinsulinemia, while there was a significant increase in the concentration of high-density lipoprotein cholesterol in normal and hyperlipidemic rats. It was concluded that orexin-A had an important role in regulating the metabolism of glucose and lipids, treatment of hyperinsulinemia and insulin resistance, and decreasing lipid peroxidation in normal and hyperlipidemic rats.

Introduction

Orexin is a pair of neuropeptides (Orexin-A and Orexin-B) derived mainly from neurons distributed in the lateral hypothalamus (LH) (1). In 1998, two researcher’s groups discovered the new peptides in the lateral hypothalamus of the rat brain (2). One group of researchers named it "Hypocretin" derived from the hypothalamus, based on its anatomic location and the amino acid sequence similar to the gut hormone, secretin. Another group called it “Orexin” came from orexis, the Greek word meaning appetite (3). There are two type of orexin: Orexin-A (OXA), 33-amino acid, molecular mass 3562 Da, contains 4 Cys residues which form two intra-chain disulfide, and has identical sequences in human, rat, mouse, and cow (4,5). Orexin-B (OXB), 28-amino acid, molecular mass 2937 Da, is a liner peptide, and human OXB have different sequences that of the rodents (6). Orexin-A and -B initially identified as endogenous ligands for orphan G-protein coupled receptors (GPCRs) OX1R and OX2R (7). The widespread of orexin neurons and receptors in tissues shows the important role of this hormone. OXA controls body weight through regulation of food intake according to the levels of plasma fuel to maintain energy homeostasis (3) thus, OXA protects against obesity (8) also OXA has a certain role in the metabolism of glucose, improvement of insulin receptors function, preserving insulin sensitivity, regulating lipid metabolism and controlling sleep-waking cycle (8,9). Orexin-A increases the cytoplasmic calcium Ca²⁺, Orexin-A initially binds to the receptors which in turn activate G-protein and

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subsequently enhances the influx of Ca^{2+} through channels of the plasma membrane (3,10). The aim of the research was study the effect of isolated OXA from the human plasma on the metabolism of lipids and glucose in normal and hyperlipidemic rats, since there are a few studies in Iraq about OXA in experimental animals.

**Materials and methods**

**Samples**

Fresh plasma (50 ml) was obtained from one healthy male person with age (37 years) with the assistance of the blood bank in Mosul city (the plasma was taken ready and frozen).

**Organic solvent precipitation**

Cold acetone at 4°C (40:60v/v) was used to precipitate proteinious material (11). Cold acetone added to plasma gradually with slowly stirring at 4°C for 60 min. The mixture was left for 24 h in the refrigerator at 4°C. Cooling centrifuge for 30 minutes at 12000g was used to isolate the precipitated protein, which was dissolved in the lowest volume of distilled water. The protein and orexin-A concentration were estimated (12,13). The protein solution then kept in a tight test tube for a subsequent step.

**Gel filtration chromatography**

The column used in this technique has a dimension of 2*60 cm and filled with a gel (Sephadex G-50). The protein solution (prepared previously) was applied to this column, and the fractions were collected at a flow rate 58 ml/h. Protein and orexin- A concentration were estimated at each step of isolation.

**Lyophilization technique**

Peaks A and B obtained from the column was dried using freeze-drying, which was performed in the department of pathological analysis, technical institute, Northern University in Mosul.

**Orexin A assay**

Orexin-A level determined by a competitive-enzyme linked immunosorbent assay (ELISA) technique (12) using Elabscience biotechnology, Inc. kit (USA). This assay performed in an immunity laboratory in Al-Salam hospital in Mosul city.

**Determination of protein concentration:**

Protein concentration used standard bovine serum albumin by modified Lowry method (13).

**The animals**

Albino rats were obtained from the Animal House, College of Veterinary Medicine, University of Mosul. Twenty fourth healthy male rats with age 10±1 weeks and weight 200-250 g were divided into four groups (6 each), housed in cages under standard environmental conditions with providing water and pelleted food ad libitum.

**Induction of hyperlipidemia: High fat diet**

The high-fat diet was prepared by adding five grams of cholesterol, 1 g of Cholic acid, 10 ml of coconut oil, 1 kg of standard diet (14). Rats fed on the high-fat diet for 14 weeks and lipid profile was determined every week.

**Experimental design**

The dose used for intraperitoneally injection of isolated orexin-A hormone was 1μmol/kg bw/d (15). The rats were divided randomly into four groups, each contained six rats and all groups were treated for one month. Control group (CG), the first group was normal rats fed on a standard diet and injected intraperitoneally with physiological saline solution. Normal group with Orexin- A (NGO), the second group was normal rats fed on a standard diet and injected intraperitoneally with isolated Orexin-A (peak B). Hyperlipidemic group (HG), the third group was fed on a high-fat diet and after induction of hyperlipidemia; it was injected intraperitoneally with physiological saline solution and served as control hyperlipidemic group. Hyperlipidemic group with orexin A (HGO), the fourth group was fed on a high-fat diet and after induction of hyperlipidemia; it was injected with isolated Orexin-A (peak B).

**Collection of blood**

The Blood samples were collected from the four groups after fasting for 16 h using a capillary tube without anticoagulant via the orbital sinus puncture technique. Serum separated and used to estimate the following biochemical analysis (16).

**Biochemical analysis**

Collected sera were being aliquot to several aliquots for biochemical analysis, which includes the following tests; Fasting blood glucose was determined immediately using the Spinreact kit (Spain) by spectrophotometer at 500 nm (17). Insulin concentration was measured by Monobind ELISA kit USA (18). Total cholesterol was determined by enzymatic colorimetric method using the BIOLABO kit (France) by spectrophotometer at 500 nm (19). Triglycerides TG were determined by enzymatic colorimetric method using the BIOLABO kit (France) by spectrophotometer at 500 nm (20). Very low-density lipoprotein-cholesterol (VLDL-C) was calculated using the equation: VLDL-C [(mmol/L) = TG (mmol/L)/2.2] (21). High-density lipoprotein-cholesterol (HDL-C) was estimated by precipitation method using the BIOLABO kit (France) by spectrophotometer at 500 nm (22). Low-density lipoprotein-cholesterol (LDL-C) was calculated using Friedewald equation [LDL-C (mmol/L) =Total cholesterol-HDL-C-TG/2.2] (17). Malondialdehyde (MDA) determined...
using Thiobarbituric acid test (23), MDA react with Thiobarbituric acid to produce colored compound measured at 532 nm. Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) activity were determined by the Reflotron Plus system using reflation strips (24,25). Uric acid was determined by enzymatic colorimetric method using the Spinreact kit (Spain) by spectrophotometer at 520 nm (26).

Statistical analysis
Data were analyzed by SPSS software and expressed as mean ± SE. To compare between more than two treatments, one-way ANOVA and Duncan-test were used, and the results considered to be significant at P≤0.05 (27).

Results
Isolation and purification of orexin A from human plasma
The protein precipitate solution obtained from plasma by cold acetone contains a level of orexin-A 402.7 pg/ml compared to plasma 313.9 pg/ml, while there wasn't any level of orexin-A in the filtrate thus, it was neglected.

The gel filtration chromatography technique
Gel filtration chromatography was used to separate the protein precipitate solution obtained by precipitation method using cold acetone from human plasma. As shown in, the elution of proteinous precipitate solution shows two peaks A and B. the elution volume of peaks A and B were 71.1, 155.1 ml respectively. Only the obtained peak B has a high level of orexin-A. The results in a table 1 showed purification steps of orexin-A hormone. The level of orexin-A was increased from 313.9 pg/ml in plasma to 402.7 pg/ml in protein precipitate solution to 614.3 pg/ml in peak B, while the protein concentration was decreased (Figure 1) (Table 1).

Effect of isolated orexin A on some clinical parameters in normal and hyperlipidemic male rats
The intraperitoneal injection of 1μmol/kg b.w. /day of isolated orexin-A in normal and hyperlipidemic rats showed the following results:

The effect of orexin A on lipid profile and malondialdehyde
The results in tables 2 and 3 showed that there was a significant decrease in TC, TG, VLDL-C and LDL-C concentration in NGO compared to CG after 4 weeks and in HGO compared to HG after 2 and 4 weeks of treatment.

On the other hand, the results in a table 4 showed a significant increase in HDL-C concentration in NGO compared to CG after 2 and 4 weeks and in HGO compared with HG after 4 weeks of treatment. Also the results showed a significant decrease in malondialdehyde concentration in NGO compared to CG after 4 weeks and in HGO compared to HG after 2 and 4 weeks of treatment (Table 4).

Table 1: Partial purification of orexin A in human plasma

<table>
<thead>
<tr>
<th>Purification Steps</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total con. of OXA (pg)</th>
<th>Total specific Con. of OXA (pg/mg)</th>
<th>Recovery %</th>
<th>Times of purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>50</td>
<td>410</td>
<td>15695</td>
<td>38.2</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Proteinous precipitate solution</td>
<td>35</td>
<td>214</td>
<td>14096</td>
<td>65.8</td>
<td>89.8</td>
<td>1.7</td>
</tr>
<tr>
<td>Gel filtration /Sephadex G-50 (peak B) after Lyophilizer</td>
<td>20</td>
<td>9.3</td>
<td>12286</td>
<td>1321.1</td>
<td>78.2</td>
<td>34.5</td>
</tr>
</tbody>
</table>

Table 2: Effect of isolated orexin-A on TC and TG concentration after 2 and 4 weeks of treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>TC conc. (mmol/L) Mean ± S.E</th>
<th>TG conc. (mmol/L) Mean ± S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-treatment zero time</td>
<td>Post-treatment</td>
</tr>
<tr>
<td></td>
<td>Week 2</td>
<td>Week 4</td>
</tr>
<tr>
<td>CG</td>
<td>4.0±0.26</td>
<td>3.92±0.21</td>
</tr>
<tr>
<td>NGO</td>
<td>3.98±0.1</td>
<td>3.33±0.26</td>
</tr>
<tr>
<td>HG</td>
<td>5.01±0.29 b</td>
<td>*6.0±0.18 b</td>
</tr>
<tr>
<td>HGO</td>
<td>5.14±0.26 b</td>
<td>*3.96±0.26 b</td>
</tr>
</tbody>
</table>

* indicate significant differences at (p≤0.05) between time zero and treatment groups. Small different letters indicate significant differences between control and treatment groups at (P≤0.05), SE: stander error.
The effect of Orexin-A on Blood glucose (BG) and insulin concentration

The results in a table 5 showed a significant decrease in glucose concentration in NGO compared with CG and in HGO compare with HG after 2 and 4 weeks of treatment. While, there was a non-significant increase in insulin concentration in NGO, and HG has hyperinsulinemia. Also, there was a significant decrease in insulin at HGO compared to HG after 2 and 4 weeks of treatment.

The effect of Orexin-A on AST and ALT activity and uric acid concentration

As shown in a tables 6 and 7 there was a non-significant difference in AST, ALT activity, and uric acid concentration at NGO and HGO after treatment with Orexin-A.

Figure 1: Elution of protein precipitate solution obtained from plasma by cold acetone on Sephadex G-50. The dimension of the column is 2×60 cm at the flow rate is 58 ml/h.

Table 3: Effect of isolated orexin-A on VLDL-C and LDL-C concentration after 2 and 4 weeks of treatment

* indicate significant differences at (p≤0.05) between time zero and treatment groups.
Small different letters indicate significant differences between control and treatment groups at (P≤0.05).

<table>
<thead>
<tr>
<th>Groups</th>
<th>VLDL-C conc. (mmol/L) Mean ± S.E</th>
<th>LDL-C conc. (mmol/L) Mean ± S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-treatment zero time</td>
<td>Post-treatment week 2</td>
</tr>
<tr>
<td>CG</td>
<td>0.86±0.02</td>
<td>0.8±0.08</td>
</tr>
<tr>
<td>NGO</td>
<td>0.88±0.09</td>
<td>*0.63±0.04</td>
</tr>
<tr>
<td>HG</td>
<td>1.11±0.01 b</td>
<td>*1.28±0.02 c</td>
</tr>
<tr>
<td>HGO</td>
<td>1.21±0.03 b</td>
<td>*1.01±0.02 b</td>
</tr>
</tbody>
</table>

Table 4: Effect of isolated orexin-A on HDL-C and MDA concentrations after 2 and 4 weeks of treatment

* indicate significant differences at (p≤0.05) between time zero and treatment groups.
Small different letters indicate significant differences between control and treatment groups at (P≤0.05).

<table>
<thead>
<tr>
<th>Groups</th>
<th>HDL-C conc. (mmol/L) Mean ± S.E</th>
<th>MDA (μmol/L) conc. Mean ± S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-treatment zero time</td>
<td>Post-treatment week 2</td>
</tr>
<tr>
<td>CG</td>
<td>1.29±0.11</td>
<td>1.21±0.09</td>
</tr>
<tr>
<td>NGO</td>
<td>1.22±0.01</td>
<td>*1.54±0.02 c</td>
</tr>
<tr>
<td>HG</td>
<td>0.92±0.01 b</td>
<td>*0.8±0.01 b</td>
</tr>
<tr>
<td>HGO</td>
<td>0.9±0.01 b</td>
<td>*0.98±0.02 b</td>
</tr>
</tbody>
</table>

Table 5: Effect of isolated orexin-A on blood glucose and insulin concentration after 2 and 4 weeks of treatment

* indicate significant differences at (p≤0.05) between time zero and treatment groups.
Small different letters indicate significant differences between control and treatment groups at (P≤0.05).

<table>
<thead>
<tr>
<th>Groups</th>
<th>BG conc. (mmol/L) Mean ± S.E</th>
<th>Insulin con. (μlU/ml) Mean ± S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-treatment zero time</td>
<td>Post-treatment week 2</td>
</tr>
<tr>
<td>CG</td>
<td>5.83±0.11</td>
<td>5.91±0.11</td>
</tr>
<tr>
<td>NGO</td>
<td>5.62±0.14</td>
<td>*4.98±0.20 b</td>
</tr>
<tr>
<td>HG</td>
<td>7.47±0.15 b</td>
<td>*8.06±0.13 c</td>
</tr>
<tr>
<td>HGO</td>
<td>7.75±0.09 b</td>
<td>*6.12±0.05 b</td>
</tr>
</tbody>
</table>

* indicate significant differences at (p≤0.05) between time zero and treatment groups.
Small different letters indicate significant differences between control and treatment groups at (P≤0.05).
The treatment of rat groups with isolated Orexin-A for one month caused a significant decrease in TC, TG, VLDL-C and LDL-C concentration, these results are agreement with (28). This might be due to the role of OXA in activation of AMP-activity protein kinase (AMPK) by increasing the concentration of intracellular calcium (Ca^{2+}) (1,29). AMPK inhibits HMG-CoA reductase and acetyl-CoA carboxylase which involved in the synthesis of cholesterol and de novo pathway of fatty acid and TG synthesis respectively (30), or because OXA decreases hormone-sensitive lipase (HSL) and inhibits lipolysis in adipose tissue (31), also OXA increases lipogenesis by decreasing the release of glyceral from adipose tissue and increasing glucose uptake (32). Also, it can be due to that Orexin-A promotes adiponectin secretion which increases LPL activity and VLDL-C receptors and decreases Apo CIII, so increases catabolism of VLDL-C and reduces serum TG (33).

On the other hand, the rats treated with isolated Orexin-A showed an increase in concentration of HDL-C and this might be due to that Orexin-A increases LPL activity which in turn increases HDL-C (32), or due to that Orexin-A promotes expression and secretion of adiponectin which correlates positively with HDL-C (34). In addition, there was a significant decrease in malondialdehyde concentration and this agrees with Butterick et al. (35) that Orexin-A decreases lipid peroxidation by its neuroprotective mechanisms due to the induction of the transcription factor hypoxia inducible factor-1α (HIF-1α) in the hypothalamus which decreased lipid peroxidation (36).

Glucose concentration decreased in treated rats and that constant with Skrzypski et al. (34), Orexin-A promotes glucose uptake and synthesis of glycogen in skeletal muscle. Orexin-A suppresses the secretion of glucagon thus, Orexin-A regulates the concentration of glucose and dysfunction in Orexin-A is an effective factor in homeostasis of glucose and induce of diabetes (8), while insulin increased slightly in NGO when treated with isolated Orexin-A (34). Feeding with the high-fat diet for long-time might cause hyperinsulinemia in HG, which constant with Barclay et al. (37). High-fat diet can cause reduction in the expression of Orexin-A in the hypothalamus, which exacerbates insulin resistance (3). Furthermore, HGO showed a significant decrease in insulin compared to HG after treatment and that is in agreement with Kaczmarek et al. (38), Orexin-A decreases hyperinsulinemia, enhancing insulin sensitivity and attenuates apoptosis of pancreatic β-cells by decrease the activity and production of caspase 3, which induces β-cells death (38).

While there were non-significant differences in AST, ALT activity, and uric acid concentration at NGO and HGO after treatment with Orexin-A, and this might be due to the time of treatment that might be not enough or the dose was not sufficient.

**Discussion**

The treatment of rat groups with isolated Orexin-A for one month caused a significant decrease in TC, TG, VLDL-C and LDL-C concentration in the hypothalamus which decreased lipid peroxidation (36). Glucose concentration decreased in treated rats and that constant with Skrzypski et al. (34), Orexin-A promotes glucose uptake and synthesis of glycogen in skeletal muscle. Orexin-A suppresses the secretion of glucagon thus, Orexin-A regulates the concentration of glucose and dysfunction in Orexin-A is an effective factor in homeostasis of glucose and induce of diabetes (8), while insulin increased slightly in NGO when treated with isolated Orexin-A (34). Feeding with the high-fat diet for long-time might cause hyperinsulinemia in HG, which constant with Barclay et al. (37). High-fat diet can cause reduction in the expression of Orexin-A in the hypothalamus, which exacerbates insulin resistance (3). Furthermore, HGO showed a significant decrease in insulin compared to HG after treatment and that is in agreement with Kaczmarek et al. (38), Orexin-A decreases hyperinsulinemia, enhancing insulin sensitivity and attenuates apoptosis of pancreatic β-cells by decrease the activity and production of caspase 3, which induces β-cells death (38).

While there were non-significant differences in AST, ALT activity, and uric acid concentration at NGO and HGO after treatment with Orexin-A, and this might be due to the time of treatment that might be not enough or the dose was not sufficient.

**Conclusion**

It was concluded that Orexin-A (isolated from human plasma) had an important role in the control of glucose and lipids metabolism, treatment of hyperinsulinemia and increasing insulin sensitivity in normal and hyperlipidemic rats. Orexin-A had a critical role in decreasing lipid peroxidation.

**Acknowledgment**

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**Conflict of interests**

The authors declare no conflict of interest.


تأثير أوركسين - أ المعزول من البلازما على تنظيم النواتج الأيضية في ذكور الجرذان

رنا فاضل جاسم 1 و ذكري علي عوش 2

قسم الكيمياء، كلية التربية للبنات، جامعة الموصل، الموصل، العراق
قسم الكيمياء، كلية العلوم، جامعة الموصل، العراق

الخلاصة

تضمن البحث محاولة عزل وتنقية الأوركسين - أ من البلازما باستخدام عدة تقنيات حياتية. كما تم دراسة تأثير الأوركسين - أ المعزول على بعض المتغيرات السريرية لدى ذكور الجرذان السليمة والمصابة بارتفاع الدهون. حقق الأوركسين - أ المعزول مستوى عالياً من الدهون في الدم وبيروكسيدات الدهون. وقد لوحظ مستوى عالياً للوركسين - أ فقط في الحزمة المفصولة بواسطة تقنية كروماتوغرافيا الترشيح الهلامي (باستخدام سيفاديكس ج-50) مع عدد مرات تنقية يصل إلى 34.5 مرة. كما تم دراسة تأثير الأوركسين - أ المعزول على بعض المتغيرات السريرية لدى ذكور الجرذان السليمة والمصابة بارتفاع الدهون. حقيقة أن الأوركسين - أ المعزول بطريقة واحدة مالكرو مول/كغم من وزن الجسم يوم داخل الغشاء البريتوني لمدة شهر واحد. أظهرت النتائج المستحيلة قبل العلاج وبعد أسبوعين وأربع أسابيع من العلاج، وجد انخفاض معنوي في تركيز الكولستيرول الكلي والكليبريدات الثلاثية والبروتين الدهني للكولسترول وألي الكثافة وواط الكثافة جداً والكلوكوز والمالوناديد إهيدروكسيك والكولسترول. بينما كان هناك ارتفاع معنوي في تركيز البروتين الدهني للكولسترول أعلى الكثافة لدى الجرذان السليمة والمصابة بارتفاع الدهون. تستنتاج الدراسة الدور الهام للأوركسين في تنظيم البروتين الدهني للكولسترول ومقاومة انزيمات الأوزون ومقاومة الدهون وحمض بروكسيد الدهون لدى الجرذان السليمة والمصابة بارتفاع الدهون.

وقد لوحظ مستوى عالي للأوركسين - أ فط في الحزمة بمفصلة بواسطة تقنية كروماتوغرافيا الترشيح الهلامي (باستخدام سيفاديكس ج-50) مع عدد مرات تنقية تصل إلى 34.5 مرة. كما تم دراسة تأثير الأوركسين - أ المعزول على بعض المتغيرات السريرية لدى ذكور الجرذان السليمة والمصابة بارتفاع الدهون. حقق الأوركسين - أ المعزول مستوى عالياً من الدهون في الدم وبيروكسيدات الدهون. وقد لوحظ مستوى عالياً للوركسين - أ فقط في الحزمة المفصولة بواسطة تقنية كروماتوغرافيا الترشح الهلامي (باستخدام سيفاديكس ج-50) مع عدد مرات تنقية يصل إلى 34.5 مرة. كما تم دراسة تأثير الأوركسين - أ المعزول على بعض المتغيرات السريرية لدى ذكور الجرذان السليمة والمصابة بارتفاع الدهون. حقيقة أن الأوركسين - أ المعزول بطريقة واحدة مالكرو مول/كغم من وزن الجسم يوم داخل الغشاء البريتوني لمدة شهر واحد. أظهرت النتائج المستحيلة قبل العلاج وبعد أسبوعين وأربع أسابيع من العلاج، وجد انخفاض معنوي في تركيز الكولستيرول الكلي والكليبريدات الثلاثية والبروتين الدهني للكولسترول وألي الكثافة وواط الكثافة جداً والكلوكوز والمالوناديد إهيدروكسيك والكولسترول. بينما كان هناك ارتفاع معنوي في تركيز البروتين الدهني للكولسترول أعلى الكثافة لدى الجرذان السليمة والمصابة بارتفاع الدهون. تستنتج الدراسة الدور الهام للأوركسين في تنظيم البروتين الدهني للكولسترول ومقاومة انزيمات الأوزون ومقاومة الدهون وحمض بروكسيد الدهون لدى الجرذان السليمة والمصابة بارتفاع الدهون.