Some characteristics of the uterine progesterone receptor and the effects of norethindrone on conception in the rock hyrax (*Procavia capensis)*

by

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Ene vir die dassie

Doer bo op die skurwe krans
duisend myl van die hemeltrans
sit twee dassies teen die son
en luister hoe Awie trompetter speel
dassiedier vat jou plesier
moenie wag tot môre
dassiedier jy's nou nog hier
wie weet waar's jy môre
koes vir die klou op die skurwe krans
duisend myl van die hemeltrans

Boerneef
(Uit: Pallisandryne)

ABSTRACT

The mean dissociation constant (± SEM) of the uterine progesterone receptor for progesterone in eight rock hyraxes, culled at Elandsrand Mine Reserve, was 1.78 nM (±0.107). The receptor also showed a high relative binding affinity (RBA) for the synthetic hormones norethindrone (%RBA = 185), levonorgestrel (%RBA = 179) and RU486 (%RBA = 221). The binding of norethindrone is similar to that of the African elephant and thus allows for the use of the hyrax as a biological model for the development of a contraceptive for the elephant as an alternative to elephant culling. Norethindrone and cholesterol (placebo) implants in 18 captive hyraxes resulted in zero pregnancies for the seven animals receiving norethindrone, five pregnancies in the eight animals receiving cholesterol and three pregnancies in the three animals receiving no implants. Circulating oestradiol-17β and progesterone concentrations of the mid-pregnant and non-pregnant animals were similar to those in free ranging hyraxes, suggesting that norethindrone had no effect on the availability of these hormones. Histological investigation of selected organs (adrenals, liver, spleen, uterus and ovaries) showed that the effects of norethindrone were limited to the endometrial and ovarian tissue. The endometrium showed atrophic glands and proliferating stromal cells while the ovaries were inactivated. This indicated that norethindrone possibly interferes with both the uterine environment and ovulation, thus preventing conception. This study indicates that norethindrone has contraceptive abilities in the rock hyrax that can potentially be extrapolated to the African elephant, in the search for a contraceptive to manage high population growth rates.

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CHAPTER 1

INTRODUCTION

The rock hyrax (*Procavia capensis*) is also known as the "dassie" or "cony" from the Bible "...the rocks are a refuge for the conies..." --Psalm 104 :18. In Hebrew it is called "shafan" or "shaphan", which translates loosely into "(the) hidden one", and in Arabic "wabara" or "wbr", which translates into "(the) animal covered with thick hair".

Reproductive biology of the rock hyrax

The rock hyrax is a small herbivorous subungulate that occurs commonly in montane and rocky areas throughout most of southern Africa. Fluctuations in hyrax population densities are common, even in areas where their natural predators have been introduced (Kolbe 1967). In some areas there is a need to control their numbers as they have become pest species (Eley 1994).

In South Africa hyraxes are seasonal breeders and males and females have a short period of sexual activity during the second half of the summer (Millar 1971). The time of mating and parturition is influenced by latitude, with colonies from lower latitudes characterized by a delay in mating and thus parturition (Millar 1971). Hyraxes also have an unusual long gestation period of 270 days (7.5 months) for their body size of 2-3kg. (Millar 1971). The males are true testicond and the females exhibit an oestrus cycle of 13.42 ±1.31 days (Gombe 1983). Litter sizes vary from one to six, and middle-aged females (2-8 years) have significantly larger litters than younger and older females (Fourie & Perrin 1987). Females attain puberty when 16-17 months old (Fourie & Perrin 1987).

The hyrax is a gregarious species and colony size varies according to the availability of food and space (Skinner & Smithers 1990). A territorial male can monopolize 3-17 females (Hoeck, Klein & Hoeck 1982). The social organization is that of a female-bonded group with a single territorial male,

and a few peripheral males associated with the polygynous group (Fourie & Perrin 1987). Juveniles stay within the colony until old enough to disperse. Males are forced to disperse as females disperse voluntarily at a later age (Skinner & Smithers 1990).

The hyrax and the African elephant

Greyling, van Aarde and Potgieter (1997) measured the binding affinities of the progesterone receptor in the uterus of the African elephant. They found no significant difference in the binding affinities across the various reproductive stages of the elephant. Their study also revealed that 5α-pregnane-3,20-dione has a relatively high binding affinity (43%) for the progesterone receptor in the elephant. Similar studies by Meyer, Jewgenow & Hodges (1997) found the relative binding affinities of 5α-pregnane-3,20-dione and that of progesterone to be equivalent in the elephant endometrium. Both studies concluded that 5α-pregnane-3,20-dione may be of greater biological importance in maintenance of pregnancy than progesterone (see Hodges, Heistermann, Beard & van Aarde 1997; Greyling *et al.* 1997; Meyer *et al.* 1997). Greyling *et al.* (1997) identified the synthetic hormones norethindrone and levonorgestrel as potential contraceptives based on their relatively high binding affinities (293% and 24% respectively) for the progesterone receptor in the uterus of the African elephant. To further investigate this possibility, *in vivo* trials need to be run on several elephant cows. These trails will involve treatment of the cows with the synthetic hormones and close monitoring of the effects it might have on the reproductive system and behaviour. Because the effects of these treatments are not presently known such trails on elephants may for ethical reasons not be acceptable.

The controversy surrounding the conservation of the African elephant cumbers the use of live specimens in the development of a contraceptive as an alternative to culling. To circumvent this, we opt to find a biological model for the elephant in the form of the rock hyrax.

The phylogenetical relationship between the elephant and the hyrax is one of disagreement and debate. The Paenungulate Hypothesis (the grouping the hyrax, elephant and dugong) was first

suggested by Cope in the late 1800's. An opposing hypothesis, however associates hyraxes with Perissodactyls (Fisher 1989). Morphological data link the Hyracoidae with either the Perissodactyla or the Tethyteria (elephant and dugong) (Shoshani 1991). Molecular data however, only support the latter (Shoshani 1991) and classify *Procavia* as a sister group to *Loxodonta* and *Dugong* (Springer & Kirsch 1993; Shoshani 1991) and amplifies the monophyly of the Paenungulata. In addition to this a close serological relationship (Weitz 1953) and similarities in the electrophoretic behaviour of haemoglobin (Buettner-Janusch & Buettner-Janusch 1964) between the hyrax and the African elephant have been identified. Of importance to the present study, however, is that a phylogical relationship exists between these two species.

Preliminary investigations, also done in our laboratory, suggested that there might be similarities between the progesterone receptor of the elephant and the hyrax. Pilot competitive binding assays in the hyrax showed similar binding affinities for progesterone and norethindrone and weaker affinities for the 5α-reduced metabolites at 50% binding saturation (S. Mecenero, pers. comm.). This suggests that the hyrax receptor might react biologically in the same way as that of the elephant when exposed to progesterone antagonists. Thus, potentially a contraceptive for the elephant could be developed and assessed on the hyrax as an intermediate step before experimenting on elephants. If possible side-effects or failure is to occur, the effect should be measurable in the hyrax and may be extrapolated to the elephants.

Contraception as a wild life management tool

The conflict between humans and wildlife is not a new phenomenon, and the management of wildlife populations is often complicated by political, practical and economical concerns. Current methods of controlling large numbers of animals include poisoning, fumigation, shooting, trapping, mustering, commercial harvesting and translocating. Because most of these methods are based on killing it is becoming increasingly unacceptable and unethical. Oppose to that, contraception offers a

more humane and potentially effective method of controlling animal numbers (Artois 1997). The most popular types of female contraception for wildlife investigated to date, include either the use of antibodies against sperm recognition and attachment (immunocontraception) or the use of synthetic steroid hormones (orally or implanted) that prevent the implantation of the blastocyst or suppress ovulation.

The ideal contraceptive should: cost effectively cause a decrease in population size, be easy to administer, reversible, biologically active for a long period in a living animal, biologically inactive in a dead animal, non-toxic, have minimal effects on the behaviour and social status of an individual and require minimal handling of the target animal. This is a tall order when considering that fertility control may not always cause a reduction in population size, when processes such as improved survival of juveniles or adults and territorial behaviour overrides the effect of lower fertility (Sinclair 1997). Other drawbacks of contraception is that it is impractical for large game species in comparison to hunting or culling which is species-specific, efficient, reversible and cheap (Artois 1997; Whyte, van Aarde & Pimm 1998). The adequate dose rate for a long-term administration of a drug also poses difficulties (Remfry 1978). The most important drawback probably is the cost-effectiveness of the techniques.

Some success using steroid progestins as contraceptives has been reached on the population of free-ranging lions (*Panther leo*) in Etosha National Park (Orford, Perrin & Berry 1988), where the long-acting contraceptive melangesterol acetate prevented pregnancy. This procedure was reversible and apparently did not affect the behaviour of the animals. The overpopulation of white-tailed deer has also triggered the search for a steroid contraceptive. The orally administered microencapsulated estrogen, diethylstilbestrol (DES), prevented pregnancy in the deer, but the need for high doses and the likelihood of prompt rebreeding after abortion makes it not useful in population control (Matshke 1977a). Two oral synthetic progestins (MGA and DRC 6246) were unsuccessful in preventing pregnancy in white-tailed deer and supported the hypothesis that exogenously administered progestins are not accumulated in sufficient quantities in the body fat to provide prolonged hormonal storage

(Matschke 1977b). Steroid implants (estrogen DES and progestin DRC-6246) successfully prevented pregnancy in white-tailed deer but the short life expectancy and high costs of the implants and capturing of animals made them impractical for controlling free-ranging deer (Matschke 1977c; Matschke 1980).

Immunocontraception using porcine zona pellucida (PZP) antigen has also been intensely investigated in white-tailed deer and feral horses. Turner, Liu & Kirkpatrick (1992; 1996) demonstrated that the use of this method in white-tailed deer can produce contraception for at least one breeding season. In the feral horse contraception was also effective for one year and booster injections prolonged the effect, the method was also reversible (Turner, Liu, Rutberg & Kirkpatrick 1997). However, Kirkpatrick, Liu, Turner, Naugle & Keiper (1992) found that three consecutive years of PZP treatment may interfere with normal ovarian function by depressing oestrogen secretion. Another serious side-effect of this method was illustrated by Paterson, Koothan, Morris, O'Byrne, Braude, Williams & Aitken (1992) in marmosets. The use of high titer antibodies against PZP was inevitably associated with the progressive depletion of the primordial follicle pool within one or two years. This ovarian pathology makes the method unpopular since the treatment removes animals permanently from the gene pool and is then in effect similar as culling (Whyte *et al.* 1998).

Contraception may also play an important role in the management of diseases and pest species (Peterson 1991). Because fertility control does not depend on natural mortality to reduce numbers, it seems wise to use conventional control in combination with contraception in long-lived species (Barlow 1997). Mathematical models exist to determine, before hand, the efficiency of a contraceptive effort on a specific population (Barlow 1997, Pech, Hood, McIlroy & Saunders 1997). This is of importance when a long-acting contraceptive is considered. However, these models usually lack field data and can only be used as a guideline because the specified criteria for success often is stringent and difficult to achieve.

Attempts at controlling the fertility of the African elephant had recently made progress when some public outcry put a halt to the annual culling program in the Kruger National Park. The destructive feeding behaviour of African elephants is said to be harmful to the biodiversity (Cumming, Fenton, Rautenbach, Taylor, Cumming, Dunlop, Ford, Hovoka, Johnstohn, Kalcounis, Malangu & Portfors 1997). In the Kruger Park this is also a reality as the rising number of elephants is turning the park into a wasteland that poses a thread to the biodiversity. Two contraceptive methods were tested on free-ranging elephant cows as an alternative to culling in the Kruger National Park. The hormonal attempt of slow-release oestrogen implants was terminated soon after social problems where detected in the herd (Butler 1998, Whyte & Grobler 1998). The problems included prolonged heat in the cows that resulted in constant unwanted attention from the bulls and negligence of the calves by the cows. The immunocontraceptive attempt, however, looked more promising in preventing pregnancy by creating a hostile environment for sperm binding to an antibody-saturated ovum (Butler 1998). But, the detrimental side-effects of this method, as mentioned above, has not been investigated in the elephant cows and still poses a problem. The present study attempts to provide an acceptable progestin-based contraceptive for the elephant, but is still in its developing phase were extensive research is vital before testing it on free-ranging elephant cows is even considered.

Although the practicality of using a contraceptive as a wildlife management tool in large elephant populations, as in Kruger National Park, may seem slim (Whyte *et al.* 1998), this should not hinders nor cumbers the research in this particular field, as it will contribute to the management of small game reserves.

Aims of the study

The present study investigates the effects of antiprogestins on the reproductive system of the hyrax as a model for potential similar developments in the African elephant. The binding affinity of the uterine progesterone receptor of the hyrax will be compared to that of the African elephant in terms of

ligand specificity. Should one of these antiprogestins show a contraceptive potential in the hyrax, hyraxes will be treated with it and the effects will be closely monitored. The information gained in this study will be a step in the ultimate development of a contraceptive for the African elephant. The objectives of the present study are as follows:

- to determine the binding affinity of the uterine progesterone receptor of the rock hyrax for progesterone.
- to investigate the ligand specificity of the uterine progesterone receptor of the rock hyrax.
- to determine the effect of norethindrone implants on the reproduction of the rock hyrax.
- to investigate the possible histopathological effects of norethindrone implants in the rock hyrax.

CHAPTER 2

THE BINDING AFFINITY AND LIGAND SPECIFICITY OF THE UTERINE PROGESTERONE RECEPTOR

Introduction

Progesterone in general

Steroids are a class of organic compounds that are found throughout the whole animal and plant kingdom. Among these compounds there are two groups of hormones, the sex hormones and the adrenocortical hormones. The sex hormones comprise of progestogens, androgens and estrogens. Progesterone is the most common of the progestins and in mammals it is referred to as the "hormone of pregnancy" (Heap & Flint 1979). In short, progesterone maintains implantation of the blastocyst by preparing the uterus (endometrial proliferation), inhibits ovulation during pregnancy (blocks release of pituitary gonadotropins), maintains the developing conceptus, and increases lobular-alveolar growth of the mammary gland. At the end of gestation, parturition and lactation may also be regulated by a decrease in secretion rate of progesterone (Heap & Illingworth 1977). Extra genitally, progesterone is thermogenic and causes a rise in body temperature of 0.5 to 1 °C in women. Other effects of progesterone include promotion of water excretion and vascular contraction (Cheesman 1982). Large doses of progesterone are hypnotic and some of its derivatives can be of an anaesthetical use (Heap & Flint 1979). Progesterone has also been called "the useless hormone" because it rarely acts alone (Heap 1973). This synergism between progesterone and estrogen has been reviewed many times (Heap 1973, Heap & Illingworth 1977).

The biosynthesis of all steroid hormones starts off with the conversion of three molecules of acetate to cholesterol. From this cholesterol gets converted to pregnenolone (enzyme system is situated in adrenocortical mitochondria), this conversion is often seen as the control point of the entire steroid

biosynthesis process (Fotherby 1964). Progesterone is metabolized from pregnenolone by means of oxidation (Cheesman 1982). See Appendix I for details.

Progesterone is a 21-carbon with the basic steroid structure – four ring system of cyclopentanoperhydrophenanthrene (Cheeseman 1982) - with a double bound between C_4 and C_5, and carbonyl groups at C_3 and C_{20}. Sites of progesterone synthesis in mammals include the ovaries and placenta. Progesterone metabolism produces many closely related metabolites. At least 26 metabolites originate from the reduction of the C_4 double bound and the C_3 and C_{20} oxo-groups of progesterone, but oxidation could theoretically yield nearly 1000 metabolites (Heap & Flint 1979). The main metabolites include 5α-pregnanedione, 5β-pregnandione, 5β-pregnane-3α,20α-diol, 20α-hydroxypregn-4-en-3-one, 3β-hydroxy-5-pregnan-20-one and 17α-hydroxyprogesterone. See Appendix I for details. From here a commonly asked question arises: " Is progesterone metabolized to analogous compounds more active than their parent substance?" (Heap & Illingworth 1976).

Although progesterone is secreted in large quantities during pregnancy and is essential for maintaining pregnancy, this is not true for all animals. In some cases, such as the African elephant and the rock hyrax the role of progesterone seems to be reduced. These animals have low plasma progesterone concentrations during pregnancy (Heap, Gombe & Sale 1975, Hanks & Short 1972). In the corpus luteum of the rock hyrax the progesterone concentration is appreciable, but in the plasma very low concentrations were measured during pregnancy (Heap *et al.* 1975). In the African elephant progesterone concentrations are very low in both cases (corpus luteum and plasma) (Hanks & Short 1972; Plotka, Seal, Zarembka, Simmons, Teare, Phillips, Hinshaw & Wood 1989; McNeilly, Martin, Hodges & Smuts 1983; De Villiers, Skinner & Hall-Martin 1989). In both these species pregnancy may be supported by a metabolite of progesterone, rather than progesterone itself (Heap *et al.* 1975). This possibility has been intensely investigated in the African elephant. Heistermann, Beard, van Aarde & Hodges (1994) and Hodges *et al.* (1997) reported high levels of 5α-dihydroprogesterone in the corpus

luteum, these levels exceed those of progesterone by two orders of magnitude. In the plasma, 5α-dihydroprogesterone concentrations were also significantly higher than those of progesterone (Ford 1999; Greyling, Ford, Potgieter & van Aarde 1998). This suggests that the metabolite, 5α-dihydroprogesterone, may be of greater importance than progesterone during gestation in the elephant (Greyling *et al.* 1997, 1998).

The hyrax has an unusual combination of characteristics. It is small (3.5 kg) but has a long gestation period (7.5 months) and low progesterone concentrations during pregnancy. Rapidly decreasing levels of progesterone in freshly collected hyrax blood lead to the suggestion that the hyrax utilizes erythrocytes to metabolize progesterone (Heap *et al.* 1975; Makawiti, Osaso & Gombe 1991). The metabolites formed are mainly 5α-dihydroprogesterone and 5β-dihydroprogesterone (Makawiti *et al.* 1991, Mecenero 1998). This suggests that these metabolites may be more important than progesterone during pregnancy. The extent of progesterone metabolism also changes with the reproductive status, pregnant females converting more progesterone than non-pregnant females and males (Makawiti *et al.* 1991).

Progesterone receptor

Progesterone is transported from the site of synthesis to the target tissue. The uterus is the main target site, but besides that progesterone also targets cells in the Fallopian tubes and oviduct, the cervix, the mammary glands, central nervous system, temperature and respiratory centers and the pituitary gland (Krett, Edwards & Horwitz 1988). At all these sites, the progesterone must first be recognized by the cells, before a response can be brought about. The biochemical structure that recognizes progesterone by binding to it is the progesterone receptor.

The progesterone receptor was first discovered in 1969 by O'Malley, McGuire, Kohler & Korenman (1969) and has since been detected in many mammals. It has been studied in greatest detail

in the chick oviduct (Sherman, Corvol & O'Malley 1970; Schrader, Toft & O'Malley 1972). Research on the uterine progesterone receptor has recently been completed for the African elephant (Greyling *et al.* 1997 and 1998), but no such information is available for the hyrax.

In general all steroid receptors are acidic (P_I ~ 5.0), asymmetric, globular proteins, possibly metalloproteins with sedimentation values of 6-8S (low salt) and 3-4S (high salt) when exposed to sucrose density gradient centrifugation (Jensen & De Sombre 1972; Gorski & Gannon 1976). In the study on the chick oviduct it appears that the progesterone receptor composes of two types of steroid binding proteins. The two forms are termed subunits A and B, this has also been identified in the rabbit and the human (Loosfelt, Atger, Misrahi, Guichon-Mantel, Meriel, Logeat, Bearous & Milgrom 1986; Misrahi, Atger, d'Auriol, Loosfelt, Meriel, Fridlandsky, Guichon-Mantel, Galibert & Milgrom 1986). The natural cellular existence of these two units remain controversial (Krett *et al.* 1988). The whole progesterone receptor, however, exists as two different forms: 1) Untransformed (8S) progesterone receptor (prior to hormone binding) and 2) Transformed (4S) progesterone receptor (after hormone binding). Both forms may be complexed with additional proteins (Krett *et al.* 1988).

A generalized model for the steroid hormone-receptor interaction has been presented by various scientists (Gorski & Gannon 1976). This general model (see Appendix II for diagram) predicts that free or protein bound progesterone (or any steroid hormone) passes from the extracellular space to the cytoplasm (probably by simple diffusion) (King 1982). Activation of the progesterone receptor (in cytoplasm) is associated with dramatic conformation changes brought about by progesterone. The progesterone converts the progesterone receptor from a non-DNA binding, untransformed state to a DNA-binding transformed state where the progesterone is complexed with the progesterone receptor. (Detail to follow). This whole complex is then translocated into the nucleus of the cell. Here it binds to the acceptor and effector sites at the promotor region of progesterone responsive genes. This changes the gene expression so that transcription and translation of proteins that alter cell function proceeds (King 1982).

The most popular theory on the activation of the receptor by progesterone is that of Baulieu (1989). It states that prior to hormone (progesterone) binding the progesterone receptor is in the 8S form (untransformed) and attached to two other proteins: 1) a 90kD heat shock protein, hsp90, and 2) a nuclear protein, p59 (sometimes referred to as the 72kD protein (Krett *et al.* 1988)). In this untransformed state the receptor cannot bind to the DNA because the hsp90 caps the DNA binding domain of the receptor. The p59 binds hsp90. (See Appendix III for details). Hormone binding induces a conformational change in the ligand binding domain of the receptor. This change is frequently described as allosteric (Gorski & Gannon 1976). This change results in the dissociation of hsp90 and p59. Now the DNA binding domain of the receptor is exposed and the receptor can bind to the hormone response elements of the DNA (Baulieu 1989).

The biochemical aspect of the two receptor forms and the effect of the hormone have also been investigated. Logeat, Le Cunff, Pamphile & Milgrom (1985) described the untransformed state of the receptor as a solubilized, "cytosolic" receptor that undergoes a phosphorylation reaction in the absence of the hormone. It can thus be termed a phosphoprotein. The second phosphorylation step is hormone dependant and results in the transformed or DNA bound, "nuclear" receptor and can be termed a polyphosphoreceptor (Logeat *et al.* 1985). The role of the hormone here again seems to be to prepare the receptor for DNA binding. The succession of events leading to hormone action may be summarized as follows:

Receptor (newly synthesized)

↓ basal phosphorylation

Phosphoreceptor (cytosolic, untransformed, non-DNA binding)

hormone → ↓ hormone-dependent phosphorylation

Polyphosphoreceptor (nuclear, transformed, DNA binding)

↓

Gene regulation

Hormone-dependent transformation is a very rapid process (Krett *et al.* 1988). The conformation changes (mentioned earlier) and the phosphorylation reactions are not necessarily mutually exclusive. Binding and dissociation of proteins and ATP binding and phosphorylation may all occur simultaneously (Krett *et al* 1988).

Progesterone analogs and contraception

The steroid binding domain of the receptor appears to be confined to a relatively small (2-4 x 10^4Da) portion of the receptor molecule (Sherman, Pickery, Rollwagen & Millar 1978) The primary force that promotes binding between the receptor and the steroid is a hydrophobic "pocket" that the receptor forms around both planar faces of the steroid (King 1982). The binding specificity is determined by the hydrophobic side groups of the steroid, the size of these groups and the conformation (planarity) of steroid ring A (King 1982).

In light of the above it is clear that the binding success of a steroid to a receptor depends greatly on the molecular structure of such a steroid. This lead to the production of molecules that chemically resembles the binding part of progesterone, and like progesterone it binds tightly to the progesterone receptor. The only difference is that these molecules block the hormones' usual effects. Molecules of such nature are termed antagonists. Thus, a progesterone antagonist is a compound that occupies the progesterone receptor without inducing the effects of progesterone (synthesis of proteins in the uterus essential to the maintenance of pregnancy) (Ulman, Teutsch & Philibert 1990). Such a progesterone antagonist should then interrupt or prevent pregnancy if it competes successfully with progesterone and "wins" occupancy of the receptor more often than progesterone. The receptor shows a higher binding affinity to such a molecule than to progesterone itself. The correct treatment with this antagonist may lead to the creation of a contraceptive for a particular species.

Many synthetic progestins have been produced and are commercially available. However, the reactions of these potential contraceptives are species and tissue specific and depend on the

characteristics of the uterine progesterone receptor of the specific animal. Research is needed to determine the parameters of the different antagonists before choosing the best option. The present chapter deals with these aspects of the rock hyrax. The binding affinity of the progesterone receptor for progesterone will be measured and compared in terms of relative binding affinity to the binding resembled by progesterone analogs to determine a possible contraceptive. These findings will be compared to that of the African elephant to evaluate the potential use of the hyrax as a biological model for the development of a contraceptive for the elephant.

Materials and methods

Animals and tissue

Twenty-four female hyraxes, shot as part of a population control initiative, were collected at Elandsrand Mine Reserve (27° 45' S 27° 24' E), South Africa, between November 1997 and January 1998. Their uteri were excised, cut into smaller pieces and placed on dry ice within 20 minutes of death. After wrapping the uterine pieces in aluminum foil it was snap frozen in liquid nitrogen. The samples were stored at –70 °C until processed. Sixteen of the females collected were non-pregnant, 6 pregnant and 2 lactating.

Preparation of cytosols

To extract the progesterone receptor from the tissue, a method similar to that described by Greyling *et al.* (1997) was used. The uterine tissue was cut into paper thin slices while on crushed dry ice. For homogenization a 1:5 ratio (m/v) of tissue:$TEDAG_{10}$ buffer was used. The $TEDAG_{10}$ buffer consisted of 10mM Tris HCL (Sigma, St. Louis, USA), 1.5mM EDTA (Sigma, St. Louis, MO), 5mM dithiotreitol (Sigma, St. Louis, MO), 1mM sodium azide (Sigma, St. Louis, USA), and 10% (m/v) glycerol (Sky Chem, Alberton, South Africa) with the pH adjusted to 7.4 at 4°C. To inhibit protease activity associated with the breaking up of cells, Leupeptin (Sigma, St. Louis, USA) at the final

concentration of 10mM was added to the $TEDAG_{10}$ buffer before homogenization. Homogenization (TP Ultra Turrax; Janke & Kunkel, Staufen, Germany), was conducted in three burst of 10 seconds duration, alternated with a 50 seconds cooling period on crushed ice. The collagen rich homogenate was centrifuged (1.05×10^5 g , 30 min, 4 °C) and the clear cytosol was drawn off using a pre-cooled Pasteur pipette. Cytosols were stored at -70 °C until further analysis. The concentration of cytosolic proteins was assayed by the Bradford method (Bradford 1976).

Equilibrium binding assays

Specific binding was determined in duplicate aliquots (100 µl) of cytosol incubated with a concentration series (0.25nM-64nM) of [1,2,6,7-^{3}H]progesterone (^{3}H-P; specific activity 95Ci/mmole; Amersham International, Buckinghamshire, UK). The different concentrations were made up in $TEDAG_{10}$ buffer. Non-specific binding was determined by adding 1000-fold (0.25mM-64mM) excess of unlabelled progesterone (Sigma, St. Louis, MO) to each concentration of labelled progesterone. All incubations had a final volume of 400 µl by the addition of appropriate volumes of buffer. Total radioactive counts were determined by counting 50 µl of the labelled concentration series suspended in 350 µl $TEDAG_{10}$ buffer. Incubations lasted 2 hours at 4 °C after which 100µl of $TEDAG_{60}$ buffer (similar to $TEDAG_{10}$ buffer, but 60% glycerol) was added to all the tubes. Another incubation of 2 hours at 4 °C followed. The addition of 500µl cold dextran coated charcoal (DCC) terminated all reactions. DCC consisted of 0.5% (wt/v) pre-washed, activated charcoal (Merck, Darmstadt, Germany), 0.1% (wt/v) gelatin (Carraggenan Type 1; Sigma, St. Louis, MO) and 0.5% (wt/v) dextran T70 (Pharmacia, Uppsala, Sweden) in $TEDAG_{10}$ buffer. Cold $TEDAG_{10}$ buffer (500µl) was added to total counts tubes. After the termination of the reactions the suspensions were vortexed and left at 4 °C for 10 minutes and then centrifuged (2500 x g; 15 min; 4 °C). The radioactivity in 500µl of the supernatants was counted by adding 4ml scintillation cocktail (Ultima Gold XR, Packard, Meriden,

USA) to a confidence limit of 95% and an uncertainty of 1% using a Tri-carb liquid scintillation counter (CARB 1500; Packard, Meriden, USA).

By means of the COMBICEPT 2000CA software program (Packard Instrument Company, Illinois, USA) Scatchard and saturation curves with the Rosenthal-correction for non-specific binding were drawn. Results from the Scatchard plots were subjected to least square regression analysis and only data with correlation coefficients ≥ 0.9 were considered reliable.

The equation of the saturation curve that is produced by this method is based on the Law of Mass Action and is analogous to the Michaelis-Menton equation used in enzyme kinetics and has the same graphical shape (Figure 1). The data have been transformed to a linear shape to prepare the Scatchard plot (Figure 2). However, it is important to note that there was no significant difference in the dissociation constants obtained by two plots ($t = 0$, $n = 8$, $p = 0.852$). To determine the inter-assay coefficient of variation for the K_d value of progesterone, frozen duplicate samples of the same tissue were included in each assay. The coefficient of variation between assays was 19.6 % and within an assay < 12 %.

Competitive binding assay

Duplicate cytosol aliquots (100µl) were incubated for 30 minutes at 20 °C with 50 µl of [1,2,6,7-^{3}H]progesterone (^{3}H-P; specific activity 95Ci/mmole; Amersham International, Buckinghamshire, UK), which was made up in $TEDAG_{10}$ buffer to a final concentration of 8nM, in the presence of 50µl of increasing concentrations (0.3125mM - 0.1mM) of the following competitors: progesterone (Sigma, St. Louis, MO), norethindrone (Sigma, St. Louis, MO), promegestone (New England Nuclear, Massachusetts, USA), RU486 (donated by Roussel Uclaf, Paris, France), levonorgestrel (donated by SA-Druggists, Port Elizabeth, South Africa), 4-pregnen-20α-ol-3-one

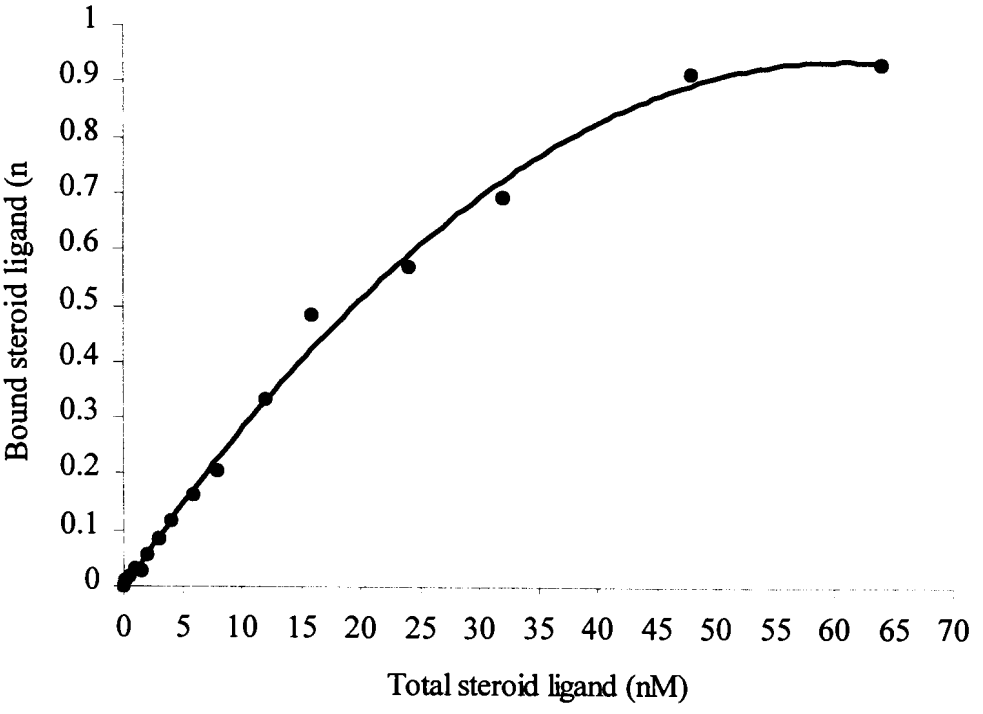


Figure 1. Example of a saturation curve (15 points) of the uterine progesterone receptor progesterone of the hyrax.

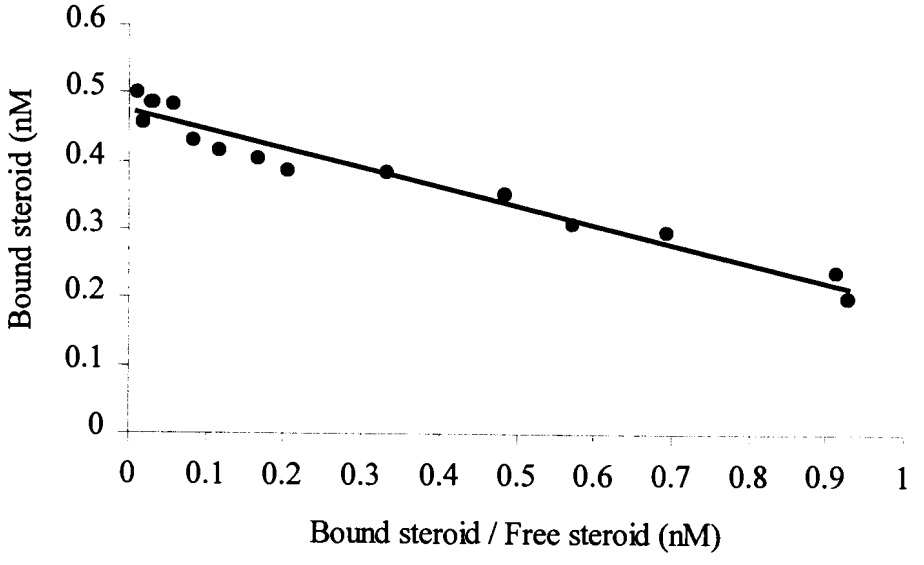


Figure 2. Example of a Scatchard plot (15 points) of the uterine progesterone receptor and progesterone of the hyrax.

(Sigma, St. Louis, MO), 17α-hydroxyprogesterone (Sigma, St. Louis, MO), 5α-pregnane-3α-ol-20-one (Sigma, St. Louis, MO) and 5α-pregnane-3,20-dione (Sigma, St. Louis, MO). All volumes were adjusted to 400µl by the appropriate volumes of buffer. To determine the non-specific binding a set of tubes containing cytosol, [1,2,6,7-^{3}H]progesterone and a 1000 fold excess of unlabelled progesterone was included in each assay. Total binding was determined by tubes containing [1,2,6,7-^{3}H]progesterone and cytosol and total counts by tubes containing only [1,2,6,7-^{3}H]progesterone and buffer. Reactions were terminated with DCC and counted for radioactivity as described for the equilibrium binding assays.

If the specific binding of the assay did not account for > 60% of the total binding, the tissue was regarded as degraded and the results were not used. Displacement curves were constructed by plotting the percent specific bound [1,2,6,7-^{3}H]progesterone against the $\log_{10}$ of the concentration of the competing ligand. These displacement curves were subjected to least square regression analyses and only data with correlation coefficients ≥ 0.9 were considered reliable for the application of the Rodbard equation (1973) to calculate relative binding affinities (RBA). The RBA of each compound was determined at the 50% competition level relative to progesterone. The intra-assay coefficient of variation of the assays (n = 120) never exceeded 5%. To determine the inter-assay coefficient of variation a three-point equilibrium binding assay, using frozen duplicates of the same sample, was included in every assay. This resulted in a inter-assay coefficient of variation of 5,1% (n = 16).

Results

The mean dissociation constant (K_d value (±SEM) of progesterone for the uterine progesterone receptor obtained from both saturation curves and 15-point Scatchard plots was 1.78 nM (±0.107) (n = 8). Promegestone showed the highest competitive binding ability (K_d = 0.691nM; %RBA = 272)

Table 1. Mean ± SE relative binding affinity and apparent K_d values of potential competitors for the uterine progesterone receptor in the hyrax. Sample sizes are presented in brackets.

Competitor	Apparent K_d (nM)	Relative binding affinity (%)
Promegestone	0.691 ±0.112 (3)	272 ±42
RU486	0.859 ±0.147 (3)	221 ±41
Norethindrone	1.072 ±0.268 (3)	185 ±38
Levonorgestrel	1.003 ±0.059 (4)	179 ±11
Progesterone	**1.783 ±0.107 (8)**	**100**
5α-pregnane-3,20-dione	1.949 ±0.239 (3)	94 ±10
4-pregnen-20α-ol-3-one	39.180 ±6.500 (3)	3.553 ±0.62
17α-hydroxyprogesterone	105.758 ±19.171 (3)	1.830 ±0.405
5α-pregnane-3α-ol-20-one	281.278 ±160.59 (3)	0.385 ±0.216

(Table 1). The synthetic progestins RU486 (K_d = 0.859nM; %RBA = 221), norethindrone (K_d = 1.072nM; %RBA = 185) and levonorgestrel (K_d = 1.003nM; %RBA = 179) also exhibited a higher affinity for the progesterone receptor than the natural ligand (K_d = 1.783nM; %RBA = 100). The natural progestins 4-pregnen-20α-ol-3-one (K_d = 9.180nM; %RBA = 3.553), 17α-hydroxyprogesterone (K_d = 105.758nM; %RBA = 1.830), 5α-pregnane-3α-ol-20-one (K_d = 281.278nM; %RBA = 0.385) all showed competitive abilities weaker than that of progesterone. The binding of 5α-pregnane-3,20-dione (K_d = 1.949 nM; %RBA =94) was very similar to progesterone (Table 1). The displacement curves (Figures 3 and 4) illustrate the binding abilities of each compound relative to progesterone. The compounds that out compete progesterone on receptor level are found left to the progesterone curve on the graph and the compounds with a weaker binding performance are present right of the progesterone curve.

Discussion

Differences in the interaction of the progesterone receptor and the hormone (King & Mainwaring 1974), result in the different effects that the same hormone may have in different species (Grey & Leavitt 1987). Thus, it is important to determine the receptor characteristics of a certain species before applying a synthetic hormone, as different responses can be expected across species. In the present investigation the characteristics of the uterine progesterone receptor of the hyrax were investigated and will now be compared to that of the African elephant. Prior to this study nothing was known about the binding abilities of the uterine progesterone receptor of the hyrax.

In the African elephant norethindrone has a relative binding affinity (293%) almost three-fold that of progesterone (Greyling *et al.* 1997). In the hyrax this value is almost two-fold, suggesting that norethindrone may have contraceptive abilities in both species, as is the case in the human, rabbit and

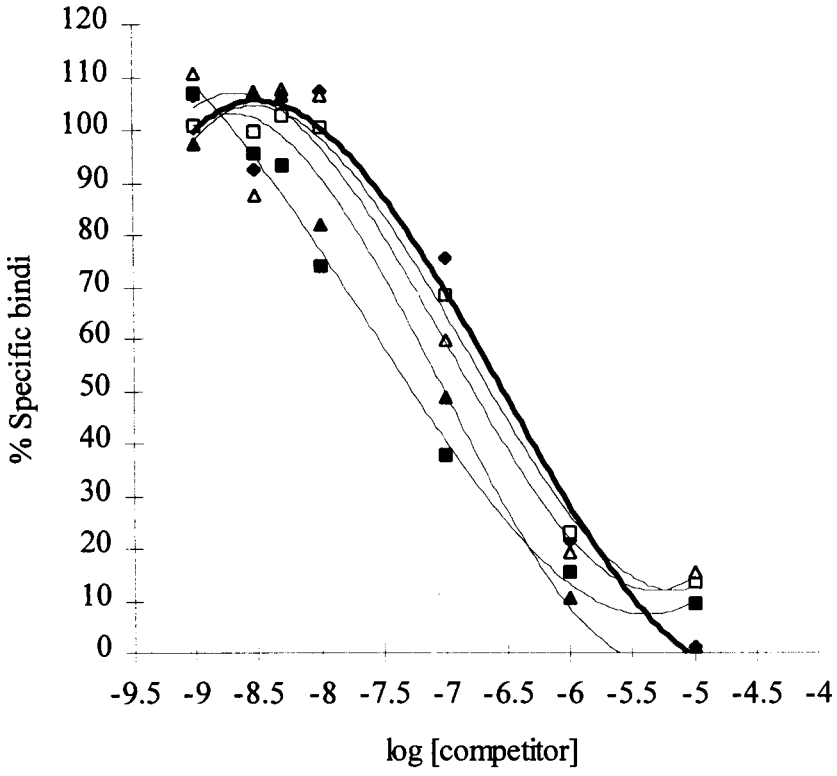


Figure 3. Displacement curves illustrating the competition of various ligands for binding to the uterine progesterone receptor in the rock hyrax: promegestone (■), RU486 (▲), norethindrone (Δ), levonorgestrel (□), progesterone (◆).

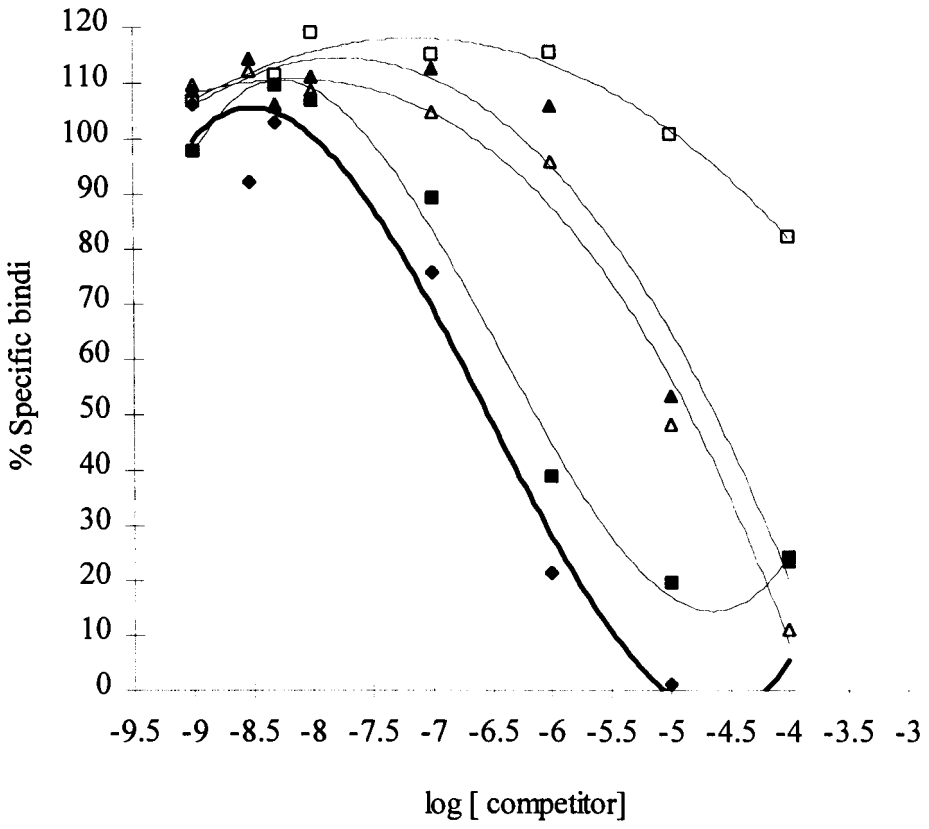


Figure 4. Displacement curves illustrating the competition of various ligands for binding to the uterine progesterone receptor in the rock hyrax: progesterone (◆), 5α-pregnane-3,20-dione(■), 4-pregnen-20α-ol-3-one (Δ), 17α-hydroxyprogesterone(▲), 5α-pregnane-3α-ol-20-one(□).

ewe (Kontula 1975). The hamster and the guinea pig however, are intolerant to norethindrone and have low affinities for this progestin (Wilks, Spilman & Campbell 1980; Maclaughlin & Westphal 1974). Thus, species differences do occur for binding affinities for norethindrone in the uterus of different animals. This cautions against the utilisation of this synthetic steroid across species before the binding behaviour has been examined individually in each species (Grey & Laevitt 1987). The binding of norethindrone in the uterine tissue of the hyrax and the elephant, however, proved to be similar and this values the use of the hyrax as a model for the elephant.

In the hyrax, whole blood metabolizes progesterone to 5α-dihydroprogesterone (Mecenero 1998) and results in circulating 5α-dihydroprogesterone concentrations up to 17 times higher than that of progesterone (Mecenero 1998). Whole blood also formed more of 5α-dihydroprogesterone during pregnancy than during non-pregnancy (Mecenero 1998). Both these findings suggest that 5α-dihydroprogesterone, rather than progesterone, may be related to the maintenance of pregnancy in the hyrax. The almost equivalent competitive abilities of 5α-dihydroprogesterone and progesterone for the progesterone receptor in the endometrium of the hyrax not only supports the biological importance of 5α-dihydroprogesterone, but also suggests the involvement of 5α-dihydroprogesterone in the maintenance of pregnancy. A similar situation exists in the African elephant with circulating progesterone levels well below that of other mammals and that of 5α-dihydroprogesterone (Hodges *et al.* 1997; Ford *et al.*; Heistermann *et al.*1994). Similar relative binding affinities for 5α-dihydroprogesterone and progesterone also exist in the endometrium of the elephant. These two species seem to exhibit hormonal characteristics and functions different to most mammals but similar to each other.

Although 4-pregnane-20α-ol-3-one is metabolised in relatively large amounts by the placenta of the hyrax and may be involved in the in the control and/or maintenance of progesterone production and metabolism during pregnancy (Mecenero 1998), this compound did not target the uterus through the

progesterone receptor. However, 4-pregnane-20α-ol-3-one targets the placenta and prevents the formation of progesterone in the cow, goat and rhesus monkey (Wiener 1976). Similar low relative binding affinities for 4-pregnane-20α-ol-3-one have also been recorded in the uterus of the African elephant (Greyling *et al.* 1997).

In the hyrax 17α-hydroxyprogesterone is metabolised from progesterone in the luteal tissue (Mecenero 1998) and 5α-pregnane-3α-ol-20-one is produced by both red and white blood cells (Heap *et al.* 1975; Mecenero 1998). These two natural hormones are also the main progesterone metabolites excreted in the faeces of the hyrax (personal observation) suggesting that they are waste products with no apparent function. The poor performances of 17α-hydroxyprogesterone and 5α-pregnane-3α-ol-20-one on receptor level in the hyrax support this by suggests that these two compounds may not be important in maintaining uterine functions. Both of these natural steroids also seems of minor value to the uterine progesterone receptor of the elephant (Greyling *et al.* 1997).

The synthetic steroid, RU486, has been reported to be a successful competitive antagonist of progestin action in human (Schaison, George, Lestrat, Reinberg & Baulieu 1985; Ulmann *et al.* 1990), rhesus monkey (Shortle, Dyrenfurth & Ferin 1985), calf (Moudgil, Lombardo, Hurd, Eliezer & Agarwal 1986), rabbit and rat uteri (Gray & Leavitt 1987). This was also the case in the hyrax, but not in the African elephant (Greyling *et al.* 1997), the golden hamster (Gray & Leavitt 1987) and the chick oviduct (Moudgil *et al.* 1986, Groyer, Bouc, Joab, Radanyi, Renoir, Robel & Baulieu 1985). The inability of the hamster progesterone receptor to bind to RU486 has been connected with the high sensitivity of the receptor for the 11 and 17 structural positions of the steroid (Gray & Leavitt 1987). However, the high binding of promegestone to the progesterone receptor of the elephant indicates no sensitivity for the 17 position, but the poor performance of RU486 may indicate an inability to bind to bulky structures on the 11 position. Levonorgestrel, another human contraceptive (Schwarz, Pierce, Walden & Knopp 1992; Masson, Franssen, Hilditch & Powell 1993; Woods, Estherann, Havens,

Merola & Emans 1992) has a high binding affinity for the progesterone receptor in the hyrax, but a low binding affinity in the elephant (Greyling *et al.* 1997). When considering the chemical structure of this progestin in the light of the above it is possible that the bulky structures on either the 11 and/or the 13 positions may indicate that the uterine progesterone receptor binding site of the hyrax is not as sensitive to these areas as that of the elephant when binding to these progestins. See appendix IV for chemical structures.

Though the uterine progesterone receptor characteristics of the hyrax and the elephant show some differences they share a high affinity for norethindrone on receptor level. The contraceptive abilities of norethindrone can now be tested *in vivo* on hyraxes and the results obtained from such an experiment can be useful in the search for an acceptable contraceptive for the African elephant.

Conclusion

The uterine progesterone receptor of the hyrax has a higher relative binding affinity for the synthetic progestins norethindrone, levonorgestrel and RU486 than for the natural ligand progesterone. This suggests that any of these progestins may prevent pregnancy when administered to a female. The natural progestins, 4-pregnen-20α-ol-3-one, 17α-hydroxyprogesterone, and 5α-pregnane-3α-ol-20-one all had relative binding affinities below that of progesterone. The whole blood metabolite 5α-pregnane-3,20-dione however, had values similar to that of progesterone and this might indicate the involvement of this hormone in the maintenance of pregnancy. When the results of the synthetic hormones are compared to the characteristics of the African elephant uterine progesterone receptor, RU486 highlights the differences between the species and norethindrone and levonorgestrel the similarities. Norethindrone, in particular, is very similar to the reaction of the elephant uterine progesterone receptor and thus tends to have contraceptive abilities in both species. This parallelism favours the use

of the hyrax as a biological model for the elephant in the development of a contraceptive as an alternative to culling.

CHAPTER 3

THE EFFECT OF NORETHINDRONE IMPLANTS ON REPRODUCTION IN THE HYRAX

Introduction

Structure and metabolism of norethindrone

Norethindrone (19-norethisteronr-17-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one) is part of a series of synthetic steroid hormones similar to progesterone. Norethindrone is a 20-carbon and has a basic steroid structure. The only structural difference between progesterone and norethindrone is found on the C17 and C19 positions. See appendix IV for structures. The metabolism of norethindrone was most comprehensively studied in the human by oral administration of norethindrone (Braselton, Lin, Mills, Ellegood & Mahesh 1977). The metabolites of norethindrone include 5β-dihydronorethindrone; 3α,5α-tetrahydronorethindrone; 3α,5β-tetrahydronorethindrone; 3β,5β-tetrahydronorethindrone and 3β,5α-tetrahydronorethindrone and is found unconjugated or conjugated either with a sulfate or a glucuronide (Braselton *et al.* 1977). The major route of metabolism involves reduction of the Δ^4 – 3-ketone structure, resulting in the formation of a saturated double bond plus a 3-hydroxyl group (Breuer, Dardenne & Nocke 1960). The contraceptive potential of the norethindrone metabolites have not been investigated.

Contraception

The contraceptive abilities of norethindrone are widely described in humans (Wiese, Marker & Holma 1976; Olind, Moo-Young, Gupta, Weiner & Johansson 1979; Woods *et al.* 1992; Masson *et al.* 1993), mice (Pal & Guha 1990), guinea pigs (Maclaughlin & Westphal 1974) and rhesus monkeys (Jha, Murugesan & Farooq 1995). At doses lower than needed for contraception and in combination with other steroids, norethindrone also has useful therapeutic applications in humans (Taitel &

Kafrissen 1995). In humans norethindrone is commercially used as a post-coital contraceptive for termination of early pregnancies, induction of parturition and medical abortions. However, the mechanism through which this progesterone analog prevents contraception is not clearly known. It was suggested that norethindrone either has estrogenic activity or is converted to a metabolite with such activity (Edgren, Peterson & Jones 1966; McPherson, Costoff, Elridge & Mahesh 1974; Stanczyk & Roy 1990) and that this characteristic causes a steady and highly secretory state in the uterus which may interfere with implantation and/or gamete transport (Pal & Guha 1990). The estrogenic behaviour of norethindrone is further illustrated by the ability of the human liver to transform norethindrone to ethinylestradiol (Yamamoto, Yoshiji, Yasuda, Shiroshita, Kitawaki, Fuji, Urabe, Honjo & Okade 1986). In humans, norethindrone can be taken orally or can be administered to the body through slow-release, subcutaneous implants. It is also known that high doses of norethindrone influences the hypothalamic-pituitary-ovarian function (McPherson *et al.* 1974; Baird 1993; Spitz & Agranat 1995; Singh, Saxena, Raghubanshi, Ledger, Harman & Leonard 1997). High doses of norethindrone inhibits ovulation by suppressing the luteinizing hormone surge responsible for ovulation. In humans norethindrone implants resulted in acyclic patterns of oestrogen, suppressed progesterone levels and anovulation (Wiese *et al.* 1976; Singh *et al.* 1997). Jha *et al.* (1995) recorded normal bleeding patterns in four out of five rhesus monkeys receiving norethindrone implants and concluded that the circulating norethindrone levels were to low to produce anovulation. This characteristic of norethindrone is not as widely researched as its use in termination of early pregnancies, but seems to be a pre-coital method through which pregnancy can be prevented.

In this chapter the contraceptive abilities of norethindrone is tested on a group of female hyraxes. The possible effects of norethindrone on ovarian function are investigated through the measurement of circulating progesterone and oestradiol-17β concentrations. Due to the similarities between the interaction of the uterine progesterone receptor and norethindrone in the African elephant

and the hyrax the results obtained here may be indicative of the effects that norethindrone treatment may have on elephant reproductive output.

Materials and methods

Animals

All procedures were approved by the Ethics Committee of the Faculty of Biological Sciences at the University of Pretoria. Eighteen female and seven male hyraxes captured at Elandsrand Mine Reserve, were kept in an outdoor enclosure that comprised of five cages (4.5m x 2.5m). Each cage contained a sleeping box, adequate shade and branches to climb onto. Their diet consisted of *ad libitum* water, antelope cubes (Epol, South Africa), dried lucerne with fresh lettuce or cabbage leaves provided twice a week. The cages were cleaned weekly. The females were in groups of three or four individuals per cage. Fifteen of the females, kept in four different groups, were separated from the males until the implantation of the norethindrone pellets. Three females, kept as a group were in the company of a male at all times and received no implants.

Preparation of hormonal implants

Norethindrone (Sigma, St. Louis, MO) and cholesterol (Sigma, St. Louis, MO) pellets to the total weight of 125-135mg were prepared by melting and dropping the liquefied steroid into cold, sterile water. The pellets were implanted subcutaneously, on the dorsal part of the neck of the females. The treatment group consisted of seven females each receiving norethindrone implants and the control group of eight females each receiving cholesterol implants. Only after the implantation of the pellets were males introduced to the cages of the females, one male in the company of three of four females. After three months all the females were killed by injecting 2ml/kg euthanasia (Etha-naze solution, Centaur Laboratories, Bryanston, South Africa) into the heart of animals sedated with an intra muscular injection of a 1:1 mixture of ketamine hydrochloride (100 mg/ml; Kyron Labs, Pretoria, South Africa)

and xylazine hydrochloride (100mg/ml; Bayer, Isando, South Africa) at the dose of 1.25 ml/kg. The implanted pellets were removed, dried, and a release rate calculated as the daily reduction in weight of the pellets.

Uteri were dissected, inspected for the presence of foetuses and the gestational age (days) was determined using the equation $t = (\sqrt[3]{w} / 0.047) + 41$, where w = total fetal weight in grams (Millar 1971). Fetal age was used to distinguish between early- (< 78 days) and mid-pregnant (78 to 154 days). No late-pregnant (> 154 days) animals were recorded. The Fisher exact test (Williams 1994) was used to determine if there was any significant difference in pregnancy rates of the treatment and control groups.

Radioimmunoassay of progesterone and oestrogen

Before culling, blood was collected from each sedated animal through cardiac puncture with a hypodermic syringe. The blood was stored on ice in a glass tube (10ml) containing 500µl heparin (600 iu/ml). The whole blood was centrifuged (4°C, 1200 rpm, 15min) and the plasma removed and stored at -20°C until assayed.

Progesterone

The radioimmunoassay used in the present study is similar to that of Van Aarde (1985). Triplicate plasma aliquots (100µl) were extracted in glass test tubes (10ml) using 4ml petroleum ether (Merck, Darmstadt, Germany) and vortexed for 10 min. The extracts were separated from the organic phase through freezing(- 40°C, 1 h) and the organic phase was dried down in a waterbath (37°C) under a stream of nitrogen. 100µl PBS was added to the dried tubes that were vortexed for 1 min. The phosphate buffered saline (PBS; pH~6.9) consisted of 0.1% w/v gelatin powder (uniLAB, Saarchem, Krugersdorp, South Africa), 2.25% w/v di-sodium hydrogen orthophosphate dodecahydrate

($Na_2HPO_412H_2O$.AR; univAR. Saarchem, Krugersdorp, South Africa), 0.61% w/v sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_42H_2O$.AR; univAR. Saarchem, Krugersdorp, South Africa) and 0.9% w/v sodium chloride CP (NaCl; Associated Chemical Enterprises c.c., South Africa). Buffer blanks, ether blanks and quality controls (known amounts of progesterone) in PBS were included in each assay. To determine the efficiency of the extraction process 100µl [1,2,6,7-^{3}H]progesterone (~20 000 dpm/100µl PBS) was added to triplicate aliquots (100µl) of a plasma pool, vortexed and incubated in a waterbath (10 min, 37°C). These samples (recovery estimates) were extracted as described above, but the organic phase was decanted into scintillation vials and once dried, 4ml scintillation cocktail (Ultima Gold XR, Packard, Meriden, USA) was added and the radioactivity counted with a scintillation counter. All extracts were corrected for losses incurred during extraction.

A series of progesterone concentrations (7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000, 2000 pg progesterone/100µl PBS) prepared in triplicate was included in the assay. To each of the reconstituted plasma, buffer and ether blanks, quality controls and standards 100µl antiserum were added (1522; supplied by Prof. R.P. Millar, Department of Chemical Pathology, University of Cape Town, Cape Town, South Africa), raised in a rabbit against progestrone-6-(O-Carboxymethyl)oxime bovine serum albumin, the final dilution of the antibody was ~1:56 000. After vortexing the tubes for 30 sec on a multi-tube vortexer (Model 2601, Scientific Manufacturing Industries, USA) and leaving it at room temperature for 10 min., 100µl [1,2,6,7-^{3}H]progesterone (~20 000 dpm/100µl PBS) was added and the tubes were vortexed again for 30 sec and left at 4°C for ~16h. The non-specific adsorption method using cold dextran charcoal (750µl, 4°C) was used to separate bound and free progesterone. The dextran charcoal consisted of 0.156% w/v activated charcoal (Merck, Darmstadt, Germany) and 0.0156% w/v dextran T70 (Pharmacia LKB, Biotechnology AB, Upsala, Sweden) and was stored at 4°C for 24h prior to use. After adding the dextran-coated charcoal the tubes were vortexed (20 sec), incubated (4°C, 15 min) and centrifuged (4°C, 2500rpm, 15min). The supernatants were decanted into

scintillation vials and 4ml scintillation cocktail was added. The vials were vortexed and incubated at room temperature for 2-3h before the radioactivity was counted. A computer package supplied by Packard (SECURIA^{TM} PLUS RIA/QC Software Package and PC-Data Acquisition and Analysis System, United Technologies, Packard, Illinois, USA) was used to calculate hormone concentrations.

The cross-reactivity of the antibody (as determined by the supplier) was as follows: progesterone 100%; 11β-hydroxyprogesterone 48.3%; 11α-hydroxyprogesterone 26.2%; 5-pregnane-3,20-dione 25.1%; pregnenolone 5.2%; 17β-hydroxyprogesterone 2.6%; 11-deoxycorticosterone 1.9%; 11-deoxycortisol 1.7%; 3-hydroxy-5-pregnane-20-one 0.4%; 20-hydroxy-4-pregnane-3-one 0.3%; cortisol < 0.1%; testosterone, Δ-4-androstenedione, oestradiol-17β and oestrone < 0.001%. Only one assay was conducted and the sensitivity thereof was 35pg progesterone/ml and the non-specific binding 4.6%. The intra-assay coefficients of variation were 5.04 % and 3.22% for 500 pg and 250 pg progesterone/ml, respectively. Recovery estimate was 85%. In our laboratory serially diluted samples of pooled plasma produced a displacement curve parallel to the standard curve.

Oestrogen (Oestradiol -17β)

The radioimmunoassay used in the present study is similar to that described by Van Aarde (1985) was used to determine plasma concentrations of oestradiol-17β. Duplicate plasma aliquots (250 µl and 500 µl) were extracted with 3 ml and 5 ml diethyl ether (Merck, Darmstadt, Germany), respectively, in glass extraction tubes and extracted as described for the progesterone radioimmunoassay. Duplicate aliquots (100 µl each) of ether blanks and quality controls (31.25 pg and 125 pg oestradiol-17β/100 µl PBS) were included in the extraction and assay process. Plasma, ether blank and quality control extract residues were reconstituted in 100 µl PBS. A series of standards (7.8 to 2000 pg oestradiol-17β/100 µl PBS) and a PBS blank was included in each assay in triplicate. Antiserum (E29BI; supplied by Prof. R.P. Millar, Department of Chemical Pathology, University of Cape Town, Cape Town, South Africa)

was raised in a rabbit against a conjugate of oestradiol-6-(O-Carboxymethyl)oxime:BSA and 100 µl aliquots were added to the reconstituted extracts and the standards at a working dilution of 1:25 000 in PBS (final antibody dilution was ~1:260 000). The mixture in each tube was vortexed for 1 min, after which 100 µl [2,4,6,7-^{3}H]oestradiol-17β (~20 000 dpm/100 µl PBS) was added to each tube and vortexed for 1 min. and left at 4°C for ~16h. Dextran-coated charcoal (750 µl) was added to each tube at 4°C to separate the bound steroid from the free steroid. The tubes were vortexed for 20 sec, incubated for 12 min at 4°C and centrifuged (4°C, 2500 rpm, 15 min). Supernatants were decanted into scintillation vials, radioactivity counted and hormone concentrations determined as for the progesterone assay. All values were corrected for extraction efficiency.

The cross-reactivity of the antibody against oestradiol-17β (as determined by the supplier) was as follows: oestradiol-17β 100%; oestrone 0.01%; cortisol 0.005%; deoxycorticosterone 0.002%; pregnanediol and corticosterone 0.001%; 17α-hydroxypregnenolone, androstenedione, progesterone and testosterone < 0.001%. Sensitivity of the assay was 30pg oestradiol-17β/ml (n = 1). PBS blank was 30pg oestradiol-17β/ml (n = 1). Non-specific binding was 3.24% (n = 1) and specific binding of the antiserum was 60.96% (n = 1).The intra-assay coefficients of variation were 7.32% and 1.72% (n = 1) for 0.03125 ng and 1.250 ng oestradiol-17β/ml, respectively. The recovery estimate was 80%. In our laboratory serially diluted samples of pooled plasma produced a displacement curve parallel to the standard curve.

Statistical analyses of hormone assays

The Mann-Whitney U and the Kruskal-Wallis (Sokal & Rohlf 1969) tests were used to determine differences between the treatment and control groups and between animals in different the reproductive stages. In all cases significance was taken at the 95% level.

Results

The mean release rates of the norethindrone and cholesterol pellets were 0.515 mg/day (±0.051) and 0.098 mg/day (±0.013), respectively. None of the females that received the norethindrone implants showed any signs of conception. Five out of the eight females in the cholesterol implant group were in the early stage of pregnancy at the time of the culling and the other three were not pregnant. The Fisher exact test shows a significant difference between these two groups at the 95% significance level (p = 0.0256). At the time of culling the three females that received no implants were all in a mid-stage of pregnancy.

Plasma progesterone concentrations

The plasma progesterone concentrations of free-ranging and captive mid-pregnant females did not differ significantly ($U = 2$; $p > 0.05$) and ranged from 0.7 to 5.1 ng/ml. (Table 2). Values for non-pregnant females receiving norethindrone and cholesterol also did not differ significantly ($U = 2$; $p > 0.05$) and ranged from 0.7 to 2.4 ng/ml and are similar to the value (0.8 ng/ml) recorded for free-ranging non-pregnant females by Mecenero (1998) (Table 2). The early pregnant females receiving cholesterol had circulating progesterone concentrations ranging from 0.9 to 1.2 ng/ml and were slightly lower than the two values (1.7 and 5.9 ng/ml) for free-ranging early pregnant females, presented by Mecenero (1998) (Table 2).

Plasma oestrogen concentrations

Plasma oestradiol-17β concentrations in free-ranging and captive mid-pregnant females were similar ($U = 1$; $p > 0.05$) and ranged from 66.4 to 95.1 pg/ml (Table 2). Mean values for non-pregnant females receiving norethindrone and cholesterol also did not differ significantly ($U = 0$; $p > 0.05$) and

Table 2. Plasma concentrations (mean ± SEM) of oestradiol-17β and progesterone for different group of female hyraxes (*n* = number of individuals).

Female groups and reproductive status	Oestradiol-17β (pg/ml)	Progesterone (ng/ml)
Free-ranging mid-pregnant (*n* = 2)	95.1 and 69.7	0.7 and 2.5
Captive mid-pregnant (*n* = 3)	71.1 ± 4.1	3.1 ± 1.0
Norethindrone non-pregnant (*n* = 4)	89.6 ± 16.4	1.3 ± 0.4
Cholesterol non-pregnant (*n* = 3)	47.9 ± 8.3	0.8 ± 0.1
Cholesterol early pregnant (*n* = 3)	51.8 ± 9.3	1.1 ± 0.1
*Free-ranging non-pregnant	30.9 and 33.9	0.8 ± 0.1
*Free-ranging early-pregnant	56.2	1.7 and 5.9

*Taken from Mecenero 1998

ranged from 31.6 to 128.0 pg/ml, but these values are higher than those (30.1 and 33.9 ng/ml) recorded in free-ranging, non-pregnant females (Mecenero 1998). The early pregnant females receiving cholesterol had values ranging from 37.4 to 69.1 pg/ml and were similar to that of free-ranging early pregnant females (Mecenero 1998).

Discussion

Subdermal implants are contraceptive systems that release low, stable amounts of progestins for a period of time. Unlike injectable methods and oral contraceptives, subdermal implants have the advantages of not causing peaks in progestin levels beyond those required for effective contraception and they do not use estrogens that can pose a health risk (Darney 1994). Moreover, the ability to remove an implant at any given time makes their effect reversible and safer. These facts favour and justify the use of implants in the present study above any other method available.

The successful contraceptive abilities of the norethindrone implants is best illustrated in the *in vivo* part of the study where the number of pregnant individuals in the treatment and control groups differed significantly with none of the females treated conceiving. This study also revealed that the norethindrone pellets released about 515 µg norethindrone per day in the hyrax, a value similar to the daily release of 500 µg in rhesus monkeys, reported by Jha *et al.* 1995. In humans, daily release rates of between 187 to 300ug did not constantly suppressed ovulation and pregnancies did occur (Wiese *et al.* 1976; Coutinho 1978; Odlind *et al.* 1979a; Odlind, Weiner & Johansson 1979), however the pregnancy rate decreased with increasing amount of norethindrone implanted (Wiese *et al.* 1976). Thus, it appears that the contraceptive ability of norethindrone is dose specific and future studies on the hyrax must include the determination of an optimal dose.

In the present study the plasma concentrations of norethindrone was not measured, but it has been reported in rhesus monkeys that plasma norethindrone levels greater than 25ng/ml were

associated with contraception (Jha *et al.* 1995). Wide individual variations in serum norethindrone levels have been recorded in humans (Olind *et al.* 1979a) and rhesus monkeys (Jha *et al.* 1991) and could be due to different rates of norethindrone metabolism and elimination or the amount of sex steroid binding globulin present in the blood (Jha *et al.* 1991). However, progesterone binding globulin, which serves as a progesterone-conserving mechanism, does not appear to be present in the hyrax plasma (Gombe, Heap & Sale 1976).

In the present study, the lack of significant differences in plasma oestradiol-17β or progesterone concentrations between the two non-pregnant groups suggests that norethindrone concentrations did not alter ovarian activity or the bioavailability of these hormones in the blood. This, suggests that norethindrone did not contracept through the hypothalamus-pituitary-ovarian axis but possibly prevented implantation by inhibiting progesterone function. Schubert, Donath, Michna & Nishino (1996) also presented results that clearly demonstrated that the "typical" antiprogestational effects of progesterone antagonists are not correlated to their antiovulatory activity in rats.

In humans norethindrone implants with a daily release rate of 200-240 μg suppressed ovulation for four to five cycles by altering oestradiol and progesterone levels associated with ovulation (Wiese *et al.* 1976). A more resent study by Singh *et al.* (1997) indicated that norethindrone implants in women resulted in acyclic patterns in oestradiol concentrations and suppressed progesterone levels that brought about low incidences of ovulation in the higher dose (266.5 mg) group. Norethindrone daily release rates of between 187 and 243μg in humans proved to be only initially high enough to completely inhibit ovulation and that a higher dose should accomplish full contraceptive efficacy (Olind *et al.* 1979b). In cattle only a daily release rate of above 137μg norethindrone acetate was able to suppress oestrus (Machado & Kesler 1996). When norethindrone is used for cycle programming during *in vitro* fertilization in humans, Anderson, Stanczyk, Stein, Vijod, Paulson & Lobo (1990) found that the oral administration of 10mg norethindrone attenuated the endogenous secretion of the

luteinizing hormone (LH) and the follicle-stimulating hormone (FSH) by 59% and 50% respectively, whilst having no inhibitory effect on ovarian steroidogenesis. Thus, in this case norethindrone had a negative effect on the hypothalamic-pituitary-axis, but no effect on progesterone and oestrogen levels (Anderson *et al.* 1990). A triphasic orally administered contraceptive containing norethindrone and ethinylestradiol used in humans resulted in the suppression of all four hormone profiles (LH, FSH, progesterone and oestrogen), again ovulation was inhibited by suppression of pituitary-ovarian function (Ling, Johnson, Lea, Bent, Scott & Toews 1985). Norethindrone shows dose specific characteristics in its effect on the hypothalamus-pituitary-ovarian axis and more detailed investigations are needed to determine the effect that norethindrone might have on the hypothalamus-pituitary-ovarian axis of the hyrax.

Although this study only investigated some of the effects of norethindrone on reproduction in the hyrax, it clearly illustrates the *in vivo* contraceptive abilities of norethindrone and suggests that the method for obstructing pregnancy involves the prevention of implantation in the endometrium and not a pre-coital suppression of ovulation. It is however, important to note that the effects of a synthetic progestin on an animal can be multiple and more detailed studies on aspects such as the effects on behaviour and social structure, norethindrone metabolism, influence on hypothalamus-pituitary axis and reproductive cycling are needed.

The results from this chapter can be indicative of the effects that norethindrone can have on the reproduction of African elephants, thus we can expect norethindrone to successfully prevent pregnancy with no effect on ovarian steroidogenesis when administered to elephant cows.

Conclusion

Norethindrone and cholesterol (placebo) implants in eighteen captive hyraxes resulted in none of the seven animals receiving norethindrone falling pregnant. Five pregnancies were recorded for the eight animals receiving cholesterol and for the three animals receiving no implants The circulating

oestradiol-17β and progesterone concentrations of the mid-pregnant and non pregnant animals were the same as in free ranging hyraxes, suggesting that norethindrone had no effect on the structures producing of these hormones. This chapter indicates that norethindrone successfully prevented pregnancy in the rock hyrax. These results can be indicative of the effect that norethindrone treatment might have in the African elephant.

CHAPTER 4

THE HISTOPATHOLOGICAL EFFECTS OF NORETHINDRONE IMPLANTS ON SELECTED ORGANS OF THE FEMALE HYRAX

Introduction

The uterus of the hyrax can be classified as the long bicornuate type (Mossman 1969). Two thin, long cornua are joined at the cervical end to form a small corpus that opens in the cervix with a single ostium. It is similar to that of the pig. The histology and fine structure of the hyrax uterus and its cyclic changes is unknown at present and can only be speculated upon.

The uterus shows a prominent steroid-dependent cyclic change in structure and function. The ovarian hormones, oestrogen and progesterone are responsible for preparing the uterus for receiving the blastocyst. Oestrogen triggers the uterus through oestrogen receptors that are plentiful in the uterine tissue. An uterotrophic effect is observed in the myometrium by the increase in its contractility and excitability. In the endometrium oestrogen is responsible for cell proliferation that leads to the increase in surface area of the endometrium. Endometrial secretion also reaches a maximum at the time of the oestrogen surge. In most mammals the most crucial action of oestrogen over this period is to induce the synthesis of intracellular progesterone receptors, thus after ovulation the uterus is primed to bind progesterone and the progestagenic phase of the uterine cycle begins (Johnson & Everitt 1984). Progesterone stimulates the synthesis of the secretory material of the glands. Cellular proliferation is further increased and cells become larger and plumper. Progesterone however, depresses the excitability of the myometrium. The withdrawal of steroids towards the end of the luteal phase of the cycle collapses the endometrium and it is either reabsorbed or menses proceeds (Johnson & Everitt 1984). Again these actions and sensitivity to actions vary amongst species and were not known for the hyrax at present. In humans endometrial biopsies are characterized by preovulatory secretory cells having numerous long microvilli and a few ridge-like protrusions, ciliated cells are more frequently

observed (oestrogen response), while in the postovulatory state the microvilli are fewer and shorter and the protrusions are larger and can be judged as the progesterone response (Nilsson, Englund, Wiener & Victor 1980).

The altering of the interaction between the endometrium and ovarian hormones results in the altering of the microenvironment of the endometrium that in turn can prevent the acceptability of the endometrium to implantation. The effects of different synthetic progestins on the endometrium results in the same effects and can be described as a dose-dependent atrophy of the endometrium by reduced mitotic activity in glands, dense stroma, degradation of spiral arteries and dilation of veins (Slayden, Zelinski-Wooten, Chwalisz, Stouffer & Brenner 1998; Heikinheimo, Hsiu, Gordon, Kim, Williams, Gibbons & Hodgen 1996; Brenner & Slayden 1994). The effects of progestins on the endometrium morphology can be generalized to the basic suppression of the endometrium.

The influence of the administration of an antiprogestin has never been studied in the hyrax before. Results from other species, however shows that it can lead to the disruption of the ovarian cycle and prevent successful conception. The effects of these antiprogestins on the ovaries seem to be dose-dependent, with relatively high doses suppressing ovulation (Jha *et al.* 1991 and 1995; Spitz & Agranat 1995; Singh *et al.* 1997). McPherson *et al.* (1974) demonstrated that the action of synthetic progestational agents in altering gonadotrophin secretions is potentiated by the presence of endogenous estrogens as well as the inherent estrogenic activity of antiprogestins. This further suggests that antiprogestins, such as norethindrone, may interfere with the hypothalamic-pituitary-axis and result in the inactivation of traditional ovarian functions, and this will affect the morphology of the organ.

Materials and methods

After the culling of the female hyraxes, the following organs were collected: ovaries, uterus, adrenals, liver and spleen. After weighing, a section of each organ was fixated in Bouin's fluid for not more than 24 hours after which it was thoroughly rinsed in 70 % ethanol until the solution was clear.

The tissue was processed using standard histological techniques. It was imbedded into paraffin wax and sectioned at 6μm. The sections were mounted onto microscope slides and stained using heamotoxylin and eosin.

Results

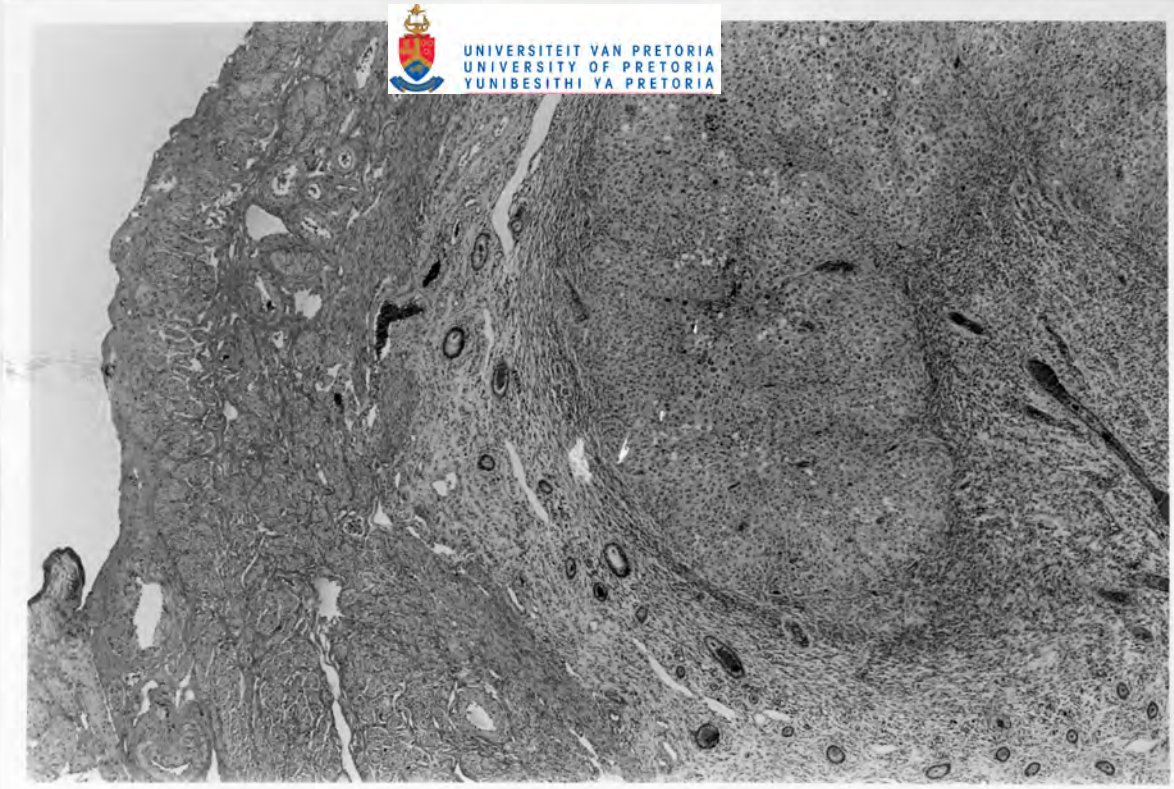
The histopathological results of each individual are summarized in Table 3. The adrenals of the norethindrone treated females show no abnormalities and where similar to that of the cholesterol treated and control groups. The liver and spleen of most of the females indicated signs of haemosiderosis (remains of broken down blood in the liver), where some of the treated females (cholesterol and norethindrone) showed scars associated with chronic cholangiohepatitis (inflammation of the bile ducts). The ovaries of both the control and cholesterol treated females were functional with normal folliculogenesis and active corpora lutea present. The ovaries of the norethindrone treated females lacked follicular activity, had no corpora lutea but revealed the presence of corpora albicantes (involuted corpora lutea). The uteri of the control and cholesterol treated animals appeared normal and functional with no evident scars. The uteri, or more specific the endometrial tissue, of the norethindrone treated females, however, had clear indications of atrophic glands and proliferation of, possibly, the stroma cells. This phenomenon was present in all the females treated with norethindrone in different degrees of severity. The stromal hyperplasia (increased growth of the stromal cells due to cell division) present in the endometrial tissue of the norethindrone treated females are represented in Figure 5 (a) and (b).

Table 3. Histopathological effects of norethindrone on selected organs of female rock hyraxes.

Animals	Organs			
	Ovaries	Uterus	Adrenals	Liver and spleen
No treatment				
A	Active. Corpora lutea present	No effect	No effect	No effect
B	Active. Corpora lutea present	No effect	No effect	Haemosiderosis*
C	Active. Corpora lutea present	No effect	No effect	Haemosiderosis
Cholesterol treatment				
22/21	Active. Corpora lutea present	No effect	No effect	Cholangiohepatitis**
41/42	Active. Corpora lutea present	No effect	No effect	Haemosiderosis
48/47	Active. Corpora lutea present	No effect	No effect	Haemosiderosis
33/34	Active. Corpora lutea present	No effect	No effect	Haemosiderosis
32/31	Active. Corpora lutea present	No effect	No effect	Haemosiderosis
49/50	Active. Corpora lutea present	No effect	No effect	Haemosiderosis
40/39	Active. Corpora lutea present	-	-	-
Norethindrone treatment				
25/26	Inactive, no follicles present, corpus albicans*** present	Atrophic endometrial glands and proliferation of endometrial cells	No effect	Haemosiderosis
30/29	Inactive, no follicles present, corpus albicantes present	Stromal hyperplasia**** present	No effect	Haemosiderosis
27/28	Follicle development and corpus albicantes present	Atrophic endometrial glands and stromal hyperplasia	No effect	No effect
36/35	Corpus albicantes present	Atrophic endometrial glands and stromal hyperplasia	No effect	Cholangiohepatitis and haemosiderosis

* Remains of broken down blood in liver
**Inflammation of the bile ducts
***Involuted corpus luteum
**** Increased growth of stromal cells due to cell division

(a)



(b)

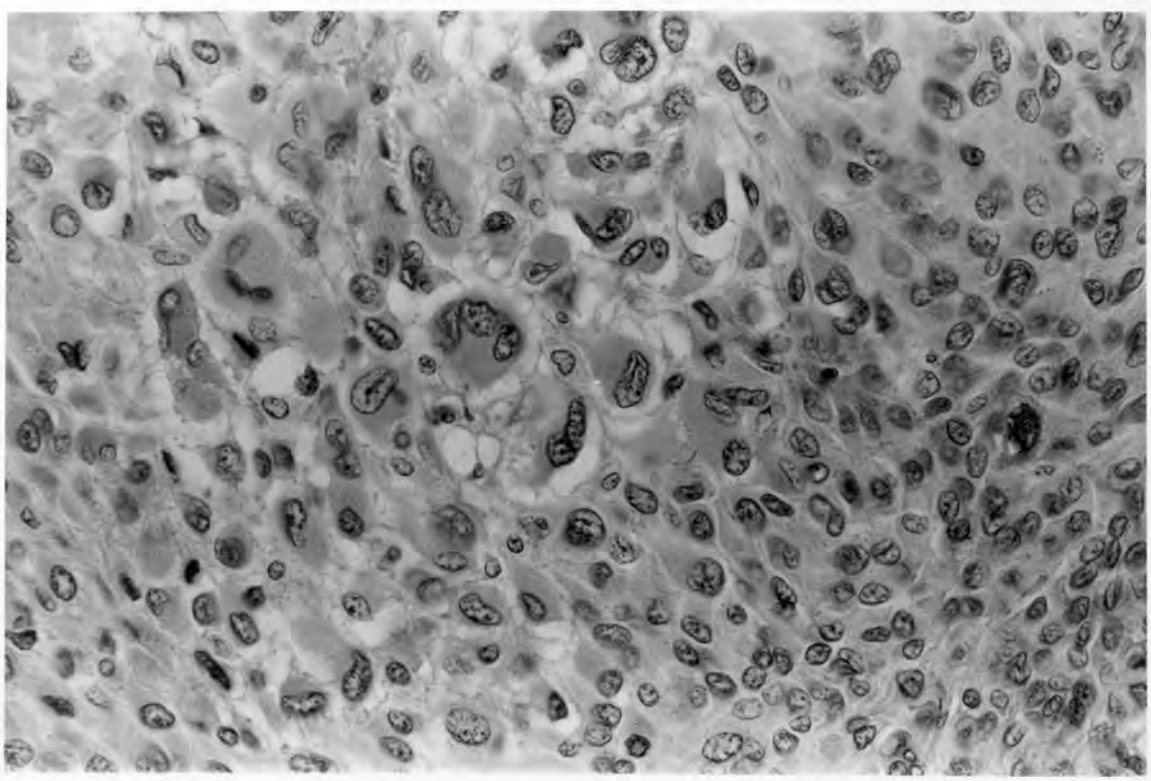


Figure 5. Endometrial tissue of the norethindrone treated females showing a section of the endometrium with stromal hyperplasia (a) and enlargement of the proliferated cells of the endometrium (b).

Discussion

The effects of norethindrone on the human endometrium as reported by Moghissi, Syner & McBride (1973) includes that the endometrium becomes thin with a compact stroma, sparse glands and an absence of stromal oedema. Other progestins, such as RU486 and ZK 137 316, showed similar affects with the generalized effect of suppression of the endometrium associated with dose-dependent atrophy of endometrial tissue and reduced activity of the glands (Gemzell-Danielsson, Swahn, Westlund, Johannisson, Seppala & Bygdeman 1997; Slayden *et al.* 1998). Some of the results presented in this study seem to agree with this general pattern of endometrial effects caused by antiprogestins. Atrophy of the endometrial glands was significant in all the females receiving norethindrone implants. Katkam, Gopalkrishnan & Chwalisz (1995) reported that bonnet monkeys treated with a low dose of an antiprogestin developed endometrial tissue showing retardation with a decreased glandular diameter. The disruption in endometrial glandular secretory differentiation, associated with antiprogestins such as norethindrone, may block implantation, since even a minor change in secretory activity may cause a hostile environment (Davies, Anderson, Mason & Jacobs 1990).

The high degree of endometrial cell proliferation recorded in hyraxes treated with norethindrone during the present study has never been mentioned or recorded in other species receiving an antiprogestin. Slayden *et al.* (1998) showed a clear dose-dependent reduction of rhesus monkey endometrium with an increase in stromal compaction that resulted in the thinning of the endometrium. The results of Gemzell-Danielsson *et al.* (1997) from human intake of an antiprogestin also suggested retarded endometrial development. The results obtained in the present study suggest a histopathological reaction to norethindrone. The endometrial cells that proliferated in the hyrax could not be identified. The pathological implications of such an increase in endometrial cells can be severe although there is currently no evidence to link norethindrone, used as an antiprogestin, with carcinoma of the cervix or endometrium (Graham & Fraser 1982). It has been speculated that norethindrone could provide some

protection against such illnesses of the endometrium (Graham & Fraser 1982). It is here suggested that the abnormal growth of the endometrial tissue and the diminution of the endometrial glands are mechanisms through which norethindrone have created an environment that prevents the implantation of the blastocyst, as implantation is a well-timed event relying on the acceptability of the endometrium.

A scanning electron microscopy investigation of the fine structure of rat endometrium after oral norethindrone administration indicated that flat wave like mucous folds of the uterus became more thick and highly convoluted and that the uterus was also highly secretory (Pal & Guha 1990). These results are coherent with the present study and appear to be paradoxical to other findings of antiprogesterone actions. The high estrogenic activity exhibit by norethindrone (Edgren *et al.* 1966; McPherson *et al.* 1974; Stanczyk & Roy 1990; Yamamoto *et al.* 1986) may be responsible for the increase in endometrial cells and that in this affected state the uterus my interfere with implantation or gamete transport (Pal & Guha 1990), and as a result prevent pregnancy.

The ovary is another organ that can be morphologically influenced by the administration of an antiprogestin as the hormone balance, that includes progesterone, is responsible for the correct functioning of this organ. In the present study the ovaries of the females treated with norethindrone were inactive and differed from those of the other two groups (control and cholesterol treatment group). Signs of corpora albicantes were recorded indicating previous ovarian activity. No other histopathological scars were present and the ovaries appeared to be in a stable, inactive state. However, apparent normal plasma progesterone and oestradiol- 17β concentrations (see Chapter 3) suggest that ovarian function was not affected by the norethindrone treatment. From the histological data represented in this chapter this does not seem to be the case. Norethindrone appears to inhibit folliculogenesis and ovulation. These contradicting results can be due to the two-way effect of norethindrone on reproduction. The antiovulatory activity and the antiprogestational effects (in the endometrium) of norethindrone is associated with a high dosage of antiprogestin, while lower dosages only triggers the antiprogestational effects in the endometrium and fails to suppress ovulation

(Schubert *et al.* 1996; Jha *et al.* 1991; 1995). Moreover, this two-way effect of an antiprogestin is not correlated (Schubert *et al.* 1996). It is also important to note the findings of Anderson *et al.* (1990) that concluded that when norethindrone is used for cyclic programming in *in vitro* fertilization, it does not inhibit ovarian steroidogenesis but does affect the hypothalamic-pituitary axis. However, the most probable explanation for the unaffected hormone concentrations measured in the females with inactive ovaries, may be that the circulating progesterone and oestradiol-17β measured in these animals are secreted by an organ other than the ovaries. This may be the case for the unaffected progesterone concentrations measured, as Mecenero (1998) clearly indicated that the red and white blood cells of the hyrax are capable of producing progesterone from pregnenolone. These findings affects the conclusion in Chapter 3 and enable me to interpret the seemingly contradicting results by suggesting that the effects of norethindrone on the ovaries are dose-specific and can affect ovarian morphology without altering ovarian functions, but more so, can the progesterone (in the hyrax) be produced through other mechanisms than ovarian secretion. The levels of progesterone and oestradiol-17β recorded here may also represent background concentrations and thus mean nothing.

Norethindrone implants had no effect on the other organs under investigation. The adrenals were unaffected and appeared normal in all the female groups. The treatment groups (norethindrone and cholesterol) showed incidents of chronic cholangiohepatitis that can possibly be linked to the increased levels of cholesterol that resulted in the altering of cholesterol metabolism. This reaction may lead to the development of gall bladder stones and predispose to the development of chronic inflammation of the bile ducts. This problem can probably be reduced with the altering of release rate of norethindrone that will in turn change the dosage to a lower and more harmless state. The haemosiderosis (remains of blood break down in the liver) experienced by all the females seems to be normal and can not be linked to a specific group and thus not considered as an direct effect of the norethindrone treatment.

The results from the present chapter indicated that norethindrone has effects on the uterine and ovarian morphology that may explain the prevention of conception in the rock hyrax. It is less likely that the results indicated a pathological state in the reproductive organs but rater a mechanism through which norethindrone creates an environment hostile to conception.

Conclusion

Sections of selected organs (adrenals, liver, spleen, ovaries and uterus) were exposed to histological procedures and investigated for possible histopathological effects that could be norethindrone induced. Effects of norethindrone were identified in the uterine and ovarian tissue and too a lesser extend in the liver and spleen. The endometrial tissue of the females treated with norethindrone exhibited atrophy of the glands and proliferation of cells (unidentified) possibly indicating the antiprogestational mechanism of norethindrone or, less likely, a pathological state associated with norethindrone treatment. The ovaries of the norethindrone administered females appeared to have reduced folliculogenesis that suggests the inhibition of ovulation. Although this seems contradictory to the findings in the hormone production of the ovaries, it is possible that the effects of norethindrone on the ovaries is dose-specific and can effect ovarian morphology without altering ovarian function, or that the measured hormones concentrations originated from a different source than the ovaries. It is also possibly that the measured levels of hormone is background concentrations and means nothing. The results can possibly be extrapolated to the African elephant and indicates expected effects of norethindrone when administered to elephants. The study may question the safety of the use of norethindrone as a contraceptive in the elephant and hyrax, but also opened up more specific research opportunities that may change our present doubts concerning contraception and possibly explain the mechanism through which norethindrone functions.

CHAPTER 5

SYNTHESIS

The conservation of the African elephant is a confounding dichotomy. Although listed as an Appendix I species by CITES, and thus considered as endangered, over abundant populations in conservation areas often necessitate culling to control their numbers. The elephant, having few natural predators, a long life expectancy and destructive feeding behaviour can at high population densities pose a threat to the biodiversity of a conservation area by turning areas of high diversity into wastelands with little or no conservation value (Cumming *et al.* 1997). The management of elephant populations by culling is now becoming unacceptable by the public who sees these acts as inhumane and unethical. Alternative methods of controlling large populations include the use of antifertility agents that can prevent pregnancy and cause a natural decline in numbers.

The antifertility method that the present study investigates involves the administration of a synthetic progestin that inhibits the binding of progesterone to the progesterone receptor in the uterus of the female. Thus, all progesterone functions are blocked and implantation can not take place (Spitz and Agranat 1995). Competitive binding assays on uterine tissue of the African elephant showed that the synthetic progestin norethindrone has a relative binding affinity almost three-fold that of progesterone (Greyling *et al.*1997), suggesting that norethindrone out competes progesterone on receptor level and thus may have contraceptive abilities in this species. Considering the issues surrounding the conservation of the elephant it is not possible to test this compound on live elephants yet, thus I investigated the use of the rock hyrax as a biological model in the development of a contraceptive for the elephant.

These two species are not just phylogenetically related (Shoshani 1991; Springer and Kirsch 1993) but also share similarities in their uterine progesterone receptor characteristics. This study showed that norethindrone has a relative binding affinity for the uterine progesterone receptor, almost

double that of the natural ligand. This finding not just illustrates another similarity between these two species, but suggests that the hyrax can be used as a biological model in the study of norethindrone as a possible contraceptive in the African elephant.

Both these species exhibit a characteristic that is unique compared to any other mammal species. They exhibit low plasma progesterone levels (Heistermann *et al.* 1994; Ford *et al.* 1997; Mecenero 1998) during gestation, suggesting that a hormone other than progesterone maintains pregnancy, or a high sensitivity for progesterone (Heap *et al.* 1975). However, the plasma concentrations recorded for the progesterone metabolite, 5α-pregnane-3,20-dione is magnitudes higher than that of progesterone in both the elephant and the hyrax (Mecenero 1997; Hodges *et al.* 1997; Ford *et al.* 1997) proposing that this hormone has a biological importance. Greyling *et al.*(1997) reported that 5α-pregnane-3,20-dione has a high relative binding affinity for the uterine progesterone receptor of the elephant and in the present study the relative binding affinity of 5α-pregnane-3,20-dione for the uterine progesterone receptor in the hyrax proved to be almost similar to that of progesterone. This poses the possibility that 5α-pregnane-3,20-dione rather than progesterone functions as the "hormone of pregnancy" in the hyrax and the elephant by triggering the uterine progesterone receptor, further supporting biochemical similarities on the reproductive level.

However, important differences also exist between the African elephant and the rock hyrax. The synthetic hormones RU486 and levonorgestrel are widely used as contraceptives in humans (Schaison *et al.* 1985, Ulmann *et al.* 1990; Schwarz *et al.* 1992, Masson *et al.* 1993, Woods *et al.* 1992). Both these progestins showed a low binding affinity to the progesterone receptor in the uterus of the African elephant (Greyling *et al.* 1997). This differed from the results for the hyrax in the present study. Both RU486 and levonogestrel out competed progesterone and had high relative binding affinities for the uterine progesterone receptor. When the chemical structures of these progestins are considered, the difference in binding characteristics between the elephant and the hyrax can possibly be explained by

the progesterone receptor binding site of the elephant being more sensitive to the bulky structures of RU486 and levonorgestrel on the 11 and 13 positions.

The natural progestins 4-pregnen-20α-ol-3-one, 17α-hydroxyprogesterone and 5α-pregnane-3α-ol-20-one seemed of minor value to the uterine progesterone receptor in the African elephant (Greyling *et al.* 1997) and the hyrax (present study) by exhibiting low relative binding affinities. The destiny of 17α-hydroxyprogesterone and 5α-pregnane-3α-ol-20-one in the hyrax is believed to be the feaces where these compounds are the main progesterone metabolites excreted.

The uterine progesterone characteristics of the hyrax that were investigated in this study proved similarities and differences with that of the African elephant. The most important finding, however, is that both species share a high affinity for norethindrone on receptor level, this favours, again, the use of the hyrax as a model in the search of a contraceptive in elephants.

To *in vivo* examine the contraceptive abilities of norethindrone in the hyrax, as a model for the elephant, the effect of norethindrone implants on the reproductive output was determined. With a daily release rate of 515 μg norethindrone no pregnancies were recorded in the treated animals. The control group received cholesterol implants and five out of the eight females conceived. These results confirmed the antifertility abilities of norethindrone in the hyrax and can be indicative of the results expected in treated elephant cows.

The circulating oestradiol-17β and progesterone concentrations of the non-pregnant females in the treatment and control groups did not differ significantly implying that the norethindrone implants did not alter bioavailability of these hormones. This finding suggests that norethindrone did not contracept through the hypothalamus-pituitary-axis, but rather prevented pregnancy through the inhibition of progesterone functions and blocking implantation in the uterus. Norethindrone however, has proved to have an effect on ovulation in humans (Wiese *et al.* 1976) and rhesus monkeys (Jha *et al.* 1995) by altering circulating progesterone and oestradiol levels. This effect has also been proved to be

dose-specific (Jha *et al.* 1991; Wiese *et al.* 1976) with lower doses of norethindrone not being able to suppress ovulation successfully. Gonadotrophin secretion has also been reported to be altered by norethindrone treatments by suppressing FSH and LH levels (McPherson *et al.* 1974, Anderson *et al.* 1990) that brings about the suppression of ovulation.

Norethindrone may also have an effect on the morphology of organs in the body of a treated animal. Selected organs (adrenals, spleen, liver, uterus and ovaries) of all the females were histologically investigated for possible indications of side-effects associated with norethindrone administration. The only indications found were limited to the uterine and ovarian tissue and the other organs seemed unaffected by norethindrone. The endometrial tissue of the uterus reacted to norethindrone in two ways, namely the atrophy of the glandular tissue and the proliferation of the stromal tissue. The latter effect does not coincide with the expected reaction of the endometrium towards antiprogestin treatment (Gemzell-Danielsson *et al.* 1997; Slayden *et al.* 1998), but reflects an opposite reaction. This may be due to a pathological state that is developing in the uterus, or more likely, portrays the estrogenic activity associated with norethindrone (Edgren *et al.* 1966; McPherson *et al.* 1974; Stanczyk & Roy 1990; Yamamoto *et al.* 1986) as the mechanism through which conception is inhibited. The inactivation of the ovaries that was observed in the norethindrone treatment group indicates that norethindrone was also interfering with the hypothalamus-pituitary system that, through the secretion of FSH and LH, maintains ovarian morphology and function. This reaction is common in females treated with relatively high dosages of antiprogestins and can possibly be eliminated through a decrease in dosage (Jha *et al.* 1991 and 1995). The inactivation of the ovaries also indicated that the unaffected circulating progesterone and oestrogen concentrations that were measured in the norethindrone treated females originate from a different source than the ovaries. The possibility that these two effects of norethindrone can be pathological and even detrimental should not be discarded, but investigated in more detail before norethindrone can be labeled a safe contraceptive in the hyrax or the African elephant.

To summarize: The present study investigated the characteristics of the uterine progesterone receptor of the hyrax and compared it to that of the elephant as recorded by Greyling *et al.* (1997). The most important similarity between these two species was revealed to be the high binding affinity of the progesterone receptor for the synthetic hormone, norethindrone, in both species. The hyrax can thus be used as a biological model in the testing of norethindrone as a contraceptive in the African elephant. The *in vivo* testing of the antifertility abilities of norethindrone on female hyraxes proved successful. Histology revealed that norethindrone targets both the ovaries and the uterus in its efforts to inhibit pregnancy. Similar findings can thus far be expected in the African elephant, but before norethindrone can be labeled a safe contraceptive in either species, the following research is suggested:

- Monitoring the effect of norethindrone on the hypothalamus-pituitary-axis.
- The determination of an optimal dose for contraception.
- More histopathological investigations.
- Investigating the reversibility of the effects associated with norethindrone.
- Monitoring effects of norethindrone on behaviour and social structure.
- Experimenting on captive African elephants.

SUMMARY

The aims of this study was to determine the binding affinity of the hyrax uterine progesterone receptor for its natural ligand, progesterone and to investigate the ligand specificity of this receptor for selected natural and synthetic hormones. Further, the effect of norethindrone implants on the reproduction of the hyrax was investigated together with the possible histopatological effects that this synthetic progestin might have on selected tissue. This study will not just contribute to our understanding of the reproductive physiology of the hyrax on a biochemical level, but will be relevant to the possible use of the hyrax as a biological model in the development of norethindrone as a contraceptive in the African elephant, as an alternative method of managing large population sizes.

The *in vitro* part of the study revealed, through competitive binding assays, the order of competing efficiency of progestins (natural and synthetic) for the uterine progesterone receptor as follows : promegestone > RU486 > norethindrone > levonorgestrel > progesterone > 5α-pregnane-3,20-dione > 4-pregnen-20α-ol-3-one > 17α-hydroxyprogesterone > 5α-pregnane-3α-ol-20-one. The relative binding affinity of 5α-pregnane-3,20-dione was almost similar to that of progesterone, together with this, the low plasma concentrations of progesterone and the opposing high levels of 5α-pregnane-3,20-dione suggested that 5α-pregnane-3,20-dione rather than progesterone in involved in the maintenance of pregnancy in the hyrax. The almost two-fold higher relative binding affinity of norethindrone implied that norethindrone successfully out competes norethindrone on receptor level and can thus inhibit progesterone functions and as a result of this prevent implantation of the blastocyct in the uterus lining. This apparent contraceptive ability of norethindrone in the hyrax is shared with the elephant that also revealed a high affinity for norethindrone. Thus, from a biochemical view, seems the hyrax to be a suitable biological model in the development of norethindrone as a contraceptive in the elephant.

As a result of this, were the contraceptive abilities of norethindrone on the reproduction of the hyrax tested by subdermal implants that resulted in the slow release of norethindrone into the circulatory system over a period of time. Norethindrone implants successfully prevented pregnancy in the treated animals with no females in the treatment group conceiving. The effect of norethindrone on the ovarian function of the hyrax was investigated by the measurement of circulating oestradiol and progesterone concentrations. The results suggested no altering of concentrations, implying that the norethindrone implants had no effects on the bioavailability of these hormones. The possible effects of norethindrone on the morphology of selected tissue (uterus, ovary, spleen, liver and adrenals) were histologically investigated and revealed effects on the endometrial and ovarian tissue. The endometrium reacted with the atrophy glands and the proliferation of stromal cells, while norethindrone caused the inactivation of the ovaries. This indicates that norethindrone can interact in two ways when inhibiting conception, namely, the creation of an uterine environment that rejects implantation through a unsynchronized endometrium and secondly, the suppression of ovulation by interference of the hypothalamus-pituitary system. However, the possibility that these effects may be pathological should not be excluded.

As a result of the similarities in the characteristics of the uterine progesterone receptors of the hyrax and the African elephant it is suggested that the *in vivo* findings of the effects of norethindrone in the hyrax are indicative of the effects expected in elephant cows treated with norethindrone. Although norethindrone prevents pregnancy in the hyrax it may not be a safe contraceptive due to its pronounced effects on the endometrial tissue. This study identified the hyrax as a suitable model in the development of norethindrone as a contraceptive for the African elephant, used if as such, and concludes that norethindrone cannot be used as a safe contraceptive in either the hyrax or the elephant.

OPSOMMING

Die doelstellings van hierdie studie was om die bindingsaffiniteit van die progesteroonreseptor, in die dassie se uterus, vir sy natuurlike ligand, progesteroon te bepaal en daarna die ligand spesifisiteit van die reseptor vir sekere natuurlike en sintetiese hormone te bepaal. Verder is die invloed van norethindrone implantings op voortplanting van die dassie ondersoek asook die moontlike histopatologiese effekte wat hierdie sintetiese hormoon op sekere organe mag hê. Die studie sal nie net bydra tot ons kennis van die voortplantingsfisiologie van die dassie op biochemiese vlak nie, maar is ook belangrik vir die moontlike gebruik van die dassie as 'n biologiese model in die ontwikkeling van norethindrone as 'n voorbehoedmiddel vir die Afrika olifant as 'n alternatiewe metode om hoë bevolkingsgroottes te beheer.

Die *in vitro* gedeelte van die studie, wat kompeterende bindings toetse ingesluit het, het die orde van kompeterende effektiwiteit as volg onthul: promegestoon > RU486 > norethindrone > levonorgestrel > progesteroon > 5α-pregnane-3,20-dioon > 4-pregnen-20α-ol-3-oon > 17α-hidroksieprogesteroon > 5α-pregnane-3α-ol-20-oon. Die relatiewe bindings affiniteit van 5α-pregnane-3,20-dioon was bykans identies aan die van progesteroon. Die lae plasma konsentrasies van progesteroon en daarteenoor hoë plasma vlakke van 5α-pregnane-3,20-dioon stel voor dat 5α-pregnane-3,20-dioon eerder as progesteroon betrokke is by die onderhoud van dragtigheid in die dassie. Die byna tweevoudige hoër relatiewe bindingsaffiniteit van norethindrone veronderstel dat norethindrone progesteroon suksesvol uitkompenteer op reseptor vlak en kan dus progesteroon se funksies onderdruk en as 'n resultaat hiervan implantasie van die blastosist in die uteruswand verhoed. Hierdie voorgestelde voorbehoedingsvermoë van norethindrone in die dassie is ook 'n moontlikheid in die olifant wat ook 'n hoë affiniteit vir norethindroon toon. Dus, van 'n biochemiese oogpunt, blyk die dassie 'n gepaste biologiese model te wees in die ontwikkeling van norethindrone as 'n voorbehoedmiddel vir die olifant.

As gevolg hiervan is die voorbehoedingsvermoë van norethindrone op die voortplanting van die dassie *in vitro* getoets deur subdermale implantante wat die stadige vrystelling van norethindrone in die sirkulêre sisteem oor 'n sekere tydperk bewerkstellig. Norethindrone implantante het dragtigheid suksesvol verhoed deurdat geen van die behandelde wyfies dragtig geword het nie. Die invloed van norethindrone op die ovariale funksie van die dassie is getoets deur die sirkulerende konsentrasies van oestradiol en progesteroon te meet. Hierdie resultate het getoon dat geen veranderinge in die konsentrasies plaasgevind het nie en dit impliseer dat norethindrone implantante geen invloed op die bio-beskikbaarheid van hierdie hormone gehad het nie. Die moontlike invloed van norethindrone op die morfologie van sekere organe (uterus, ovarium, milt, lewer en byniere) is deur middel van histologiese prosedures ondersoek en het 'n invloed in the uterale en ovariale weefsel aangetoon. Die endometrium het gereageer met die atrofie van die kliere en die proliferasie van stromale selle, terwyl norethindrone stasis van die ovaria veroorsaak het. Hierdie bevindinge dui daarop dat norethindrone dragtigheid op twee maniere kan voorkom, naamlik deur 'n uterus-omgewing te skep wat onaanvaarbaar is vir implantasie as gevolg van 'n ongesinkroniseerde milieu en tweedens, deur die onderdrukking van ovulasie deur die versteuring van die hipotalamus-hipofise-sisteem. Die moontlikheid dat hierdie reaksies patogenies van aard kan wees moet nie buite rekening gelaat word nie.

As gevolg van die ooreenkomste in die uterus-progesteroonreseptor eienskappe van die dassie en die Afrika olifant is dit voorgestel dat die *in vivo* bevindinge van norethidrone op die dassie 'n aanduiding kan wees van die verwagte invloed op olifantkoeie wat met norethindrone behandel sou word. Al het norethindrone dragtigheid in die dassie suksesvol onderdruk, word dit nog nie as 'n aanvaarbare voorbehoedmiddel bestempel nie as gevolg van die invloed daarvan op die endometriale weefsel. Hierdie studie het die dassie as 'n gepaste model vir die ontwikkeling van norethindrone as 'n voorbehoedmiddel in die Afrika olifant geidentifiseer, ondersoek vir die doel, en tot die gevolgtrekking

gekom dat norethindrone nie 'n aanvaarbare voorbehoedmiddel vir beide die dassie of die olifant kan wees sonder verdere studies nie.

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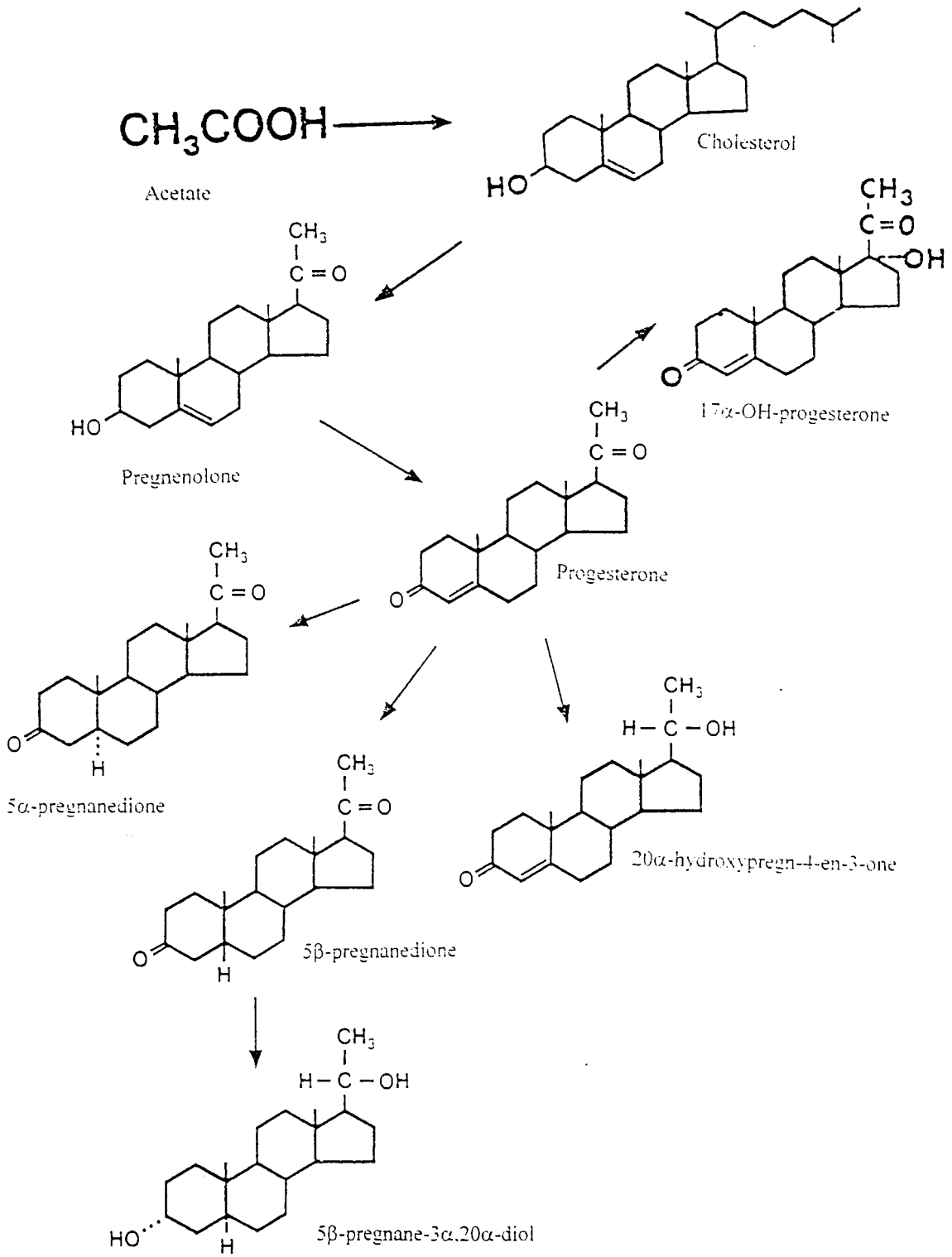
APPENDICES

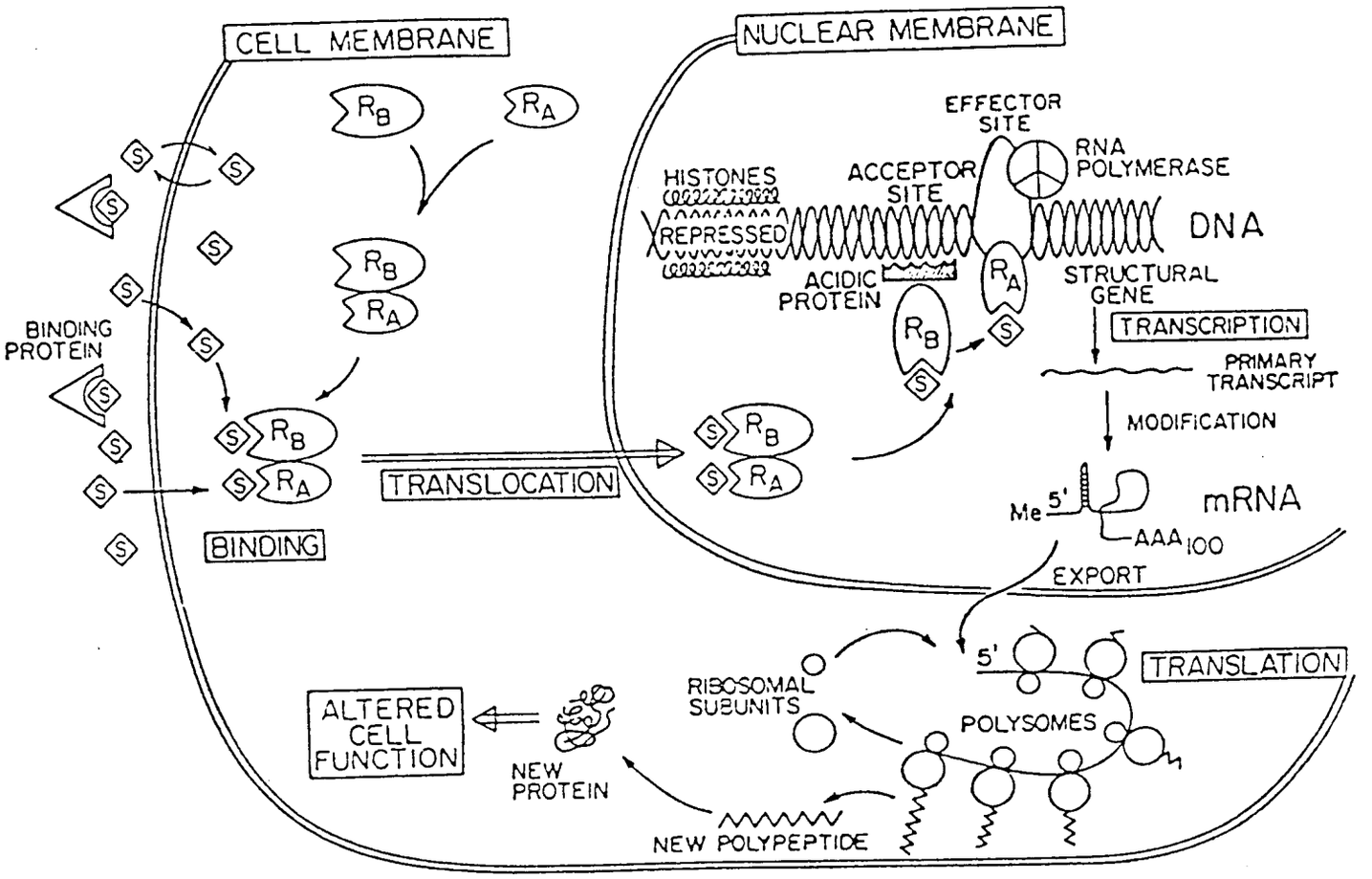
APPENDIX I

The biosynthesis of progesterone and some of its metabolites (Modified from Heap 1973)



APPENDIX II

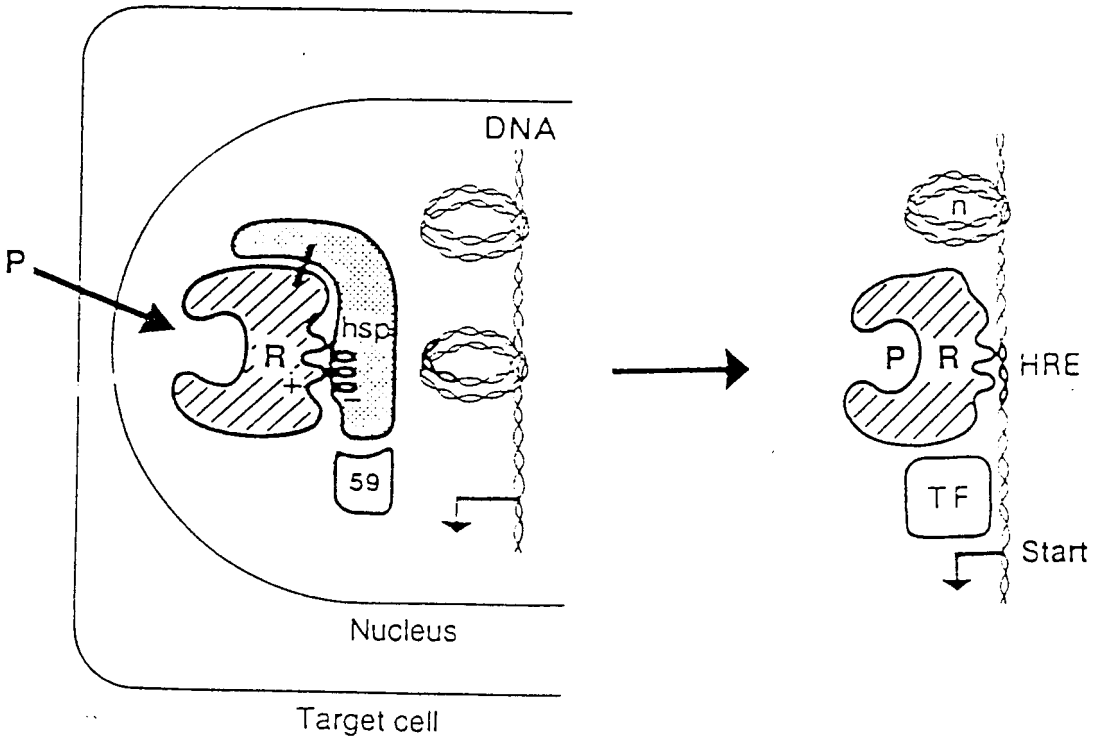
General model for the mechanism of progesterone action (from King 1982).



APPENDIX III

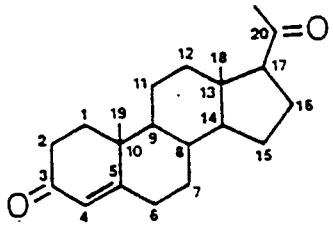
Cellular and molecular mechanisms of the interaction between progesterone and the progesterone receptor (from Baulieu 1989)

(P = progesterone, R = receptor, hsp = hsp90, 59 = p50, TF = transcription factor, HRE = hormone response element, n = nucleosone)

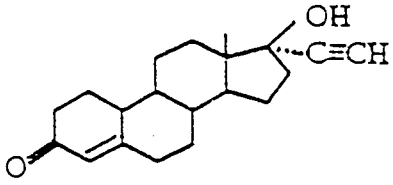


APPENDIX IV

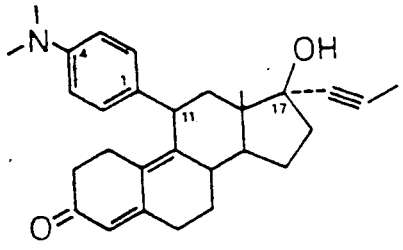
Molecular structure of progesterone and some of its antagonists.



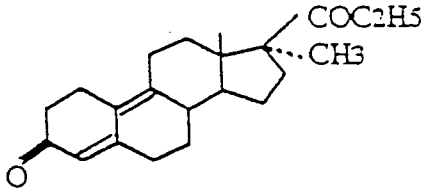
Progesterone



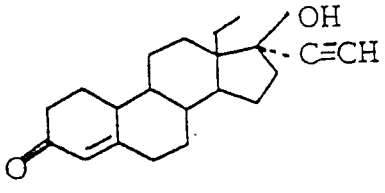
Norethindrone



RU486



Promegestone



Levonorgestrel