Radio Receptor Assay Studies of ^{125}I-Testosterone Binding to its Receptors in Benign and Malignant Human Uterine Tumors

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Abstract

A Radio receptor assay procedure was developed for the determination of testosterone receptors in two groups of human uterine tumor patients (benign and malignant). The optimum conditions of the binding of ^{125}I-testosterone to the receptors were as follows: receptor concentration: (250 µg protein), tracer testosterone concentration (0.9 ng per 100µl), time (8 hr), pH (8.0) and temperatures (4 and 25°C) for benign and malignant tumors respectively.

Incubation of unlabeled testosterone and estradiol with uterine tumor tissue homogenate inhibited the ability of ^{125}I-testosterone to bind the uterine tissue homogenate, while progesterone inhibited the binding slightly.

The use of 0.1 M of sodium halides and 0.025 M of divalent salts were shown to cause different effects on the binding in the two groups.

الخلاصة

تم تطوير طريقة لاختبار المستلم الإشعاعي لتقدير مستلمات هرمون التيستوستيرون في مجموعتين من النساء المصابات بمرض ورم الرحم (الحميد والخبيث). ودرست الظروف المثلى لارتباط هرمونّ التيستوستيرون المعلّم بنظير اليود المشع ذو العدد الكتلي 125 مع مستلماته وكالآتي : تركيز المستلمات (250 مايكرو غرام بروتين) ، تركيز الهرمون المعلّم (0.9 نانو غرام لكل 100 مايكرولتر) ، درجة الحرارة (4 درجة مئوية نسبة إلى الورم الحميد و25 بالنسبة إلى الورم الخبيث) ،الزمن (8 ساعة) والأس الهيدروجيني (8.0) في مجانس أورام المبيض الحميدة والخبيثة.

أدى إضافة هرمون الاسترادايول إلى وسط الحضن إلى تثبيط ارتباط هرمون التيستوستيرون المعلّم بنظير اليود المشع ذو العدد الكتلي 125 إلى مستلماته بينما كان تأثير هرمون البروجيستيرون قليلا. كذلك تمت ملاحظة تأثيرات مختلفة على عملية الارتباط عند استخدام 0.01 مولاري من هاليدات الصوديوم و 0.025 مولاري من الأملاح ثنائية التكافؤ

Introduction

Testosterone diffuses freely into cells of many but not all target cells. It rapidly undergoes reduction to 5α-dihydrotestosterone (5α-DHT). However, testosterone and 5α-DHT are capable of binding to a single cytoplasmic receptor but with different affinities (1-4). Within the nucleus, the SR complex binds to nonhistone protein of chromatin. This interaction leads to the formation of new messenger RNA (mRNA). The mRNA then migrates into the cytoplasm, where it attaches to ribosomes. The mRNA is translated to affect the formation of proteins that eventually bring about specific changes in the target cell (5,6).

The characterization of the binding of testosterone with its receptor is one of clinical and histopathologic prognostic factors, which is used in the patients were admitted for treatment to Al-Arabe Hospital and Saddam Medical City

under the supervision of specialists, Dr. Ryad Mohammed Salh, Dr. Raja Al-Tikreti and Dr. Nada Al-Ubadi. They were histologically proven from the supervision of specialists Dr Luay Edward and Dr. Nabel Abdulwadoad. The patients were newly diagnosed and not underwent any type of therapy. Patients that suffer from any disease that may interfere with our study were excluded.

Collection of Uterine Tissue Specimens

The tumor tissues were surgically removed from uterine tumor patients by either hysterectomy or myomectomy. 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.

Preparation of UterineTumor Tissue Homogenate

The frozen tissues were weighed, pulverized finely with a scalpel in petri dish standing on ice bath, and then homogenized at 4°C in buffer solution with a ratio of 1:5 (weight: volume), using a manual homogenizer. The buffer of Tris-EDTA (Tris-HCl 0.01M,pH 7.4, containing 0.15 mM EDTA, 2-mM mercaptoethanol and 10% glycerol) was used.

The homogenate was filtered through several layers of nylon gauze to eliminate fibers of connective tissue, then centrifuged at 2000 x g for 30 minutes at 4°C. The sediment was suspended in 10 volumes of TEMG buffer for 15 minutes at 4° C and then the suspension was used to obtain the crude nuclear fraction.

Solutions

All buffer solution was prepared (9) by dissolving the appropriate amount of salts in distilled water and the required pH was adjusted. The stock solution of 0.2 M Tris (hydroxymethyl aminomethane) was prepared; other reagents were prepared as described previously (10):

- TEMG buffer (pH 7.4): Tris (0.01 M, pH 7.4) buffer containing 0.15 mM Na2EDTA, 2 mM mercaptoethanol and 10% glycerol.
- Dxtran-coated charcoal (DCC) solution: Tris (0.01M) buffer, pH 7.4 containing 1.25- % charcoal, 0.6% dextran-70, and 0.2% gelatin.

Method

The Effect of Different Concentrations of ^{125}I-Testosterone on the Binding in Uterine Tumor Homogenate

Increasing concentrations (3.12—31.2 PM) of ^{125}I-testosterone was individually added to 200 µl (250 µg protein) of nuclear part in the first set of tubes with a final volume of 1 ml (completed with TEMG buffer).

The second set of tubes consists of the same reactants plus 200-fold excess of unlabeled testosterone. After incubation for 16 hrs at 25°C, 250 µl of DCC was added in order to adsorb the free unbound-labeled hormone. The tubes were shaken for 10 min and centrifuged for 10 min at 4°C at 2000xg. One milliliter was then taken from each supernatant and counted in gamma counter. It represents the bound hormone.

Calculations

- The percent of specific binding (SB%) was determined according to the following formula:

$$SB\% = \left[\frac{SB}{TC}\right] \times 100$$

Where:

SB: Specific binding (CPM) and

TC: Total count of ^{125}I-testosterone (CPM).

- The percent of specific binding (SB%) was plotted against the concentration of ^{125}I-testosterone.

The Effect of Testosterone Receptor Concentration on the Binding in Uterine Tumor Homogenate

One hundred micro-liter contains (0.9 ng) of ^{125}I-testosterone was added to 200 µl of increasing amounts (50, 100, 150, 200, 250 µg) of nuclear fraction in a final volume of 1 ml (completed with TEMG buffer) with and without the addition of 200 fold excess of unlabeled testosterone. At the end of incubation of (16hrs) at 25°C, the bound hormone was estimated by adding 250µl of DCC, then the tubes were shaken for 10 min and centrifuged for 10 min at 4°C at 2000xg. One milliliter was taken from each supernatant and counted in gamma counter. It represents the bound hormone.

Calculations

- The percent of specific binding (SB%) was determined according to the following formula:

$$SB\% = \left[\frac{\text{Specific binding}}{\text{Total count of labeled hormone}}\right] \times 100$$

- The percent of specific binding (SB%) was plotted against the amount of protein receptors included in each mixture.

The Effect of Different Temperature on the Binding of Testosterone to Its Receptor in Uterine Tumor Homogenate

One hundred micro-liter contains (0.9 ng) of labeled testosterone was added to 200 µl (250µg protein) of nuclear fraction in a final volume of 1 ml (completed with TEMG buffer) with and without the addition of 200 fold excess of unlabeled testosterone. After incubation for (16hrs) at 25°C, the bound hormone was estimated by adding 250µl of DCC, then the tubes were shaken for 10 min and centrifuged for 10 min at 4°C at 2000xg. 1 ml was taken from each supernatant and counted in gamma counter, the experiment was repeated at different temperatures (0, 4, 37 and 42°C), then the percent of specific binding (SB%) was calculated.

The Choice of Most Appropriate Incubation Time of ^{125}I-Testosterone Binding to its Receptors in Uterine Tumor Homogenate

One hundred micro-liters (31.2 PM) of ^{125}I-testosterone was added to 200 µl (250µg protein) of nuclear fraction of homogenate, with and without the addition of 200 fold excess of unlabeled testosterone. The volume of the mixture was completed with TEMG buffer to 1ml. The tubes were incubated at 25°C for different time intervals (2, 4, 8, 12, 14, 16, and 24 hrs). At the end of incubation, the bound hormone to nuclear receptor and the percent of specific binding (SB%) were calculated, then plotted vs. different times of incubation.

The Effect of Different pH on the Binding of ^{125}I-Testosterone to the Receptor in Uterine Tumor Homogenate.

Crude nuclear fractions (250 µg protein in 200 µl) were added to 100 µl (31.2 PM) of labeled testosterone with and without the addition of 200 fold excess on unlabeled testosterone. The volume of the mixture was made up to 1 ml with TEMG buffer of different pHs (6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, and 8.4). The tubes were incubated at 4°C for benign tumor and 25°C for malignant tumor homogenate for 8 hrs. After incubation, the bound hormone was estimated.

Calculations

The percent of specific binding (SB%) was determined at each pH.

- The percent of specific binding (SB%) was plotted against their corresponding pH.

Stability of ^{125}I -Testosterone-Receptor Complex

This experiment was carried out at the optimum conditions of labeled testosterone concentration (100 µl, 0.9 ng), protein receptor concentration (250µg in 200µl), temperature (4°C for malignant) and (25°C for benign), time of incubation (8hrs), and pH (8.0), in order to investigate the effect of temperature on receptor properties.. The bound testosterone (testosterone-receptor complex) was reincubated at different temperatures (0, 4, 25, 37 and 45°C). Between 0 and 24 hrs the remaining bound testosterone in each tube was measured as described previously.

Calculations

- The relative percent specific binding (RSB%) was estimated according to the following formula:

$$SB\ \% = \frac{(SB)_t}{(SB)_o} \times 100$$

Where:

$(SB)_t$: Specific binding of ^{125}I-testosterone at time (t) of reincubation.

$(SB)_o$: Specific binding of ^{125}I-testosterone at zero time of reincubation.

- The percent of relative specific binding (RSB%) was plotted against the time of reincubation.

Competitive Effect of Different Concentration of Unlabeled Estradiol, Progesterone and Testosterone on the Binding of ^{125}I-Testosterone to its Receptors in Uterine Tumor Homogenate

The experiment was carried out at the optimum conditions of labeled testosterone concentration (100 µl, 0.9 ng), protein receptor concentration (250µg in 200µl), temperature (4°C for malignant tumor) and (25°C for benign one), pH (8.0), and the time of incubation (8hrs). The experiment was performed by adding 100 µl of labeled testosterone to 200 µl (250µg protein) of crude nuclear fraction with or without addition of increasing concentration (5-500 nM) of unlabeled testosterone in a final volume of 1 ml (completed with TEMG buffer). After incubation for 8 hrs, the bound hormone was measured as described in section (2.4.2) [11]. The experiment was repeated with increasing concentration of unlabeled estradiol and progesterone.

Solutions

All solutions were prepared as described in section (2.1.7) and (2.4.2)[11]. Unlabeled steroids were prepared by dissolving them in small amount of absolute ethanol, then the volume completed with distilled water.

Calculations

- The percent of specific binding (SB%) was estimated and plotted against the concentration of competitor

(testosterone, estradiol, and progesterone).

Effect of Different Halides on the Binding ^{125}I-Testosterone to its Receptors

One hundred micro-liters of labeled testosterone (0.9 ng) was added to 200 µl (250µg protein) of nuclear fraction homogenate, with and without the addition of 200 fold excess of unlabeled testosterone in a final volume of 1ml (completed with TEMG buffer containing 0.1 M of each of the following halides: NaF, NaCl, and NaI, pH 8.0). The tubes were incubated for 8 hrs at 4°C for malignant tumor and 25°C for benign one, then the bound hormone was estimated as mentioned in section (2.4.2)(11).

Solutions

Halide solutions prepared in concentration of 0.1M in TEMG buffer pH 8.0, 1.0497 g of NaF in 250 ml of TEMG buffer, 1.463 g of NaCl in 250 ml of TEMG buffer and 3.750 g NaI in 250 ml of TEMG buffer.

Calculations

- The (SB%) was estimated as and the percent of specific binding was plotted against each type of halide.

Effect of Divalent Cations of the Binding of ^{125}I-Testosterone with its Receptors in Uterine Tumor Homogenate

The experiment was carried out at the optimum conditions (temperature, time, pH, ^{125}I-testosterone, and homogenate concentration) as mentioned with one exception that the reaction mixtures were completed to 1 ml with TEMG buffer containing 25 mM of different divalent salts ($MgCl_2$, $MnCl_2$, $CuCl_2$, $CaCl_2$, and $ZnCl_2$). The bound hormone was measured using a sample without the addition of any salt as a control.

Solutions

The stock solution (25 mM) of divalent salts were prepared as the following: 1.2706 g $MgCl_2$ in 250 ml of TEMG buffer, 1.2369 g $MnCl_2$ in 250 ml of TEMG buffer, 0.6936 g $CaCl_2$ in 250 ml of TEMG buffer, 0.83125 g $CuCl_2$ in 250 ml of TEMG buffer, and 0.8518 g $ZnCl_2$ in 250 ml of TEMG buffer.

Calculations

- The (SB%) was estimated the percent of specific binding (SB%) was plotted against each salt type.

Results and Discussion

Effect of ^{125}I-Testosterone Concentration on the Binding with its Receptors in Uterine Tumor Homogenate

It is one of the most important criteria of the true receptors in the saturability. To fulfil this criterion and to estimate the suitable concentration of testosterone receptors, the experiment was carried out in the presence of 250 µg of receptor protein and increasing concentration of tracer testosterone.

The results are illustrated in Fig. 1. It is shown that the specific binding of tracer testosterone with receptor protein binding is a saturable process but complete saturation however is theoretically never reached unless the amount of steroid hormone used reached infinity [12]. As shown in the Fig. 1, the nuclear testosterone receptors appeared to be saturated with hormone at a concentration that was equivalent to (0.9 ng) per 100 µl. Accordingly, in all the subsequent experiments, (0.9 ng) per 100 µl of labeled testosterone was used, since it gives highest binding.

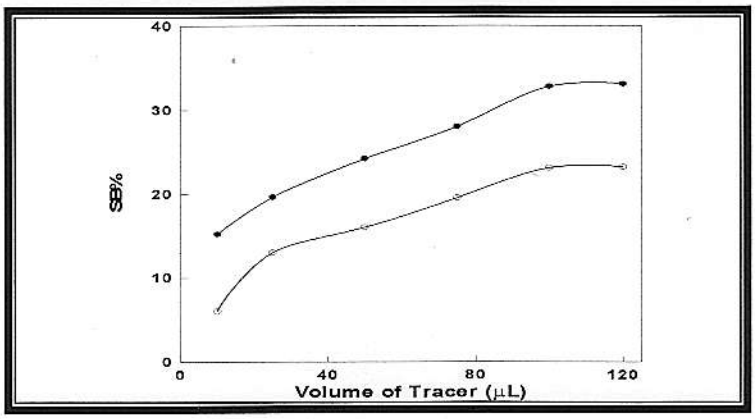

Fig.1. Effect of the concentration of ^{125}I-testosterone on the binding with, (O) benign uterine tumor homogenate, (●) malignant uterine tumor homogenate.

Effect of Testosterone Receptor Concentration on the Binding in Uterine Tumor Homogenate

In order to demonstrate whether the specific binding is proportional to the amount of protein of the receptors actually present in the incubation mixture, increasing amount of nuclear homogenate were incubated with ^{125}I-testosterone or with nonradioactive testosterone. As shown in Fig. 2, the percentage of tracer testosterone bound specifically to their receptors was increased as the amount of receptors in the incubation mixture. The specific binding was increased linearly whereas the nonspecific binding was increased somewhat less than that of specific binding. These results indicate that testosterone receptors binding are principally depended on the amount of receptor protein in the reaction mixture [13]. In all subsequent experiments, 250 μg of receptor protein in the incubation mixture was used.

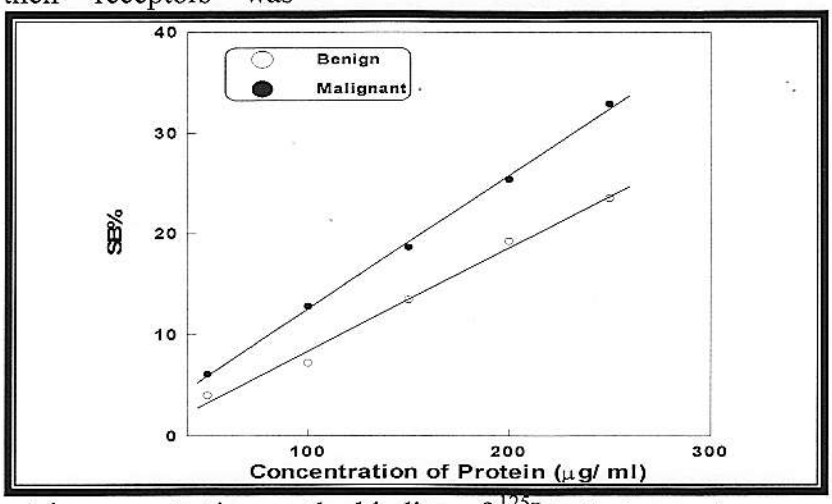

Fig.2. Effect of protein concentration on the binding of ^{125}I-testosterone to its receptors in uterine homogenate

Effect of Temperature on the Binding of ^{125}I-Testosterone to its Receptors in Uterine Homogenate

Temperature dependency of the association of radioactive testosterone to its nuclear receptors was investigated. Nuclear fractions of benign and malignant uterine tumors were incubated at different temperatures (4-42°C). Fig.3 reveal that the specific binding of ^{125}I-testosterone to its nuclear receptors was

maximal at 4°C for benign tumor homogenate and it is maximal at 25°C for malignant one. The specific binding was decreased at temperature increase after maximal value of binding. The loss of binding activity may be due to degradation of the receptor (14) and or the irreversible dissociation of the hormone-receptor complexes. According to these results, 4°C was used in all the subsequent experiments of radio receptor assay studies related to benign uterine tumor homogenate and 25°C was used in malignant homogenate.

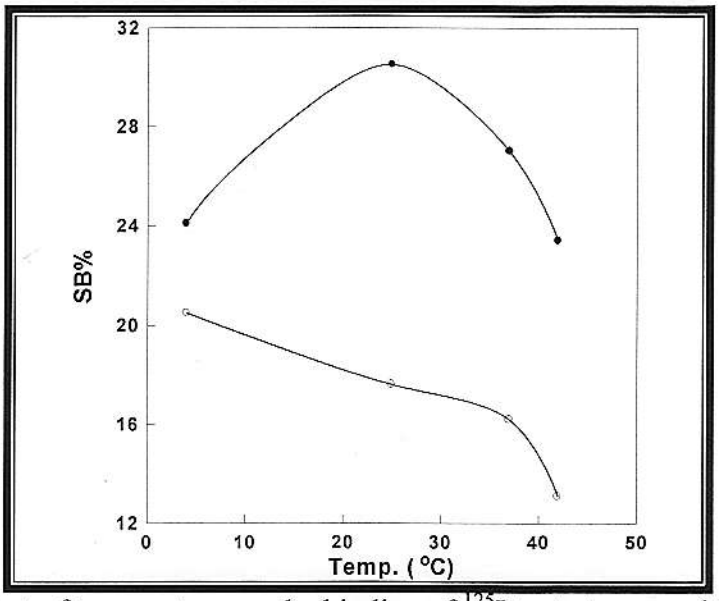

Fig.3. Effect of temperature on the binding of ^{125}I-testosterone with, (○) benign uterine homogenate, (●) malignant uterine homogenate.

Time-Course of Receptor Binding inUterine Tumor Homogenate

To examine the characteristics of the association of ^{125}I-testosterone with its receptors, the experiment was carried out at different temperatures (4, 25, 37, 42, 50, and 60°C). As seen in Fig. 4, the time course of the binding reaction after incubation of labeled hormone with or without unlabeled hormone. The specific binding of tracer hormone to its receptor was maximal at 8 hrs and 4°C for benign tumor and 25°C for malignant tumor. After 8 hrs, the results revealed decrease in specific binding. These observations may be due to either the degradation of the receptors or the irreversible dissociation of the steroid-receptor complexes. In all other subsequent experiments the 8 hrs were implemented, 4°C and 25°C were implemented for benign and malignant tumor respectively.

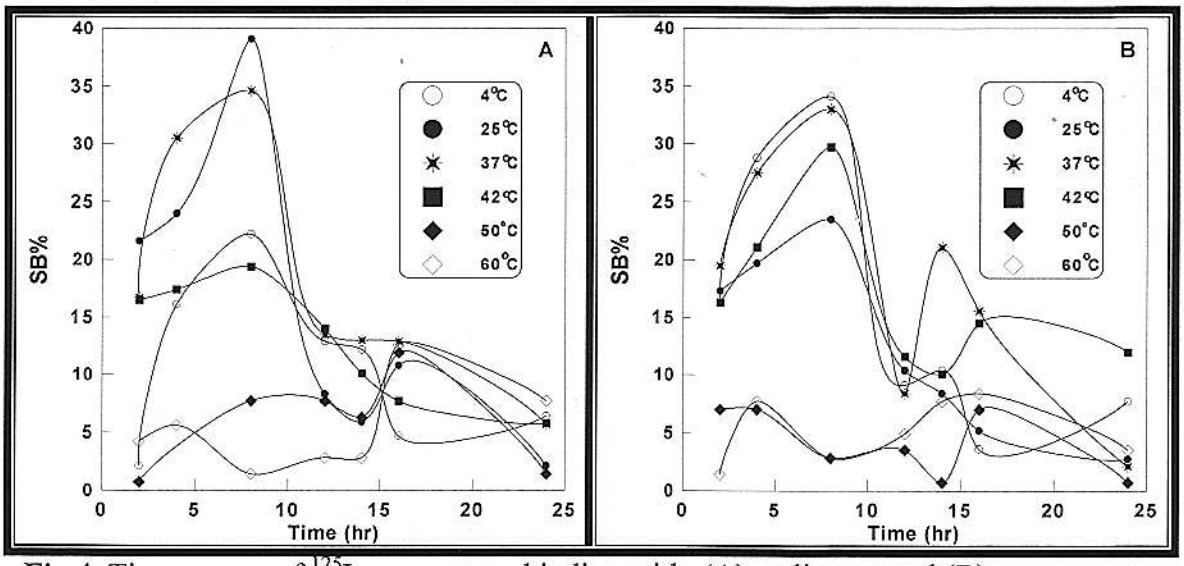

Fig.4. Time course of ^{125}I-testosterone binding with, (A) malignant and (B) benign.

The Effect of pH on the Binding of ^{125}I-Testosterone to its Receptors in Uterine Tumor Homogenate

The analysis of the influence of pH on the specific binding of tracer testosterone is illustrated in Fig. 5. The optimum pH was found to be 8.0 for the binding of ^{125}I-testosterone with its receptors. The results obtained revealed that a maximal binding occurs over a relatively narrow pH range. However, there is a sharp decline in the specific binding below the optimal pH.

The 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 processes occurring within the ionizable groups of the amino acids present in the binding domain of these receptors (15).

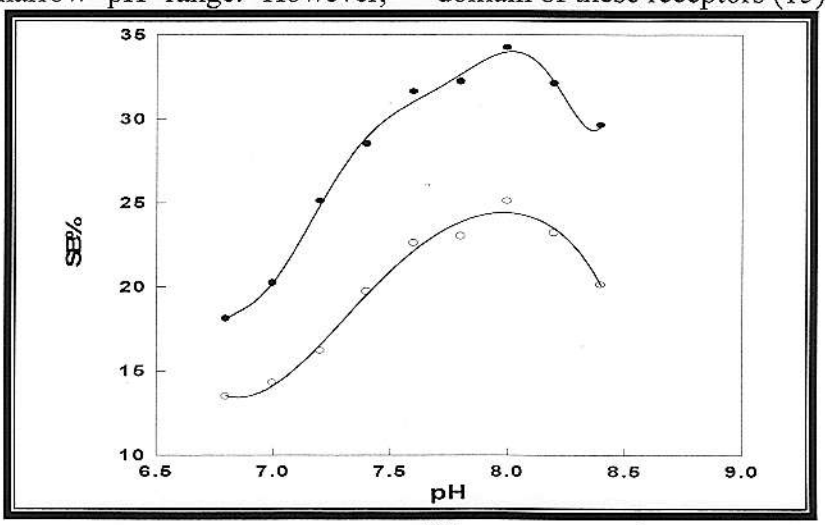

Fig.5. PH dependency of ^{125}I-testosterone binding with, (○) benign, (●) malignant tumor homogenate.

Stability of ^{125}I-Testosterone Receptor Complex

The influence of the temperature on the stability of testosterone-receptor complex as a function of time was studied. The complex was reincubated at different temperatures (0, 4, 25, 37, and 45°C) and at a certain time intervals, the remaining bound hormone was estimated. As seen in Fig. 6, the dissociation of testosterone-receptor complex was relatively increased when the temperature was also increased. The dissociation of testosterone receptor complexes is exceedingly slow at 0-4°C. When the temperature was elevated, the dissociation constant was increased as shown in the results.

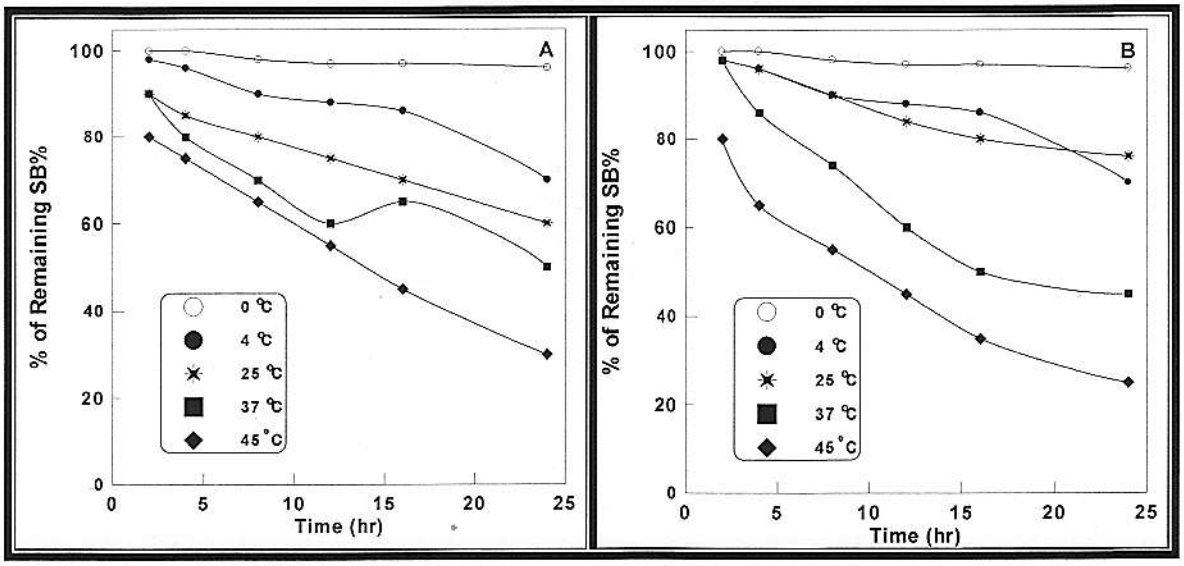

Fig.6. Stability of ^{125}I-testosterone-receptor complex at five different temperatures for A) benign, B) malignant.

Competitive Effect of Different Competitors on the Binding of ^{125}I-Testosterone with its Receptors

Figure 7 shows the effect of different competitors on the binding of ^{125}I-testosterone with its receptors. The results obtained indicate that the binding of labeled testosterone was inhibited mainly by unlabeled testosterone. The competition of estradiol is more than that of progesterone on the binding of ^{125}I-testosterone with its receptors. The amount of the fixed binding remained in the presence of competitors at the concentration of maximum displacement was considered to represent the non-specific binding (16). The displacement of tracer steroid hormone binding confirms the specificity of the nuclear receptors, which is one of the fundamental criteria of the true receptor (17).

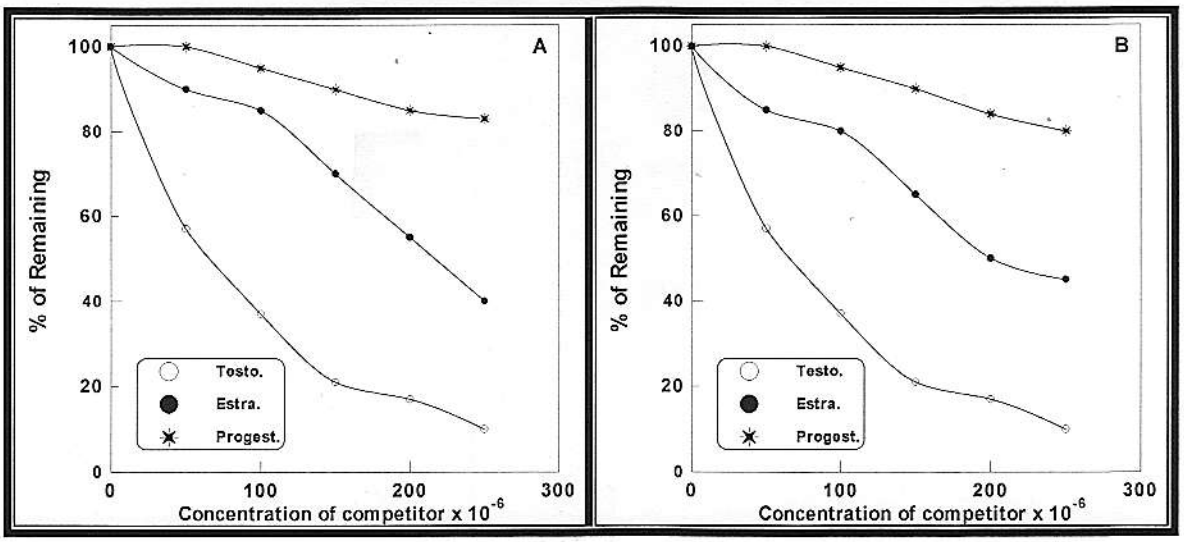

Fig.7. Binding of benign (A) and malignant (B) uterine tumor homogenate receptor with ^{125}I-testosterone in the presence of different concentration of, testosterone, estradiol, and progesterone.

Effect of Different Halides on theBinding of ^{125}I-Testosterone to its Receptors

Different sodium halides were investigated to study their action on the binding of testosterone with its receptors in uterine tumor homogenate. The sodium halides in the incubation mixture induced-activation of the percent of specific binding according to the following sequence:

NaCl > NaF > NaI

This frequency of binding indicates that activation causes subtle conformational changes in the testosterone receptor. Fig. 8 demonstrates somewhat the relationship between the halides and testosterone receptors.

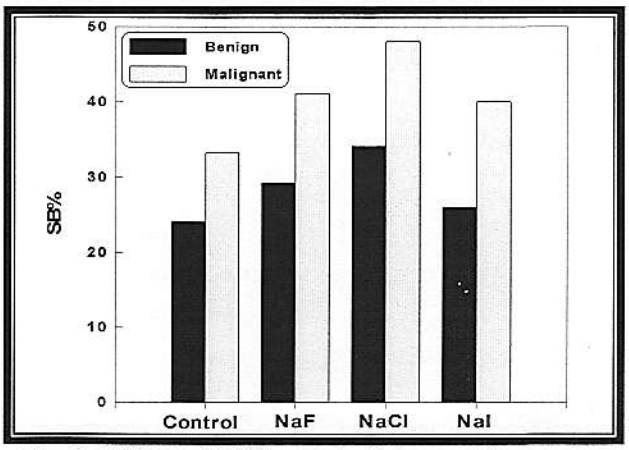

Fig.8. Effect of different halides on the extent of ^{125}I-testosterone binding with its uterine tumor.

Effect of Divalent Cations on the Binding of ^{125}I-Testosterone with its Receptors in Uterine Tumor Homogenate

Fig. 9 shows the effect of different divalent cations on the extent of binding of ^{125}I-testosterone to its receptors in the human uterine tumor. The results indicated that the testosterone binding process is sensitive to the presence of cation metal ions. $MgCl_2$ at concentration (25mM) was shown to increase the binding more than other divalent cations.

One hypothesis assumes that salts may alter the nature of the hydrophobic forces controlling the stabilization of ^{125}I-testosterone-receptor complex formed. From the results illustrates in Fig. 9, it is suggested that these salts may provide some conformational changes in the testosterone receptors and the charged groups of the binding domain of these receptors that hinder maximal binding are shielded (18-20).

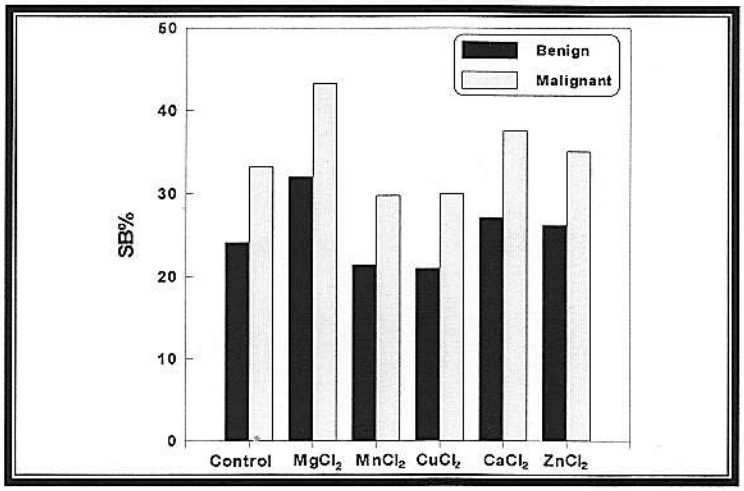

Fig. 9. Effect of divalent cations on the binding of ^{125}I-testosterone with its uterine umor. All details are described in section (2.4.3.9).

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