

Most Appropriate Conditions of the Binding of LH in Benign and Malignant Uterine Tumors Homogenate with ¹²⁵I-Anti LH Antibody.

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Date of acceptance 19/12/2006

Summary

A radiobinding assay was developed for the determination of LH in two groups of uterine tumors patients. The optimum conditions of the binding of LH in benign and malignant uterine tumor with ¹²⁵I-anti LH antibody were as follows: protein concentration of benign and malignant uterine tumor (50 µg protein). (¹²⁵I-anti LH antibody) concentration (74.93 , 93.67 µg/ml). Temperature (25 °c). Time (120 and 60 min) and pH (7.6 and 7.4) for benign and malignant postmenopausal uterine tumor respectively.

The use of 0.025 of divalent salts were shown to cause different effects on the binding in the two groups.

Introduction

The uterus is a hollow, muscular organ lying in the true pelvis between the bladder and the rectum⁽¹⁾ it can be divided into three parts; the portion above the insertion of the fallopian tubes, called the fundus; the lower constricted part, called the cervix, and the portion between the fundus and the cervix, called the body of uterus.

The uterus is supported on the both sides by four sets of ligaments⁽²⁾. The uterus wall is composed of three discrete layers: perimetrium, myometrium and endometrium⁽³⁾. The function of uterus is to house and nurture the developing fetus until the appropriate time for evacuation⁽⁴⁾. The uterus, stimulated continually by hormones, denuded monthly of its endometrial mucosa and inhibited periodically by fetuses, is subject to variety of disorders⁽⁵⁾.

The diseases of the uterus, which is non-tumorous, include endometritis, cervicitis, myometritis, parametritis, genital tuberculosis.

Benign tumors of the uterus include endometriosis, adenomyosis, endometrial polyps, leiomyomas. In addition, malignant tumors of the uterus include cancers of the cervix and endometrium⁽⁶⁾.

Experimental

Chemicals

All laboratory chemicals and reagents were of analar grade. Tris buffer, CuSO₄.5H₂O, CaCl₂, ZnCl₂, MnCl₂ were obtained from Fluka Company, Switzerland. Hydrochloric acid, were obtained from BDH limited pool, U.K. kit of radio active (¹²⁵I- anti LH antibody) was manufactured by *Immunotech, Abbeckman coulter company*. The radioactivity of (¹²⁵I- anti LH antibody) was approximately 370 KBq.

Instruments

The instruments used in this work were, LKB gamma counter type 1270-Rack gamma II, centrifuge type Hitachi, Pye-Unicam pH meter.

Patients

Two groups of postmenopausal patients were included in this study,

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group I consisted of 10 postmenopausal patients with benign uterine, (Age = 51- 60 years), group II consisted of 10 postmenopausal patients with malignant uterine tumor, (Age = 54- 62 years), with no consider to there weight or hereditary cause or mirage state or they have children or not and the main target to collect samples was those women infected with tumor.

All patients were admitted for treatment to (Medical City, Baghdad Teaching Hospital), Al-Yarmuk Teaching Hospital, Al Habibia Hospital, under the supervision of specialists, Dr. Nada Salih Ameen, Dr. Akram Al-Shareef, Dr. Wafaa Al- Omary.

They were histologically proven from the supervision of specialists Dr. Raja Al-Hadethy, Dr. Eman Alash. The patients were newly diagnosed and not underwent of any type of therapy. Patients did not suffer from any disease that may interfere with our study were excluded.

Collections of Specimens

The tumor tissues were surgically removed from uterine patients by hysterectomy. The specimens were cut off and immediately rinsed with ice-cold isotonic saline solution. They were collected individually in plastic receptacles and stored at -20°C until homogenization.

Preparations and Uterine Tissue Homogenate .

The frozen tissue was thawed, sliced finely with a scalped in petridish standing on ice bath. The slices were further minced with scissors, then homogenized at 4°C in (Triss) buffer solution (0.01M) with ratio of 1:5 (weight:volume), using manual homogenizer. The homogenate was filtered through several layers of nylon gauze, and then centrifuged at 1500xg for 15 min in a cooling centrifuge at 4

$^{\circ}\text{C}$. The supernatants were use through out our study.

Solutions

The Triss buffer solution (0.01M, pH = 7.6) was prepared by dissolving (0.303 gm) of Triss buffer in 200ml distilled water. The required pH was adjusted by adding HCl solution (0.2M), then the volume was completed to 250ml by distilled water, and it was the best binding through primary experiment.

Methods

The Effect of Different Protein Concentration of Benign and Malignant Uterine Homogenate tumors on the Binding of LH with ^{125}I –Anti LH Antibody.

- 1- Twenty-Five microliters of increasing amounts (25,50, 100,150, and 200 μg protein) of postmenopausal benign tumor homogenate was added to 25 μl (93.67 μg protein) of ^{125}I –anti LH antibody in duplicate tubes. The volume was completed to 400 μl with triss buffer pH (7.6), the assay tubes were incubated at 25°C for 90 minute.
- 2- Two additional tubes containing 25 μl of ^{125}I – anti LH antibody only (for total activity computation) were set a side until counting.
- 3- After incubation, the tubes were centrifuged for 15min in order to separate the complex formed.
- 4- Supernatant was discarded by decanting the assay tubes. Then, the tubes were inverted on a filter paper for 10min.
- 5- Rims of the tubes were swabbed with cotton piece.
- 6- Amount of bound radioactivity (C.P.M) was counted in a gamma counter for one min.
- 7- Steps above were repeated for other patient groups postmenopausal malignant tumor homogenate.

Calculations

Counted radioactivity in each tube (expressed in C.P.M) represents the

bound fraction (B), (^{125}I - anti LH antibody/LH) complex.

Counted radioactivity in the tubes containing ^{125}I - anti LH antibody only represents the total activity (T).

The B/T ratio for each tube was counted as follows :

$$(B/T) \% = \frac{\text{Sample mean counts (B)}}{\text{Total activity mean count (T)}} * 100$$

Percent of binding values B/T % were plotted against the increasing amounts of protein of the benign and malignant uterine tissue homogenate.

The Effect of Different

Concentrations of ^{125}I -Anti LH Antibody on the Binding with LH in Benign and Malignant Uterine tumors Homogenate

- 1- Increasing volumes (10,15,20,25 and 50 μl) of ^{125}I anti LH anti LH antibody containing (37.47, 56.19, 74.93, 93.67, 187.33 μg protein) respectively were each added to 25 μl (50 μg protein) of postmenopausal benign tumor homogenate in duplicate tubes. The volume was completed to 400 μl with triss buffer pH (7.6). The assay tubes were incubated at 25 $^{\circ}\text{C}$ for 90 minute.
- 2- A set of tubes containing the same increasing volumes of ^{125}I -anti LH antibody (10, 15, 20, 25 and 50 μl) only, for total activity computation, were set a side until counting.
- 3- After incubation, the tubes were centrifuged for 15min in order to separate the complex formed.
- 4- Supernatant was discarded by decanting the assay tubes. Then, the tubes were inverted on a filter paper for 10 min.
- 5- Rims of the tubes were swabbed with cotton piece.
- 6- Amount of bound radioactivity (C.P.M) was counted in a gamma counter for one min.
- 7- Steps above were repeated for other patient groups postmenopausal malignant tumor homogenate.

Calculation

- 1- B/T percent values were determined.
- 2- Percent of binding values B/T % was plotted against the increasing volume of ^{125}I -anti LH antibody.

The Effect of Different pH on The Binding of LH in Benign and Malignant Uterine tumors Homogenate with ^{125}I -Anti LH Antibody

- 1- Twenty-five microliters (50 μg protein) of postmenopausal benign tumor homogenate was added to 25 μl (93.67 μg protein) of ^{125}I -anti LH antibody in duplicate tubes and the volume was completed to 400 μl with triss buffer of different pH (7, 7.4, 7.6, 8, 8.2, 8.6 and 9), the assay tubes were incubated at 25 $^{\circ}\text{C}$ for 90 minute.
- 2- After incubation, tubes were centrifuged for 15min in order to separate the complex formed.
- 3- Supernatant was discarded by decanting the assay tubes. Then, the tubes were inverted on a filter paper for 10min.
- 4- Rims of the tubes were swabbed with cotton piece.
- 5- Amount of bound radioactivity (C.P.M) was counted in a gamma counter for one min.
- 6- Steps above were repeated for other patient groups postmenopausal malignant tumor homogenate (50 μg protein) with 20 μl (74.93 μg protein) of ^{125}I - anti LH antibody.

Calculations:

- 1- B/T percent values were determined.
- 2- Percent of binding values B/T % was plotted against their corresponding pH value.

Effect of Temperature on the Binding of LH in Benign and Malignant Uterine Tumors Homogenate with ^{125}I -Anti LH antibody

- 1- Twenty-five micro liters (50 μg protein) of postmenopausal benign tumor homogenate was added to 25 μl

(93.67 μ g protein) of 125 I –anti LH antibody in duplicate tubes, and the volume was completed to 400 μ l with triss buffer pH (7.6). The assay tubes were incubated at 10 $^{\circ}$ C for 90 minutes

2- After incubation, the tubes were centrifuged for 15min in order to separate the complex formed.

3- Supernatant was discarded by decanting the assay tubes. Then, the tubes were inverted on a filter paper for 10min.

4- Rims of the tubes were swabbed with cotton piece.

5- Amount of bound radioactivity (C.P.M) was counted in a gamma counter for one min.

6- Experiment was repeated at different temperatures (25, 37 and 45 $^{\circ}$ C).

7- Steps above were repeated for other patient groups postmenopausal malignant tumor homogenate (50 μ g protein) with 20 μ l (74.93 μ g protein) of 125 I – anti LH antibody and triss buffer PH (7.4).

Calculations

1- B/T % values were determined.

2- Percent of binding values were plotted against the different temperature of incubation.

The Choice of The Most Appropriate Incubation Time for the Binding of LH in Benign and Malignant Uterine Tumors Homogenate with 125 I-Anti LH Antibody

1- Twenty-five micro liters (50 μ g protein) of postmenopausal benign tumor homogenate was added to 25 μ l (93.76 μ g protein) of 125 I-anti LH antibody in duplicate tubes and the volume was completed to 400 μ l with triss buffer pH (7.6).

2- The assay mixtures were incubated at 25 $^{\circ}$ C for different time intervals (30, 60, 90, 120, 150 and 180 min).

3- Supernatant was discarded by decanting the assay tubes. Then, the tubes were inverted on a filter paper for 10min.

4- Rims of the tubes were swabbed with cotton piece.

5- Amount of bound radioactivity (C.P.M) was counted in a gamma counter for one min.

6- Steps above were repeated for other patient groups postmenopausal malignant tumor homogenate (50 μ g protein) with 20 μ l (74.93 μ g protein) of 125 I – anti LH antibody and triss buffer PH (7.4), temperature 25 $^{\circ}$ C for different time (30, 60, 90, 120, 150 and 180).

Calculations

1- B/T % values were determined.

2- Percent of binding values were plotted against the different times of incubation.

The Effect of Divalent Cations on the Binding of LH in Benign and Malignant Uterine Tumors Homogenate with 125 I-Anti LH Antibody.

The experiment in page (7) was repeated but instead of completing the volumes to 400 μ l with triss buffer, the volumes were completed to 400 μ l with triss buffer containing 25mM of each of the following salts: CaCl₂, CuSO₄.5H₂O, MgCl₂.6H₂O, MnCl₂.4H₂O, pH 7.6 for postmenopausal benign uterine tumor, pH 7.4 for postmenopausal malignant uterine tumor homogenate.

Solutions

The stock solutions (25mM) of divalent cations were prepared by dissolving each of the following amounts of salts in 25ml of triss buffer pH 7.6 for postmenopausal benign homogenate, PH 7.4 for postmenopausal malignant uterine tumor homogenate: (0.0693gm) CaCl₂, (0.1561gm) CuSO₄.5H₂O, (0.1271gm) MgCl₂.6H₂O, (0.1237gm) MnCl₂.4H₂O

Calculations

1- B/T % values were determined.

2- Percent of binding values were plotted against the salt concentration.

binding of ^{125}I -anti LH antibody with LH in benign and malignant uterine tissue homogenate is a saturable process but complete saturation however is theoretically never reached unless the amount of LH used reached infinity⁽⁸⁾. According to the results of this experiment (74.93 and 93.67 $\mu\text{g}/\text{ml}$ protein) of ^{125}I -anti LH antibody were used in the binding studies in the subsequent experiments.

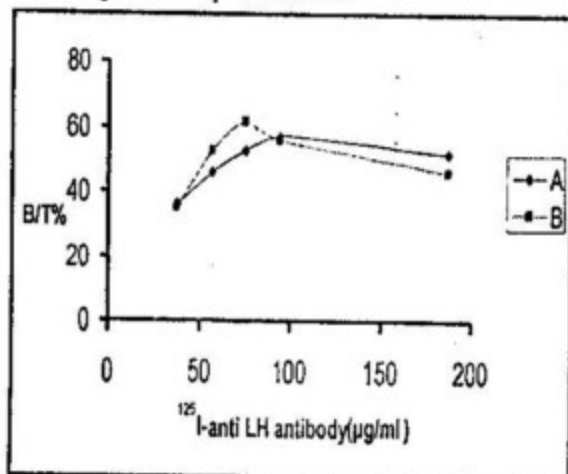


Figure (2): Effect of different concentrations of ^{125}I -anti LH antibody on the binding with LH in:

A: Postmenopausal patients with benign uterine tumor

B: Postmenopausal patients with malignant uterine tumor

The Effect of Different pH on the Binding of LH in Benign and Malignant Tumors Homogenate with ^{125}I -Anti LH Antibody

The analysis of the influence of pH on binding of LH in benign and malignant uterine homogenate to ^{125}I -anti LH antibody is stated in Figure (3). The optimum pH was found to be (7.6) for benign postmenopausal uterine tumor, while (pH 7.4) is the optimum pH for the binding of ^{125}I -anti LH antibody to the malignant postmenopausal uterine.

The same Figure (3) shows a decreasing in the binding percent (B/T %) at the pH higher or lower than the

optimum pH. These results indicate that the shift in the pH of the environment may affect the properties of the macromolecules involved in the binding. This effect includes the induction of protonation - deprotonation process occurring within the ionizable groups of the amino acids present in the binding domain of these macromolecules⁽⁹⁾.

According to the results obtained in this experiment, the pH of incubation buffer in all subsequent experiments was adjusted at (7.6 and 7.4) as optimum pH for the two different groups used in this study.

Extensive studies indicated that there were different effects of pH on the binding of ^{125}I - LH to its receptors. *Hannu et. al.* reported that the highest binding of radiolabeled hLH to luteal homogenate was seen at pH 7.0 - 7.5⁽¹⁰⁾.

The difference in the pH range suggests that difference in target tissues may possess different binding domains from the labeled hormone and may be due to the difference in the binding conditions.

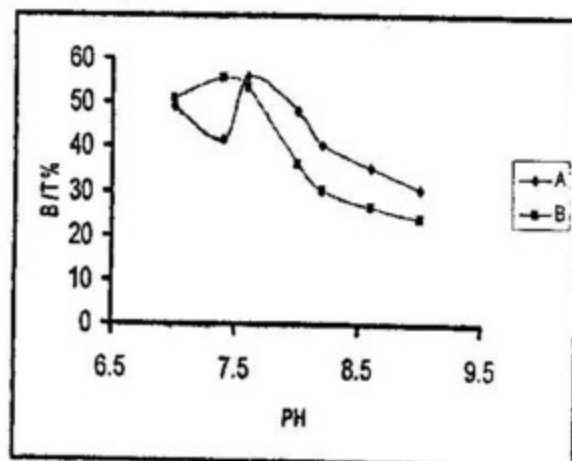


Figure (3): Effect of pH on the binding of ^{125}I -anti LH antibody with LH in:

A: Postmenopausal patients with benign uterine

B: Postmenopausal patients with malignant uterine.

Effect of Temperature on the Binding of LH in Benign and Malignant Uterine Tumor Homogenate with ^{125}I -Anti LH Antibody

The temperature dependency of the binding of ^{125}I -anti LH antibody to benign and malignant uterine tissue was investigated. Figure (4) reveals that the binding of ^{125}I -anti LH antibody to LH in benign and malignant uterine tissue was maximum at 25°C for postmenopausal patients with benign and malignant uterine tumor.

It seemed that binding percent (B/T %) was increasing when the temperature was raised to a point of maximum binding and the binding was decreased as temperature increase after maximal value of binding. The loss of binding activity may be due to degradation of the LH⁽¹¹⁾, or may be due to the irreversible dissociation of the (^{125}I -anti LH antibody/LH) complex⁽¹²⁾.

According to these results, (25°C) were used in all the subsequent experiments for benign and malignant uterine homogenate.

Our results are consistent with those obtained by others who reported that the binding was temperature dependent and that the optimum temperature for the binding of ^{125}I -anti LH antibody to LH in benign and malignant uterine tissue is 25°C ⁽¹³⁾. The difference in the appropriate incubation temperature for the binding may be due to the histologic tumor type or may be due to the fact that the fluidity and conformation of cell membranes under these different temperature will not be the same, or to the different binding conditions⁽¹²⁾.

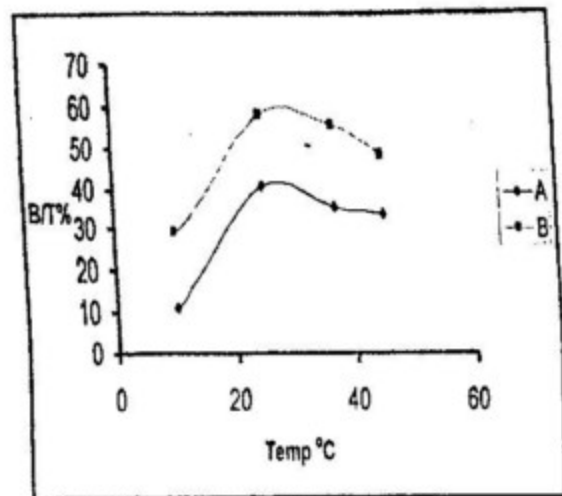


Figure (4): Effect of the temperature on the binding of ^{125}I -anti LH antibody with LH in:

A: Postmenopausal patients with benign uterine

B: Postmenopausal patients with malignant uterine

The Choice of the Most Appropriate Incubation Time for the Binding of LH in Benign and Malignant Uterine Tumor Homogenate with ^{125}I -Anti LH Antibody.

To choose the most appropriate incubation time at (25°C) the experiment was carried out at different time intervals (30-180min).

Figure (5) shows that the optimum binding of ^{125}I -anti LH antibody to LH in postmenopausal patients with benign uterine homogenate was occurred in 120min, while the optimum binding in postmenopausal patients with malignant uterine was occurred in 60min.

The degrees of binding activity may be due to either the degradation of LH or the irreversible dissociation of the (^{125}I -anti LH antibody/LH) complex⁽¹¹⁾. In view of these results, the incubation time (120min and 60min) were used in all subsequent experiments for benign and malignant tumor. Interact hormone with receptors must be a very rapid process in vivo, while in vitro hormones react very slowly. The reason for this slow

association rate is not clear, but it may depend on an alteration in the conformation of the membrane during isolation⁽¹²⁾.

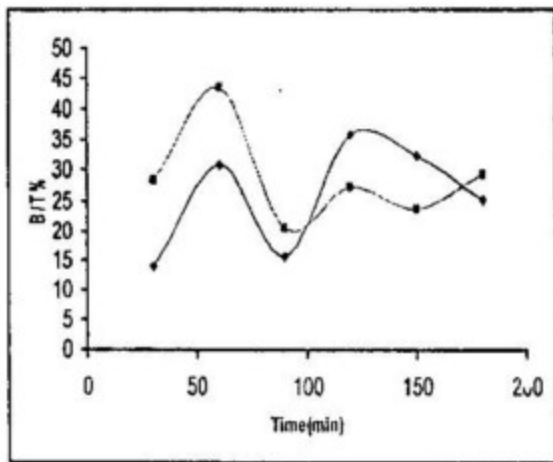


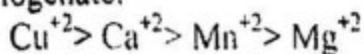
Figure (5): Time-course of ¹²⁵I-anti LH antibody binding with LH in:
A: Postmenopausal patients with benign uterine
B: Postmenopausal patients with malignant uterine

The Effect of Divalent Cations on the Binding of LH in Benign and Malignant Uterine Tumor Homogenate with ¹²⁵I-Anti LH Antibody.

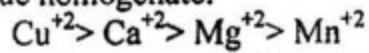
Figure (6 A & B) shows the effect of divalent cations on the binding of ¹²⁵I-anti LH antibody to benign and malignant uterine tissue homogenate. Some of divalent cations appeared to enhance the binding reaction, while others inhibit the reaction. Several cations have been used to study their action on the binding; these cations are Ca⁺², Mg⁺², Mn⁺² and Cu⁺² in a (25mM) concentration.

The presence of divalent cations in the incubation medium tend to stimulate the binding of ¹²⁵I-anti LH antibody to benign and malignant uterine tissue homogenate in the three groups according to the following:

- Postmenopausal benign uterine tissue homogenate.



- Postmenopausal malignant uterine tissue homogenate.



As shown, the maximal binding in postmenopausal benign and malignant tumor was occurred in the presence of copper (II) in the incubation mixture.

One hypothesis assumes that, the effects of those metal ions may alter the nature of the hydrophobic forces necessary for the stabilization of biological membranes and affect the hydrophobic forces controlling the stabilization of the complex formed⁽¹⁴⁾

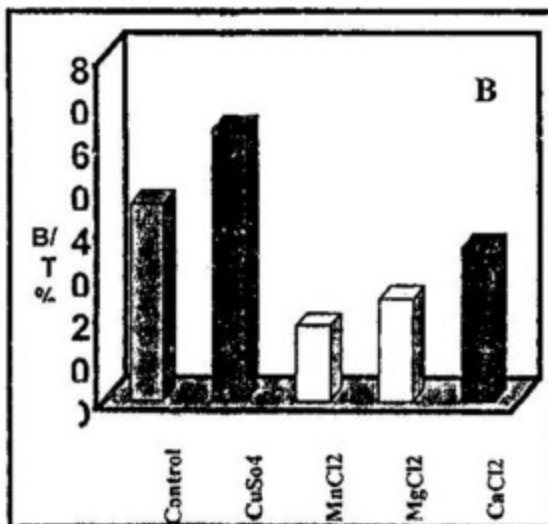
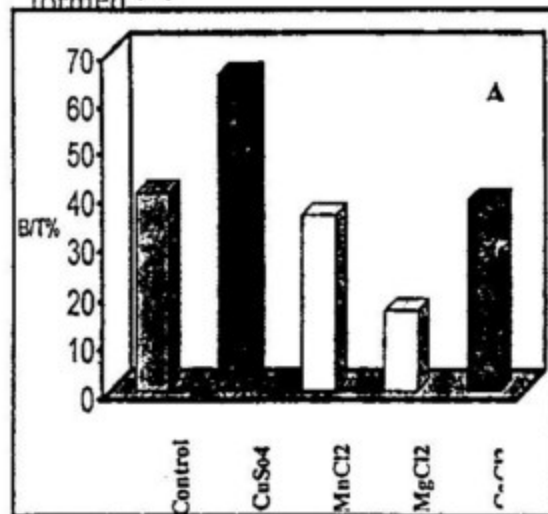


Figure (6): Effect of divalent cations on the binding of ¹²⁵I-anti LH antibody with LH in:

A: Postmenopausal patients with benign uterine.

B: Postmenopausal patients with malignant uterine.

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الظروف المثلى لارتباط الهرمون الليوتيني باورام الحميدة و الخبيثة مع
الجسم المضاد (^{125}I -Anti LH)

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الخلاصة

تم تطوير طريقة الاختبار المناعي الإشعاعي لتعيين LH في مجموعتان من المرضى المصابات بورم الرحم . الظروف العظمى لارتباط LH في الأنسجة الورمية الحميدة و الخبيثة بضده المعلم بنظير اليود المشع ذو العدد الكتلي 125 هي :-

تركيز البروتين في الأنسجة الورمية الحميدة و الخبيثة (50 مايكروغرام بروتين) . تركيز (^{125}I -anti LH antibody) (74.93 ، 93.67 مايكروغرام/ملييلتر) و الحرارة (25 °C) . الزمن (60 ، 120 دقيقة) و pH (7.4 و 7.6) للأورام الحميدة و الخبيثة لنساء بعد سن اليأس. أن استعمال 0.025 مولاري من الأملاح التثائية بينت تأثيرها في الارتباط في المجموعتان .