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Sex-hormone binding globulin from sheep serum: purification and effects of pregnancy and treatment with exogenous estradiol

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Abstract

Sex-hormone binding globulin (SHBG) is a protein that binds sex steroids in the serum of many species. SHBG binds androgens and estrogens in humans and primates with high affinity, but behaves as an androgen binding protein in other species. Here we purified SHBG from ewe and ram sera to homogeneity, by a simple and rapid method. The K_D of the purified protein was found to be 3.63 nM for testosterone and around 600 nM for estradiol. We also studied the effect of pregnancy on SHBG levels in ewes and the effect of exogenous estradiol administration either orally or parenterally on SHBG levels in rams. Basal levels of SHBG in sheep are not affected by pregnancy or exposure to exogenous estradiol. It is concluded that SHBG regulation of expression in ewes and rams differs from that in humans in that it is not affected by estrogen and possibly is species specific. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: Sex-steroid binding globulin; Sex-hormone binding globulin; Ewe; Ram; Estrogen; Sheep; Pregnancy

1. Introduction

Sex-hormone binding globulin (SHBG) is a protein that circulates in the plasma of many species including human [17,27], non-human primates [16], dog [26], cat [28], ram [20], cow [25] and many others, but is absent from mouse [20] and mature rat plasma [28]. The protein binds androgens and estrogens with high affinity in human and non-human primates, but is mainly an androgen binding protein in other species [20]. Most of the work regarding biological role, function and regulation has been done in humans and it has been found that SHBG levels increase substantially as early as 1 month of pregnancy [5]. It has been also shown in humans, that estrogen administration increases and androgen administration decreases SHBG levels [22]. These effects are mediated through gene transcription in the liver where SHBG is expressed [19]. Studies on the function and regulation of SHBG in sheep have not been performed and information regarding SHBG presence is available only in rams. Indeed, SHBG in rams has been shown to bind androgens with much higher affinity than estrogens [20]. It has, therefore, been concluded that although the bioavailability of androgens in ram plasma is controlled by both SHBG and albumin, the bioavailability of estrogens is mainly controlled by albumin. In the past few years, a number of reports in humans showed that SHBG serves not only the role of steroid carrier, but also behaves as a protein hormone with specific plasma membrane receptors in target tissues [24].

In the present study, SHBG was purified to homogeneity from ram and ewe serum by a simple and rapid

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method by means of acetone fractionation, ammonium sulfate precipitation, DEAE-cellulose chromatography and gel filtration. In addition, the effects of pregnancy in ewes and the effects of estradiol treatment in rams on basal SHBG levels were investigated.

2. Materials and methods

2.1. Chemicals

Sephadex G-200 and G-100, DEAE-cellulose, electrophoresis molecular weight markers and radio-inert steroids were products of Sigma (St. Louis, MO, USA). Acetone (pro analysis) was purchased from Merck (Darmstadt, Germany). Tritiated testosterone (sp.act. 100 Ci/mmol) and estradiol (sp.act. 120 Ci/mmol) were purchased from New England Nuclear (Dupont, Hamburg, Germany). Dextran-32 was a product of SERVA (Heidelberg, Germany) and activated charcoal of Merck (Darmstadt, Germany).

2.2. Animals

Mature domestic ewes and rams (3 years old, Chios breed, weighing ~50 and ~70 kg, respectively) farmed in the Institute of Artificial Insemination, Ionia Thessaloniki, were used. Day 1 of pregnancy was defined as the day that artificial insemination was performed. Pregnancy was verified by ultrasound. The experiments with rams were performed during spring (April–May).

2.3. Drug treatment and blood sampling

2.3.1. Intramuscular administration

Estradiol benzoate was administered in the neck region at two doses of 1 and 10 mg (in 1 ml of corn oil) to two groups of rams (six animals each) every 2nd day for 10 days. Venous blood (5 ml) was taken 1 h before the first administration of the drug and at days 3, 7, 11, 15 and 20 from the beginning of administration. A control group (six animals) was treated with vehicle only (corn oil) for the same period as the experimental groups and blood samples were collected at the same time intervals as the experimental groups.

2.3.2. Oral administration

Estradiol benzoate 1 and 10 mg was administered with the food (mixed with grain) every day for 10 days to two experimental groups of rams (six animals each) and venous blood (5 ml) was collected 1 h before the first administration and at days 3, 7, 11 and 15 from the beginning of the administration. Control animals (six animals) received the vehicle alone (corn oil) mixed with grain.

2.4. SHBG purification

A total of 25 ml of sheep serum were mixed with 37.5 ml of Tris–HCl buffer pH 8, 0.05 M containing 0.9% NaCl (buffer A), and to the total of 62.5 ml, 53 ml of cold acetone (0.85 vol.) were added dropwise at 4°C under moderate stirring. The cloudy solution was left at 4°C for 5 min, centrifuged at 10 000 × *g* for 10 min at 4°C and the heavy precipitate was discarded. To the supernatant, 22 ml of cold acetone were added dropwise at 4°C to reach a final concentration of 1.2 vol. of acetone. After a 10-min centrifugation at 10 000 × *g* at 4°C, the sediment was dissolved in 15.5 ml of buffer A, and 9.5 ml of saturated ammonium sulfate solution were added dropwise under stirring to give a 38% saturation. After a 10-min centrifugation at 10 000 × *g* at 4°C, the pellet was discarded and to the supernatant, 8 ml of saturated ammonium sulfate were added dropwise at 4°C to reach a final concentration of 53%. Then the cloudy mixture was centrifuged at 4°C at 10 000 × *g* for 10 min and the pellet was dissolved in 5 ml of buffer A. This last solution was applied to a DEAE-cellulose column (2 × 23 cm) which had been pre-equilibrated with Tris–HCl buffer pH 8, 0.05 M, and eluted with a 0–0.3-M gradient of NaCl. The active fraction was eluted at 0.15 M NaCl. SHBG was further purified by Sephadex G-100 (2.5 × 40 cm) with buffer A as running buffer. The purification was repeated three more times with almost identical results.

2.5. Protein concentration measurement

Protein concentration was measured according to the method of Bradford with bovine serum albumin as standard [1].

2.6. Electrophoresis of proteins

Polyacrylamide gel electrophoresis and visualization of proteins was performed according to standard methods [3].

2.7. Sex hormone binding globulin measurement

SHBG was measured in the serum of animal blood samples by the method of dextran-coated charcoal as previously described [23,30]. Briefly, the appropriate amount of labeled and unlabeled steroids in toluene solutions were evaporated under vacuum and then buffer A and serum were added to a final volume of 200 μl. The mixture was incubated at 4°C for 15 min and then 1 ml of dextran-coated charcoal (DCC) slurry (1% charcoal Norit A and 0.1% dextran T32 in buffer A) was added to the tubes, the contents were mixed, kept at 4°C for 5 min and centrifuged at 3500 × *g* (4°C) for 10 min to pellet down the charcoal. The radioactivity of

Table 1
Purification protocol of SHBG from ewe serum

Stage	Protein (mg)	Specific activity (pmol mg protein)	Purification	Yield
Serum (25 ml)	2500	0.7	1	100
Acetone (0.85–1.2 vol.)	85.25	18	25.7	87.6
Ammonium sulfate (38–53%)	12.5	114	162.8	81.4
DEAE	1.2	770	1100	52.8
SephadexG-100	0.5	1310	1870	26.7

400 μl supernatant sample was measured by liquid scintillation in a RACK-beta liquid scintillator (LKB, Sweden). Affinity of SHBG for steroids was determined by using increasing amounts of labeled steroid (1–100 nM) and 100 × excess unlabeled steroid for each labeled steroid concentration. Specific binding was determined and the results were plotted according to the method of Scatchard [21]. The concentration of SHBG is also reported.

All samples were determined in duplicate. The intra-assay coefficient of variation was 3.1%. A total of three samples of mean values of 70, 73 and 109 nM were determined over seven assays. The intra-assay coefficient of variation was calculated to be 3.0, 2.6 and 3.5%, respectively. Specificity of the assay was determined with purified serum albumin as the binding protein in the absence of SHBG and was found to be 0.4%. This is attributed to the fact that charcoal tends to absorb the weakly bound steroid from albumin. It was not possible to evaluate sensitivity in our system, since, as shown in a previous report from our group [30], binding of steroids to SHBG is not linear at low concentration of SHBG.

2.8. Statistics

Statistical analysis of the results was done by multiple-way analysis of variance (SHBG concentration × dose × route of administration × time) with Duncan's multiple-range F test. For the analysis of pregnancy values one-way ANOVA test was used. $P < 0.05$ was considered significant.

3. Results

A summary of the purification protocol of SHBG from ewe serum is shown in Table 1. The purification of SHBG from ram serum gave similar results (not shown). The active protein was obtained in relatively high yield and exhibited a specific activity with testosterone as ligand of 1310 pmol/mg protein. The final purification factor was 1870.

This purification procedure resulted in a preparation of SHBG that was pure and homogenous, as judged by PAGE electrophoresis (Fig. 1A). The amount of albumin in the DEAE-step was eliminated by a Sephadex G-100 filtration and thus a totally pure SHBG preparation was obtained (Fig. 1B, lane 4).

As can be seen in Fig. 2 the molecular mass of SHBG, which was estimated by Sephadex G-200 filtration, was found to be around 80 kDa. This is also confirmed by SDS electrophoresis as can be seen in Fig. 1B.

The K_D for testosterone was 3.63 nM (Fig. 3) and for estradiol around 600 nM (not shown).

The binding kinetics of pure SHBG from ram with testosterone is depicted in Fig. 4.

SHBG concentration in the serum of ewes and rams was 74.29 ± 10.58 and 118.67 ± 3.33 nM, respectively (Figs. 5 and 6).

Neither intramuscular nor orally administered estrogen treatment caused any changes on the basal levels of SHBG in ram serum (Fig. 5).

At the beginning of the 3rd month of pregnancy, no changes in the levels of SHBG in ewes were observed (Fig. 6). This period is considered as mid-term and at the same period SHBG levels in human are several-fold increased as compared to non-pregnant women.

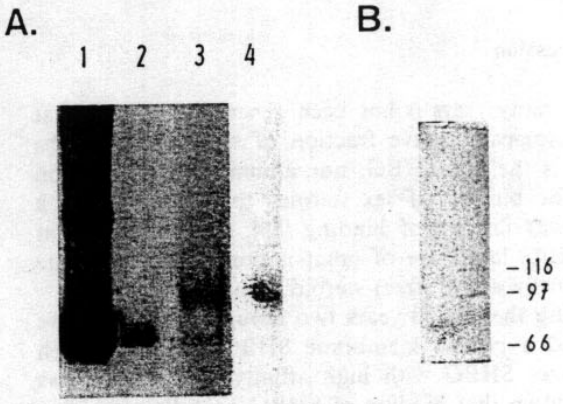

Fig. 1. (A) Native PAGE analysis of SHBG purified from ram serum. A 7.5% gel was run. Lane 1: 5 μg of crude ram serum, lane 2: 0.5 μg of albumin purified as described [30], lane 3: 0.5 μg of the DEAE step-SHBG purified from ram serum, lane 4: 0.5 μg of further purified SHBG by Sephadex G-100 filtration. (B) SDS gel of purified SHBG. A 5% gel was run and the molecular mass markers used were b-galactosidase (116 kDa), phosphorylase b (97 kDa) and serum albumin (66 kDa). The gels were stained with Coomassie blue.

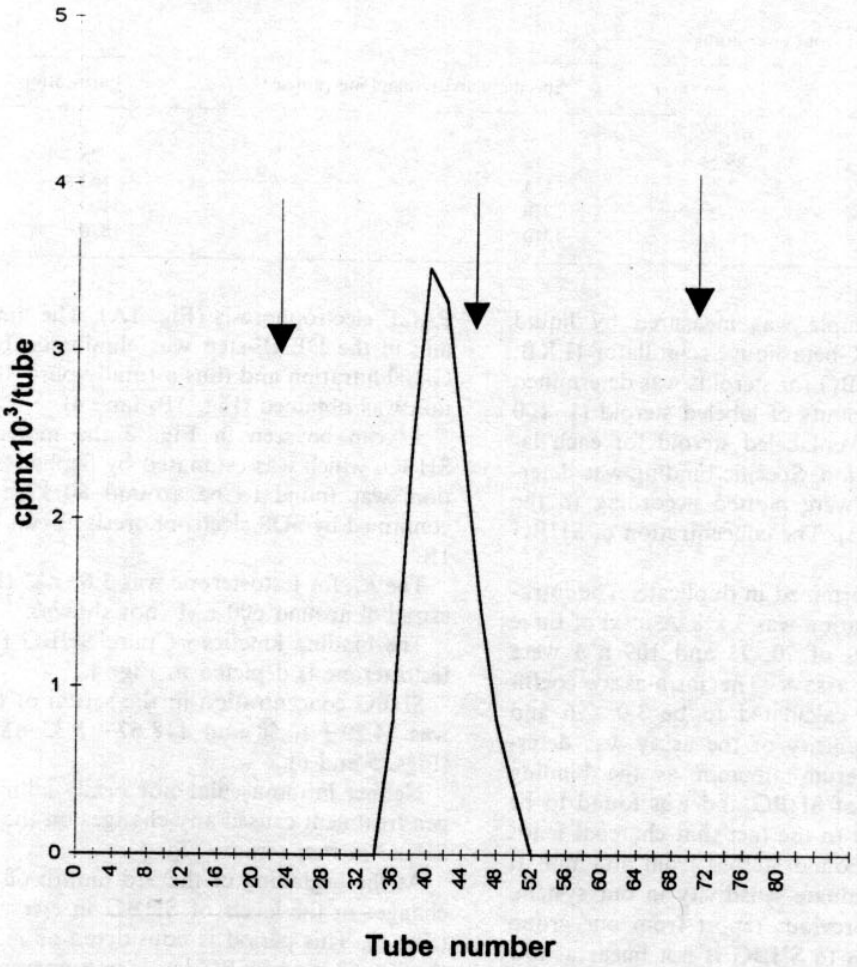

Fig. 2. Sephadex G-200 profile of pure SHBG from ram serum. A 3 x 40-cm column was used and the elution buffer consisted of Tris–HCl 0.05 M, pH 8. Fractions of 4.5 ml were collected. Arrows represent the bed volume, the elution point of albumin and the elution point of NaCl, respectively.

4. Discussion

For many years it has been generally accepted that the biologically active fraction of sex steroids in the serum is the non-SHBG, non-albumin bound fraction [29]. The binding of sex steroids to SHBG follows a non-linear fashion of binding [30], which means that the SHBG levels are of great importance to correlate biologically active (free) steroid and activity.

During the last 10 years two groups [7,8,13,24] have reported a plasma membrane SHBG receptor, which recognizes SHBG with high affinity. Recently, it has been shown that binding of SHBG on cells expressing SHBG receptor results in inhibition of cell proliferation, through signal transduction pathways [6]. Thus, the old dogma claiming that only the free steroid fraction exerts biological activity in the target tissue, has changed and it is now believed that SHBG itself exhibits biological activity, acting on cell membrane specific receptors.

Under the view point that SHBG is a hormone, SHBG gained new interest regarding gene regulation, biochemistry, physiology and biological function (reviewed in Refs. [11,14]). However, although the understanding of the function and properties of human SHBG has grown rapidly during the past 10 years, our knowledge in other species, i.e. sheep, is still poor and lacking in detail. Purification of SHBG from sheep serum has not been reported by other investigators. The only information has been obtained from Renoir et al. [20] who reported the K_D and B_{max} of whole ram serum.

In the present work we report a simple and quick (requiring 14 h) method of purification of SHBG from the serum of ewes and rams that results in a pure preparation of the active protein with the same physicochemical properties as the classical SHBG, characterized from other species [20]. The dissociation constant obtained in the present study was 3.63 nM for testosterone and around 600 nM for estradiol, similar to

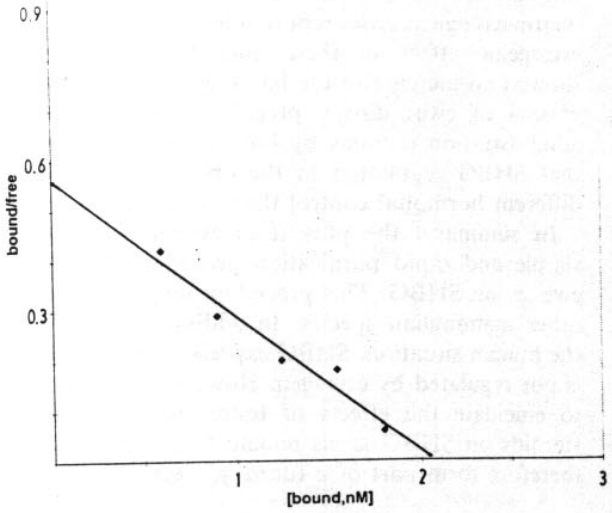

Fig. 3. Scatchard analysis of purified SHBG from ram serum. Labeled testosterone was used as described in Section 2. The K_D was estimated at 3.63×10^{-9} M $\pm 0.15 \times 10^{-9}$ M.

what has been reported for whole ram serum binding: 3.5 nM for testosterone and 534 nM for estradiol [20]. Published and unpublished observations from our group have shown that this method applies also to other species like human [30], bull and goat (manuscript in preparation) and may also apply as a method of purification of SHBG in general. The conventional techniques used so far for the purification of SHBG from other species are time consuming and are based

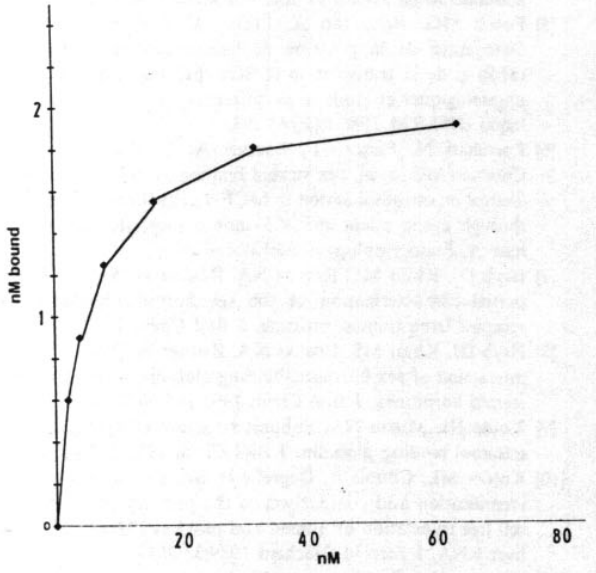

Fig. 4. Binding kinetic of pure SHBG from ram serum with testosterone. The bound hormone represents specific binding. The conditions are described in Section 2.

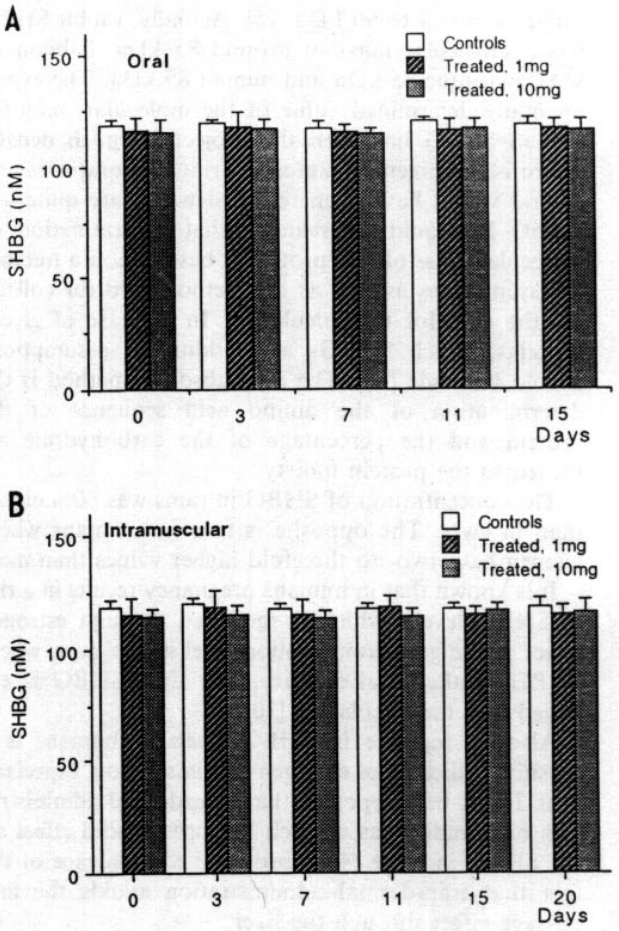

Fig. 5. Mean values $\pm$ S.D. ($n = 6$) of SHBG concentration in the serum of control and treated rams after oral (A) or intramuscular (B) administration of 1 or 10 mg estradiol benzoate.

on more complicated methods like affinity chromatography, preparative electrophoresis and require at least 2 days of work [14]. The yield of the purified protein in the present study was relatively good (26.7%). Reports from other studies in rabbit and human SHBG employing a combination of techniques, have shown a 18–32% yield in rabbit protein [9,12] and 63% yield in human protein [15]. Since rabbit SHBG is also an androgen binding protein, it is possible that purification procedures for the androgen carriers result in lower yield than human protein which binds testosterone and estradiol with high affinity. Although detailed studies have not been undertaken, the lower yield of the androgen binding proteins (the present and other studies) compared to human protein may imply that SHBGs that bind androgen much more than estrogen, are more labile than SHBGs that bind androgen and estrogen equally well.

Our data demonstrate that the molecular mass of ram and ewe SHBG was around 80 kDa. The molecu-

lar mass of SHBG characterized from other species ranges from 84 to 90 kDa [18]. Actually, rabbit SHBG has a molecular mass of around 85 kDa, baboon 84 kDa, macaque 85 kDa and human 85 kDa. The experimentally determined value of the molecular mass for human SHBG has been the subject of much debate. There is disagreement among various laboratories and several values have been reported which are quite different. It should be realized that determination of molecular mass of any protein is based upon a number of assumptions as well as the method used for collecting the data for the calculation. In the case of glycoproteins, which SHBGs are, additional assumptions should be made [12]. The only absolute method is the determination of the amino acid sequence of the protein and the percentage of the carbohydrate attached to the protein moiety.

The concentration of SHBG in rams was 60% higher than in ewes. The opposite is true for humans where women have two- to threefold higher values than men.

It is known that in humans pregnancy results in a rise in SHBG levels, which is mediated through estrogen effect at the gene transcription level in the liver, where SHBG synthesis takes place, and then SHBG is excreted into the circulation [10].

Also an increase in SHBG levels in humans is a sensitive indicator of estrogen administration, especially oral. It has been reported that transdermal administration of estradiol has a much less pronounced effect on the SHBG increase [4], a probable consequence of the fact that transdermal administration avoids the first passage effect through the liver.

In this study, we addressed the question of what is the effect of estrogen in the regulation of the SHBG levels in ewes and rams and we looked at two different situations that both have to do with increased estrogen levels in the body, i.e. an endogenous situation like pregnancy and a exogenous situation, which is estrogen

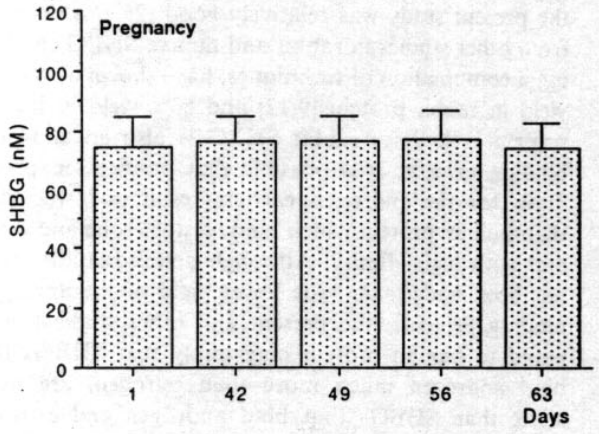

Fig. 6. Mean values ± S.D. ($n = 6$) of SHBG concentration in serum of pregnant ewes.

administration by two routes: oral which affects liver and intramuscular which bypasses first pass effect through the liver. The doses administered represent pharmacological doses reported in the literature to have estrogenic effect in these animals [2]. Our results showed no increase in the basal levels of SHBG in the plasma of ewes during pregnancy or after estrogen administration in rams by both routes. This indicates that SHBG regulation in the above species is under different hormonal control than in humans.

In summary, the present investigation describes a simple and rapid purification procedure for ram and ewe serum SHBG. This procedure might also apply in other mammalian species. In addition, in contrast to the human situation, SHBG expression in ewe and ram is not regulated by estrogen. However, detailed studies to elucidate the effects of testosterone and/or other steroids on SHBG levels remain to be established and therefore form part of a future project.

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