Binding Studies Of ^{125}I-Testosterone With Its Receptors in Uterine Tumor Homogenate

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Summary :

The sera levels of testosterone were investigated prior to surgery in 21 women with benign and 9 women with malignant uterine tumors. The sera concentrations of T were compared with these of 16 healthy age- matched controls. Non- significant lower sera T levels (P>0.1) were demonstrated in women with benign and malignant tumors. The presence of testosterone (T) receptors were investigated in one group of benign uterine tumor and one group of malignant uterine tumor. The results obtained indicate a higher incidence for these receptors in the malignant than in the benign tumors.

الخلاصة:

تم قياس مستوى هرمون (T) قبل إجراء العملية الجراحية في أمصال دم (21) امرأة مصابات بمرض ورم الرحم الحميد و تسع نساء مصابات بمرض ورم الرحم الخبيث وتمت المقارنة مع عينات من أمصال دم ستة عشر من النساء الأصحاء لغرض المقارنة . لوحظ عدم وجود نقصان معنوي واضح (P>0.1) لدى المجموعة الأولى والثانية عن مستواه لدى النساء الأصحاء .
تم التحري عن مستلمات هرمون التيستوستيرون في مجموعة من أورام الرحم الحميدة و مجموعة واحدة من أورام الرحم الخبيثة ودلت النتائج على زيادة انتشار هذه المستلمات في الأورام الخبيثة اكثر مما هي في الأورام الحميدة .

Introduction :

Although much of our knowledge on the mechanism of action testosterone has been obtained with uteri of experimental animals, yet much less is known about the interaction of testosterone with the human endometrium and the myometrium, and testosterone promotes protein synthesis in uterus and most tissues of the body[1]. High androgen concentrations may result in an abnormal secretory endometrium [2]. The steroid sex hormones enter cells by passive diffusion, bind to specific receptor proteins in the cytoplasm and then move to the nucleus where they bind to specific sites on the chromosomes. These hormones exert their effects through gene-hormone interaction, which stimulate the synthesis of specific messenger ribonucleic acid [3]. These important roles of androgen, especially testosterone, suggest that androgens are at least a co factor in the development of endometrial cancer [4]. Accordingly, the aim of the work includes the determination of the testosterone (T) level in sera of normal women and patients affected by uterine tumors and demonstration the presence of specific T receptors in the human uterine tumors.

Experimental :

Chemicals

All laboratory chemicals and reagents were of analar grade and were used without further purification. Tris (hydroxy methyl) aminomethan, $CuSO_4.5H_2O$, Na, k-tartarate, were obtained from Fluka company, Switzerland. Hydrochloric acid, Na_2CO_3, NaOH, Folin ciocalteau, and Bovine serum albumin (BSA) were obtained from BDH limited pool, U.K. Kit of radioactive testosterone (^{125}I-testosterone) was purchased from CIS Bio International (France). The activity of the labeled testosterone was approximately 5 μci.

Instruments

The instruments used in this work were, LKB gamma counter type 1270-rack gamma II, Pye-Unicom pH meter, Varian DMS 100 UV- visible spectrophotometer, LKB ultracentrifuge type 2332, Memmert water bath, Memmert incubator.

Patients

Two groups of uterine tumor patients were included in this study. Group I contained 21 patients with benign uterine tumor. Group II consisted of 9 patients with uterine cancer. The groups were matched with group control subjects (group III). All patients were admitted for treatment to Al-Arabe Hospital and Saddam Medical City under the supervision of specialists Dr. Ryad Mohammed Salh, Nada Al-Ubadi and, Raja' Al-Tikreti. They were histologically proven from the supervision of specialists Dr. Luay Edward and Dr. Nabel Abduleadoad. 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.

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 tissue were weighed, pulverized finally with a scalped 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 used was Tris-EDTA (Tris-HCl 0.01 M, pH 7.4, containing 0.15 mM EDTA, 2-mM mercaptoethanol and 10% glycerol). The homogenate was filtered through four layers of nylon gauze in order to eliminate fibers of connective tissues. The homogenate was then centrifuged at 2000xg for 30 min at 4°C. The sediment was suspended in 10 volumes of TEMG buffer for 15 min at 4°C and then suspension was used to obtain the crude nuclear fraction and the supernatant was used as crude cytosolic fraction (5).

Blood Sampling

Blood samples (5ml) were obtained from patients undergoing hysterectomy or myomectomy by venipuncture before operation. Those were left for 20 min at room temperature. After coagulation, sera were separated by centrifugation at 2000xg for 10 min and kept at -20°C until assaying.

Solutions

All buffer solution was prepared (6)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 (7).

TEMG buffer (pH 7.4): Tris (0.01 M, pH 7.4) buffer containing 0.15 mM Na_2EDTA, 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.

Methods :

Determination of protein in uterine tumor homogenate

Protein content of the homogenate of uterine tumors was determined by the *Lowry* method, using bovine serum albumin (BSA) as the standard protein (8)

Determination of ^{125}I-Testosterone Concentration

The concentration of labeled testosterone was measured according to the method of *Morris* (9). In a set of Rabbit anti-testosterone coated tubes marked from 1 to 12, 500µl of ^{125}I-testosterone was added with 100 µl of standard unlabeled testosterone of different concentration (0, 0.2, 2, 4, 8 and 16 ng/ml). In another set of the same tubes, different volumes (250, 350, 450, 550, 650, and 750µl) of ^{125}I-testosterone were pipetted. After incubation of all tubes for 2 hrs at 37°C, they were decanted and counted using gamma counter.

Calculations :

- (B) Is the bound radioactivity (CPM) which represents the counted radioactivity in the precipitated hormone-antibody complex.
- The free hormone (F) which represents unbound ^{125}I-testosterone was determined from the following formula:

$$F(CPM) = \text{total count (CPM)} - B\ (CPM)$$

- The value of the ratio (B/F) for an ordinary standard curve was plotted against the concentration of standard testosterone.
- The (B/F) values for the incubation of different amounts of ^{125}I-testosterone were also calculated, Table 1.
- The data in Tables 1 and 2 were plotted as in Fig. 1 and Fig. 2 respectively.
- Using the two curves in the two figures and corresponding equations, the amount of radioactivity corresponding to the concentration of unlabelled

- hormone can be estimated. Table 3 shows the mass of standard testosterone in ng/ml corresponding to a given amount of tracer.
- The amount of the standard testosterone was plotted against the amount of corresponding radioactivity, Table 3 and Fig.3.
- The concentration of ^{125}I-testosterone was determined from the intercept of the straight line as in Fig. 3.

Table 1. B/F values corresponding to different concentration of standard testosterone used in standard curve.

Concentration of standard (ng/ml)	B/F
0	0.785
0.2	0.766
1	0.407
4	0.238
8	0.177
16	0.115

Table 2. B/F values corresponding to different amounts of ^{125}I-testosterone used in the incubation.

Amount of ^{125}I-testosterone (CPM)	Bound radioactivity	B/F
2804	2298	4.542
3929	2632	2.028
5758	3637	1.715
7032	4282	1.557
5486	4901	1.367
9731	5391	1.242

Table 3. The mass of standard testosterone in (ng/ml) corresponding to a given amount of the tracer.

Bound radioactivity (CPM×10^{-3})	Amount (ng/ml)
24.158	1
27.968	2
29.758	3
30.863	4
34.354	5

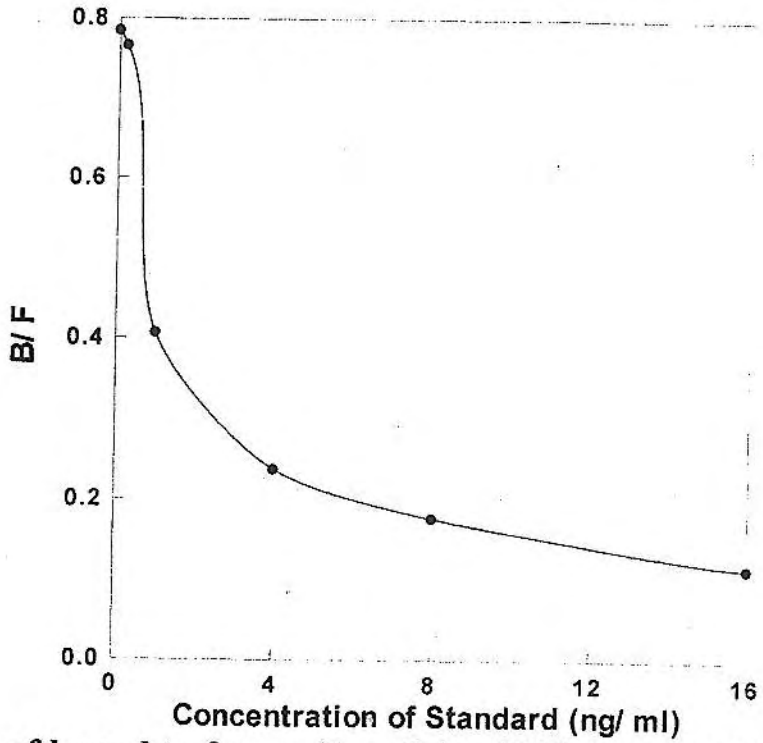

Fig.1. Ratio of bound to free radioactivity (B/F) for an ordinary standard curve, where different amounts of standard testosterone were incubated with constant amount of ^{125}I-testosterone and antibody.

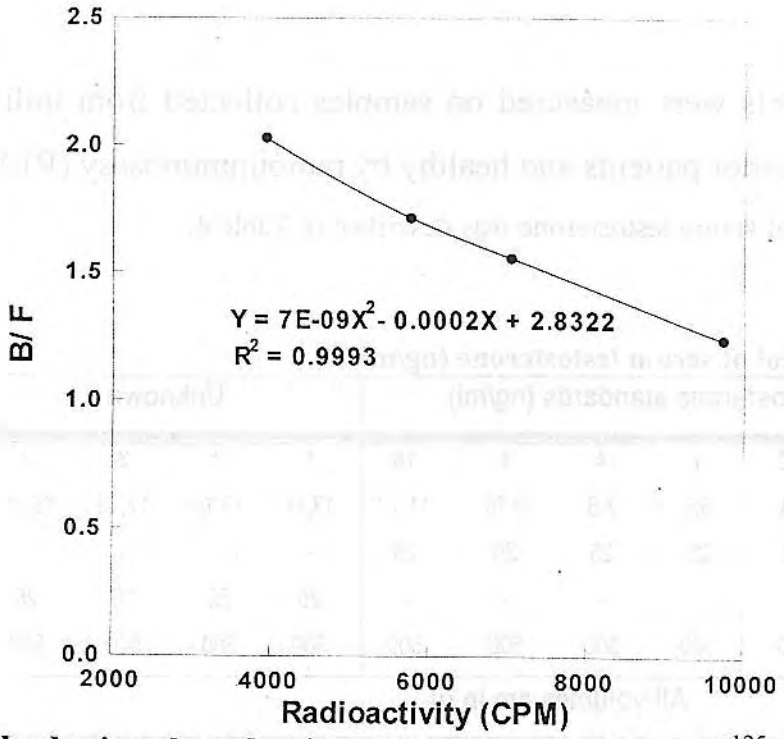

Fig.2. Antibody incubated with different amounts of ^{125}I-testosterone in the absence of unlabelled testosterone.

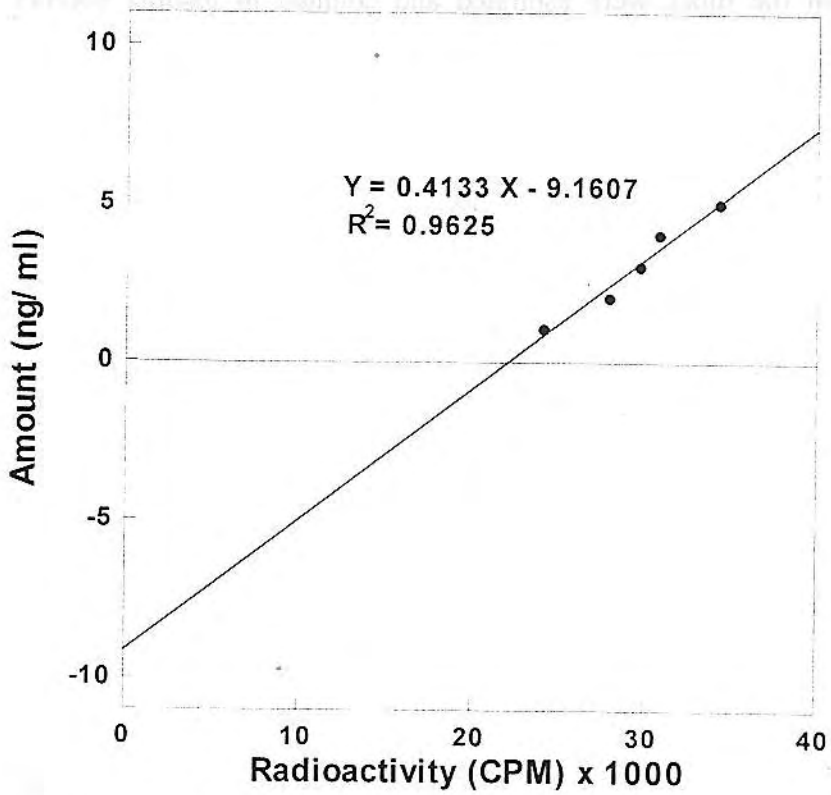

Fig.3. The mass of standard testosterone in ng/ml corresponded to the given amount of tracer.

Determination of Testosterone Levels in Sera of Uterine Tumor Patients and Controls

Serum testosterone levels were measured on samples collected from individuals of benign and malignant tumor patients and healthy by radioimmunoassy (RIA).

The assay protocol of serum testosterone was described in Table 4.

Table 4. RIA assay protocol of serum testosterone (ng/ml).

	Testosterone standards (ng/ml)						Unknown			
	0	0.2	1	4	8	16	1	2	3	4
Tube No.	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18	19,20
Standard serum	25	25	25	25	25	25	-	-	-	-
unknown serum	-	-	-	-	-	-	25	25	25	25
^{125}I-testosterone	500	500	500	500	500	500	500	500	500	500
All volumes are in µl										

The test tube rack was mixed by shaking gently by hand and all tubes then incubated in a water bath at 37°C for 60 min. Then the tubes were aspirated and counted in gamma counter for one-minute(10).

Preliminary Test of ^{125}I-Testosterone Binding to Its Receptors in Uterine Tumor Homogenate

Cytosolic and nuclear receptors were detected using two sets of experiments. The first one was carried out to determine the total binding, while the second was used for the estimation of non-specific binding. In order to detect nuclear receptors, 200 µl (250 µg protein) of crude pellet was incubated with 250 µl of labeled testosterone in duplicate tubes. The volume of the mixture was completed to 1 ml with TEMG buffer (pH 7.4), then the tubes were incubated for (16 hrs) at 25°C.

Non specific binding was accounted by preparing the same incubation mixture with the addition of 100-fold excess of unlabeled testosterone as competitor. At the end of the incubation (16 hrs), bound and unbound hormone were separated by charcoal adsorption with a suspension of dextran-coated charcoal(11).For this purpose 250 µl of dextran coated charcoal-Tris buffer solution was added. The tubes were shaken for 10 min and centrifuged for 10 min at 2000xg at 4°C. One milliliter was taken from each supernatant and counted in gamma counter. It represents the bound testosterone (^{125}I-testosterone).

In order to detect cytosolic receptor, the same steps in crude nuclear fraction are applied.

Solutions :

TEMG buffer was prepared as described previously and DCC buffer was prepared by dissolving the following compound: 1.25 g charcoal, 0.6 g dextran, and 0.2 g gelatin in 100 ml of Tris (0.01 M) buffer, pH 7.4.

Calculations

- The counted radioactivity in each tube (expressed in CPM) represents the total binding (TB).
- The counted radioactivity (expressed in CPM) in the tubes contained-labeled hormone and excess of unlabeled hormone represents the non-specific binding (NSB).
- The specific binding (SB) was calculated by subtracting the radioactivity (CPM) obtained in the presence of unlabeled hormone from that produced in the absence of unlabeled hormone.

$$SB(CPM) = TB(CPM) - NSB(CPM)$$

- The percent of specific binding (SB%) can be calculated from 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) used, in each tube.

Results and Discussion

Tissue Collection and Processing

The homogenization of the samples was carried out in a cold medium (4°C), to avoid protein denaturation and to decrease the proteolytic enzyme activity that might damage the hormone receptors, so low temperature is a part of avoiding proteolysis(12,13). The tissue homogenate was filtered through several layers of nylon gauze to remove connective tissue fragments and debris, while centrifugation at 2000 xg removed the enraptured cells and intact nuclei of the ruptured cells, leaving mitochondrial/golgi fractions and cell microcosms in the supernatant(14).

Determination of Testosterone Levels in Sera of Uterine Tumor Patients and Controls

Sera testosterone levels were measured in uterine tumor patients (benign and malignant) matched with one group of control subject. Group I contained 21 patient with uterine tumor, group II consisted of 9 patients with uterine cancer. The groups were matched with group control subjects (group III). Table 6 and Fig. 4 show the results obtained from this study.

The level of serum testosterone in-patients with benign tumors was found to be (0.76 ± 0.16) ng/ml, whereas that of uterine cancer was found to be (0.71 ± 0.24) ng/ml. But in controls, the level was found to be (0.84 ± 0.24) ng/ml. Student's T-test analysis revealed that there is no significant decrease of serum testosterone levels ($P>0.1$), in benign and malignant uterine tumors patients (15)..

No significant increase and decrease of serum testosterone levels in these two groups of uterine tumor patients were obtained. The results are nearly similar to those obtained previously by other investigator (16).

The measurement of testosterone level is important to give a good correlation with the incidence of testosterone receptors in uterine tumor. The normal value of testosterone level in female is (0.3–3.0) ng/ml (17).

Table 6. Serum testosterone levels (ng/ml) in patients of benign and malignant uterine tumor.

Group	No. of cases	Age (year)	Serum testosterone (ng/ml)
Group I Benign uterine tumor	21	53 ± 3.4	0.76 ± 0.16
Group II Malignant uterine tumor	9	42 ± 1.9	0.71 ± 0.24
Control	16	44 ± 2.1	0.84 ± 0.24

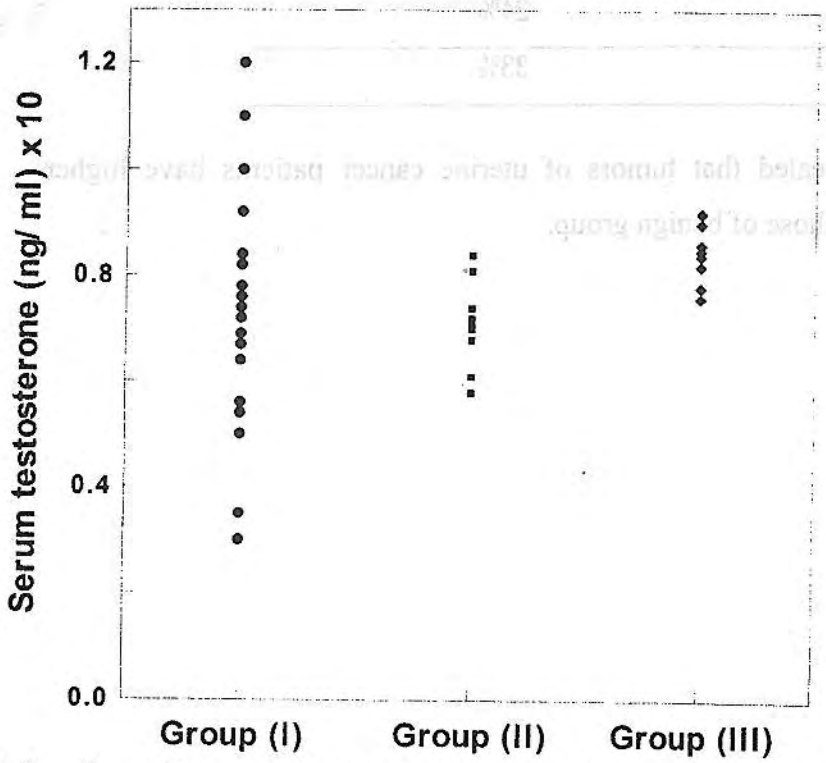

Fig.4. Distribution of serum testosterone (ng/ml) in-patients with benign (Group I), malignant uterine tumors (Group II), and controls (Group III).

Binding Studies of ^{125}I-Testosterone Binding to Its Receptors in Benign and Malignant Uterine Tumor:

Preliminary Test of ^{125}I-Testosterone Binding to Its Receptors in Uterine Tumor Homogenate

Cytosolic and nuclear testosterone receptors were investigated in (30) cases of benign and malignant uterine tumors. Cytosolic testosterone receptors were detected through the incubation of labeled testosterone with crude cytosol and the bound hormone measured by dextran-coated charcoal method. Nuclear testosterone receptors were evaluated using a nuclear traction that incubated with tracer testosterone.

The tumor was considered to have testosterone receptor if it contained more than 5% of specific binding. The specific binding in nuclear fraction was found to be 24% in benign uterine tumor patients, 33% in-patients with uterine cancer, Table 6.

Table 6.Incidence of testosterone receptors in benign and malignant uterine tumors.

Group	No. of cases	Nuclear testosterone receptors (SB%)
Benign	21	24%
Malignant	9	33%

The data obtained, in this study, revealed that tumors of uterine cancer patients have higher incidence of testosterone receptor than those of benign group.

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