Mechanism of action of cathinone from khat (*Catha edulis*) on behavioural and reproductive function in vervet monkey (*Chlorocebus aethiops*).

A thesis submitted in fulfilment of requirements for Doctor of Philosophy degree of University of Nairobi [Animal Physiology].

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This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This work is dedicated to God the Almighty for guiding me through my education and to my late father Daniel Kimungui for his incalculable contribution to my education. His words of wisdom and financial support contributed immensely to my success. May Almighty God rest his soul in eternal peace. I also dedicate this work to my mother and my beloved family for their tireless support to my studies.
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PUBLICATIONS

1. Acute and sub-chronic effects of purified cathinone from khat (*Catha edulis*) on behavioural profiles in vervet monkeys (*Chlorocebus aethiops*).

2. Dose-response inhibitory effects of purified cathinone from khat (*Catha edulis*) on cortisol and prolactin release in vervet monkeys (*Chlorocebus aethiops*).
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ABSTRACT

Biological effects of cathinone on behavioural and reproductive functions are inadequately investigated, and available reports in literature are conflicting. A number of these studies have indicated varying degree of information on effect of khat and cathinone on hypothalamo-hypophyseo-adrenocortical axis, mesolimbic system as well as neuro-endocrine disorders and histopathological lesions in testes of humans and experimental animals. This study investigated the mechanism of action of cathinone on behavioural changes in both sexes of vervet monkeys as well as neuro-endocrine alterations that influence reproductive function in males of the same species. The significance of this study was to determine whether cathinone use boosts or is detrimental to reproductive and mental health under varying degree of use. Such information would be vital to social and health care professionals and service providers, as well as policy makers, community-based health groups and international organizations. Fourteen adult vervet monkeys were divided into tests (12 animals) and controls (2 animals), and treated with escalating doses of cathinone at alternate days of each week for 4 months. Controls were administered normal saline in the same pattern as tests. Administration was done via oral gavage. One month of pre-treatment served to establish baseline values. Group II and IV animals were administered GnRH agonist alongside (-)-cathinone two weeks after treatment phase of 4 months. Composite behavioural scores of aggression, anxiety, abnormal responses, withdrawal and appetite loss were done alongside analysis of serum prolactin, cortisol, luteinizing hormone, progesterone and testosterone. Stereology of testicular subcellular organelles of Leydig cells was also done following electron microscopy of testicular tissue of the animals. For ex vivo studies, isolated Leydig cells from testis of the animals were co-incubated with luteinizing hormone and subjected to two doses of cathinone (low and
high) overnight at 37 °C. Androstenedione, progesterone and testosterone were analyzed and compared with those in control cells that were treated with incubation media alone. Gene expression of mRNA of hydroxysteroid dehydrogenase enzymes (3 beta- and 17 beta hydroxysteroid dehydrogenase type 1) was done for comparison of results with those of intracellular hormones of testicular cells. Behavioural and hormonal data were analysed for within- and between-subjects effects using Greenhouse-Geisser correction. Separate ANOVAs were performed for each behavioural and hormonal variables with Bonferroni post hoc multiple comparisons using SPSS version 14. Results indicate a dose-dependent effect of (-)-cathinone on behavioural and hormonal profiles. Composite scores of aggression, anxiety, abnormal responses, withdrawal and appetite loss increased in a dose-dependent manner. Serum prolactin, cortisol and progesterone decreased with escalating doses of (-)-cathinone. Serum LH increased with increasing doses while testosterone increased with lower doses and peaked with medium dose then decreased with high dose. The morphological picture showed disruption in smooth and rough endoplasmic reticulum, lipid droplets, mitochondria and Golgi apparatus of Leydig cells, which were either morphologically altered or reduced in number. Morphometric data showed reduced volume densities of these steroidogenic structures. Sertoli cells of treated animals were unaffected while spermatogonia, spermatocytes, spermatids and spermatozoa were variously affected by (-)-cathinone treatment. The effects observed ranged from disruption of cell membranes of spermatogonia, condensation of chromatin material in spermatocytes accompanied by intracytoplasmic vacuolation, disruption of formation acrosome granules in acrosome vesicles and failure of flagella formation at high dose of (-)-cathinone. The mRNA expression of 3 beta- and 17 beta hydroxysteroid dehydrogenase type 1 enzymes increased with low and medium doses but decreased at high dose of (-)-cathinone. The findings demonstrate that sub-chronic exposure
to (-)-cathinone at high dose causes behavioural and hormonal alterations probably via changes in presynaptic striatal dopamine system and hypothalamo-hypophyseal-adrenocortical and gonadal axis integrity. The processes of spermatogenesis and steroidogenesis appear to be affected at high dose of cathinone as evidenced by morphological alterations and suppression of steroidogenic enzyme expression of vervet monkey testis.
CHAPTER 1

1.0 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Khat is both socially and economically one of the most important plants to inhabitants of areas where it is grown, not only to countries of Eastern Africa but also of the Middle East. It is chewed as a social custom and a stimulant. Fresh leaves and shoots are chewed for their euphoriant and stimulatory effects (Al-Bekairi et al., 1991). Cathinone is the principal ingredient of khat, and is found naturally as (-)-enantiomer; the (+)-enantiomer is not found (Kalix and Braenden, 1985). The general analogy between the effects of (-)-cathinone and those of amphetamine, as well as their chemical similarity suggest that the two substances might have the same mechanism of action (Graziani et al., 2008; Houghton, 2004; Cox and Rampes, 2003) with similar potential for abuse (Kalix, 1984a).

Its impact in the body of the consumer ranges from mental, respiratory, digestive to reproductive system effects. There is a growing interest in trying to understand the underlying neural mechanisms and biological functions following use and abuse of khat among consumers. Research findings in humans and experimental animals have reported changes in sleep patterns, mood, attention, aggression, anxiety, locomotor activity, and affiliative behaviours (Kalix, 1994; Pantelis et al., 1989), learning and memory (Kimani and Nyongesa, 2008) and sexual behaviour (Tariq et al., 1990; Qureshi et al., 1988) following khat use. These behaviours are partly influenced by disorders in the hypothalamic dopaminergic system.
(Ishikawa et al., 2007) and partly due to dopaminergic activity in the mesolimbic system (Eisch and Harburg, 2006; Jones and Bonci, 2005; Rang, 2003).

Studies on neuro-behavioural functions following khat use in humans and experimental animals are scarce. Most of the findings have not given clarity to the corresponding magnitude of psychological behavioural changes reported perhaps due to the paucity of objective and, therefore, relevant human data. The framework for postulating khat/(-)-cathinone associated neuro-behavioural changes in sleep patterns, mood, attention, aggression, anxiety, locomotor activity, and affiliative behaviour has been extrapolated from results of khat studies predominantly in mice (Kimani and Nyongesa, 2008), rats (Banjaw et al., 2005; Kalix et al., 1995; Islam et al., 1990) and monkeys (Schuster and Johanson, 1979). Behavioural studies on khat and (-)-cathinone in primates are scarce. The available literature highlights on subjective effects of khat in humans such as induction of mood changes, irritability, anorexia and insomnia following long-term khat use (Al-Motarreb et al., 2002; Nencini et al., 1989). There is scanty literature on effects of khat and (-)-cathinone on consummatory behaviour in humans and non-human primates. Most available literature is on appetitive behaviour in rats (Islam et al., 1990; Eisenberg et al., 1987; Foltin et al., 1983; Zelger and Carlini, 1980; Knoll, 1979), guinea pigs (Jansson et al., 1988) and humans (Murray et al., 2008; Kalix and Braenden, 1985; Halbach, 1972). Appetitive behaviour basically concerns ability or failure to seek food while consummatory behaviour refers to eating according to the Pavlovian homeostatic model.

Mammalian reproduction is systematically controlled by nervous and endocrine systems. Steroid hormones are primarily synthesized by the ovaries and placenta (females) and testes
testicular tissue constitutes seminiferous tubules and interstitium. The seminiferous tubules are composed of Sertoli cells, various forms of spermatogenic cells and cells of the basal compartment while the interstitium is comprised of Leydig cells, fibroblasts, macrophages, blood and lymphatic vessels. Toxic agents impact each of these structures differently through diverse cellular and endocrine pathways. Contradictory findings have been reported on effects of khat and (-)-cathinone on reproductive sex hormones and semen parameters in roosters (Hammouda, 1978), testicular tissue morphology in rats (Islam et al., 1990) and humans (El-Shoura et al., 1995), steroid hormones in mice (Nyongesa et al., 2007) and rabbits (Nyongesa et al., 2008). Quite recently, studies on khat extract and (-)-cathinone in rats showed a biphasic effect on sexual motivation and potency, with high doses diminishing sexual motivation and potency while low doses enhanced motivation (Mohammed and Engidawork, 2011). On the other hand, studies in baboons demonstrated increased plasma testosterone and decreased cortisol and prolactin following khat administration (Mwenda et al., 2006). The measure on cortisol is in concordance with studies in rats (Mohammed and Engidawork, 2011). The discrepancy in results has left more questions than answers. Do khat and (-)-cathinone interfere with neuroendocrine function at the meso-striatal regions of the brain, hypothalamo-hypophyseal level with gonadotropin releasing factors or with enzymatic activity in sex steroid metabolism or structural alteration at gonadal level? Does species difference or dose schedule play role towards this variance in effects? These questions led to the objectives of the present study.
1.2 RESEARCH OBJECTIVES

1.2.1 OVERALL OBJECTIVE

- To investigate the possible mechanism of action of (-)-cathinone on behavioural and reproductive function.

1.2.2 SPECIFIC OBJECTIVES

- To determine effect of (-)-cathinone on behavioural changes in vervet monkeys
- To determine plasma levels of serum cortisol, prolactin, testosterone and progesterone in (-)-cathinone-treated animals.
- To determine serum levels of luteinizing hormone (LH) in response to gonadotropin releasing hormone (GnRH) agonist administration in (-)-cathinone-treated male vervet monkeys.
- To immunolocalize anterior pituitary cell types secreting above hormones and identify potential adenohypophyseal cell apoptosis using S100 as biochemical marker of degeneration.
- To determine the suppressive effects of (-)-cathinone on Leydig cell and germ cell function.
- To determine the effect of (-)-cathinone on the enzymatic activity in sex steroid metabolism

1.3 JUSTIFICATION OF STUDY

The mechanism of action of khat on hypothalamo-hypophyseal-adrenocortical axis influencing neural behaviour and biological function in khat consumers remains to be elucidated. Studies were advanced that increased adrenal phosphorylase activity, serum free fatty acids and urinary
17α-hydroxycorticosteroids could be attributed to stimulatory effect of khat on adrenal function (Connock et al., 2007). Furthermore, studies showed that khat increases adrenocorticotrophic hormone in humans (Nencini et al., 1984) and rabbits (Nyongesa et al., 2008). High concentrations of corticotrophin releasing factor were shown to activate the hypothalamo-hypophyseal-adrenocortical axis and other stress systems in the amygdala resulting in dys-regulated emotional state of drug addiction (Goeders and Guerin, 1996b). Persistent alterations in these systems may lead to pathophysiology that translates into psychiatric disorders such as disturbances in sleep patterns, anxiety, mood, social isolation and sexual behaviour changes manifested in psychotropic drug ‘addicts’.

The mechanism of action of khat on reproductive and neuro-endocrine functions also remains to be elucidated. Quantitative analysis of the sub-cellular architecture revealed by electron microscopy is a logical step in the investigation of the normal functioning of cells and their pathological alterations. Enzymes involved in testosterone synthesis are located in both SER and mitochondria of Leydig cells (Tamaoki, 1973). Testicular ultra-structural changes interfere with morphological expression of testosterone by Leydig cells. Previously, stereological techniques of volume of cells, nuclei, mitochondria, SER and surface area densities of SER and mitochondrial cristae were used to establish the functional activity of Leydig cells in rats (Mazzocchi et al., 1982).

The present study is designed to test the integrity of the pituitary and the central mechanism involved in the regulation of LH release in (-)-cathinone-treated male vervets. The effect on pituitary gland will be assessed by observation of plasma LH release in response to GnRH agonist in (-)-cathinone-treated vervets. Testosterone metabolism was determined by
measurement of the expression of mRNA 3 β- HSD type 1 and 17 β- HSD type 1. The isoforms of 3 β- HSD involved in mammalian testicular testosterone synthesis are type 1 and 3 (Penning, 1997) while the main isymes of 17 β- HSD in testosterone formation in mammals is considered to be 17 β-HSD type 1 and 3, which catalyzes the reduction of androstenedione to testosterone with high efficiency and are almost exclusively expressed in testis (Geissler et al., 1994). The study quantified testicular Leydig cell hypofunction by assessing the steroidogenic organelles of these cells while germ cell hypofunction was done by assessment of ultrastructural alterations following (-)-cathinone treatment.

Khat use is exclusively a human habit. However, controlled and invasive studies in humans are limited by ethical constraints. Monkeys have been used extensively as models for research in human reproduction, developmental anatomy, nutritional disorders and effects of drugs (Amaral, 2002) as well as in understanding the pathogenesis of AIDS (Ambrose et al., 2001). In the present study, the vervet monkey was chosen for use as a model animal due to its recognized utility as an old world monkey alternative to the rhesus macaque, of which there is now a critical shortage for biomedical research. Vervets are currently the most populous African non-human primates. They are similar to rhesus monkeys in behaviour and body physiology (Disotell, 2000) and are closely related to humans (Raaum et al., 2005). They are less expensive and may be accompanied by fewer health and safety risks (Gordon et al., 2005; Baulu et al., 2002). Recently, research findings established paradigms that demonstrate the use of vervet monkeys as models (other than humans) for elucidating cognitive function such as working memory and response inhibition that are relevant to human psychiatric disorders such as schizophrenia (James et al., 2007).
The present study was designed to test the integrity of the hypothalamo-hypophyseal-adrenocortical axis and the mesolimbic system involved in regulation of adrenocorticotrophic hormone release in (-)-cathinone-treated vervet monkeys. The effect on the hypothalamo-hypophyseal-adrenocortical and gonadal axes as well as mesolimbic systems were observed by measurement of serum cortisol, prolactin, progesterone, testosterone and LH levels as well as accompanying immunohistochemical localization of corticotropes, lactotropes and gonadotropes in the hypophysis. Potential hypophyseal cellular degeneration was done by use of S100. The study was also designed to test the integrity of hypothalamo-hypophyseo-adrenocortical and gonadal axes and sex steroid metabolism.

1.4 LITERATURE REVIEW

1.4.1 GENERAL OVERVIEW ON KHAT USE

Khat chewing is predominantly a male habit, but women also practice it. A general moral battle is on-going over khat consumption in East Africa and the diaspora (Klein and Beckerleg, 2007). Khat use has been associated with breakdown of marriages, prostitution and a host of other social evils (Beckerleg, 2008) while male khat consumption is usually highlighted as damaging and irresponsible. Thus, a study on consumption in Djibouti found that khat consumption, mostly by men, is associated with higher inequality in distribution of resources within the family (Borelli and Perali, 2004). In Somalia, women, particularly those in the diaspora present equal discourse in their campaign against khat consumption (Klein and Beckerleg, 2007).
The pattern of khat consumption and its socio-economic impact in Kenya is not any different. According to reports of a survey carried out during the month of December, 2008 in over one hundred homesteads in Maua area of Meru District, Kenya, married couples experience marital problems associated with heavy and long-term use of khat (Nyongesa et al., unpublished data). Majority of the women, in particular, complain of their husbands spending much of their time and money on khat consumption while neglecting family duties. Of those interviewed, 60% claimed that their husbands were not sexually active especially after heavy consumption of khat. About 70% of male khat addicts that were interviewed also reported spermatorrhea following consumption of large quantities of khat while 30% of them reported a boost on sexuality following use. This array of mixed reports parallels what has been reported on khat by various investigators. Most of the research findings both in humans and experimental animals confirm such reports (Nyongesa et al., 2008; El Shoura et al., 1995; Islam et al., 1990) but other studies report differently (Mwenda et al., 2006; Adeoya-Osiguwa and Fraser, 2005; Al-Mamary et al., 2002). Some studies have shown a biphasic effect on testosterone in mice (Nyongesa et al., 2007) and rats (Mohammed and Engidawork, 2011). These reports have been discussed extensively in later chapters of this thesis. These findings indicate that khat contains some components that may have contraceptive efficacy either on their own or in combination with other therapeutics. Such findings, however, remain to be elucidated. In the past two decades, khat use has followed immigrants from the traditional use regions around the Horn of Africa and the Middle East to western countries. Khat can now be regarded as a psychoactive plant that is being targeted for illegalization in most western countries such as the United States and most parts of Europe including United Kingdom, Sweden and the Netherlands among others (Grayson, 2008; Carrier, 2007). The fact that khat’s appearance in western countries has, nevertheless, created moral and political panic in
some circles (Carrier, 2007) and growing anxiety in the source countries because of the fear of illegalization, is not surprising because most of such economies will lose on revenue collections from khat exports. This is particularly so in countries where khat use is associated with a lifestyle and its cultivation a strategy for national development. A proper approach into investigations on khat and human health is a critical consideration whereupon vital information is provided to social and health care professionals, health service providers, community-based health groups as well as policy makers and international organizations.

1.4.2 CHEMICAL COMPOSITION

*Catha edulis* contains many biologically active alkaloids. Cathinone also referred to as S-(−)-cathinone or [S- (−) - α-aminopropiophenone] is the most potent ingredient of khat (Kalix and Braenden, 1985). It is fairly unstable, being metabolized relatively quickly to (±)-norpseudoephedrine (cathine) and (±)-norephedrine, which are more stable and less potent molecules as a result of a chemical reduction once the plant is harvested (Brenneisen and Geisshüsler, 1985). A diastereomeric pair of phenylpropanolamines is produced from this reaction, specifically (±)-norpseudoephedrine [1S, 2S-2-amino-1-phenyl-1-propanol] and (±)-norephedrine [1R, 2S-2-amino-1-phenyl-1-propanol]. Cathine and (−)-cathinone are phenylisopropylamine derivatives, which resemble amphetamine in chemical structure and biological activity (Schorno and Steinegger, 1979).

The substance (±)-norpseudoephedrine was the first to be isolated and identified from the khat plant (Wolfes, 1930) and historically labelled as cathine. Other constituents of khat include: (±)-merucathinone, (±) – merucathine and (±)-pseudomerucathinone, which occur only in khat found in the Meru area of Kenya (Kalix, 1988). There is a rapid stereo-selective metabolism
of S-(-)-cathinone to (-)-norephedrine and (+)-norpseudoephedrine following its administration in humans (Brenneisen et al., 1990; Nencini and Ahmed, 1989; Geisshüsler and Brenneisen, 1987). Metabolism of S-(-)-cathinone to (+)-norpseudoephedrine involves reduction of a keto group to an alcohol, a fairly common metabolic pathway in humans, catalyzed by liver microsomal enzymes (Guantai and Maitai, 1983) (Fig. 1-1).

![Chemical structures](image)

**Figure 1-1.** Chemical structures of S-(-)-cathinone, S,S-(+)-norpseudoephedrine (cathine) and R,S-(-)-norephedrine (MW=151.2). Cathinone is transformed mainly into cathine in khat
leaves and mainly into norephedrine by human metabolism. (Adapted from Feyissa and Kelly, 2008).

(-)-Cathinone can also undergo other reactions such as dimerization and auto-oxidation to form 3,6 dimethyl-2,5-diphenylpyrazine (Berrang et al., 1982; Szendrei, 1980). Enolization of the keto-amine segment also facilitates racemization at the C2 site while S-(−)-cathinone is in solution (Fig. 1-2). Racemization can also occur during drying process after the plant is harvested (LeBelle et al., 1993).
Studies have shown that khat contains many other chemical substances including:

- Alkaloids, terpenoids, flavonoids, sterols, glycosides, tannins (7 - 14% by weight) and more than 10 amino acids including tryptophan, glutamic acid, glycine, alanine and threonine (Szendrei, 1980; Luqman and Danowski, 1976; Halbach, 1972; Geisshülsler and Brennesien, 1987; Elmi, 1983; Crombie, 1980).
- Trace quantities of vitamins including vitamin C (15 mg/100 mg), thiamine, niacin, riboflavin and carotene (Cox and Rampes, 2003; Nencini et al., 1989; Kalix and Braendén, 1985; Luqman and Danowski, 1976).

- Elements including calcium, iron (Hattab and Angmar-Mansson, 2000; Halbach, 1972), manganese (Halbach, 1972), copper, zinc, and toxic metals like lead and cadmium (Matloob, 2003) and a negligible amount of fluoride (Hattab and Angmar-Mansson, 2000).

Composition of alkaloids in khat leaves are diagrammatically presented (Fig. 1-3):

![Figure 1-3. Summary of different classes of alkaloids in khat leaves](image)

The concentrations of (-)-cathinone vary in different parts of the plant, but abundant in young fresh leaves and shoots although it is reduced by three days after leaves have been removed
from khat tree (Balint and Balint, 1994). The potency of khat also depends on the variety of khat. In Kenya, there are different sub-species of khat namely: Ghiza, kangeta and kata. Ghiza has short stems and is said to be more potent. This is the variety that was used in the study.

In this thesis the effect of (-)-cathinone covers: the reward centers of the brain, effect on hypothalamo-hypophyseal-adrenocortical and gonadal axes as well as effect on testosterone biosynthesis \textit{ex vivo}. Literature on these systems has been highlighted for purposes of bringing the present study into the context.

1.4.3 PHARMACOKINETICS OF KHAT

During khat chewing session, leaves and barks of shoots are chewed slowly over several hours, usually 2 to 10 h at which point between 100 and 500 g of khat is chewed by a particular individual (Al-Hebshi and Skaug, 2005; Matloob, 2003; Nencini and Ahmed, 1989). The juice of masticated leaves is swallowed while residues are spat (Toennes \textit{et al.}, 2003). Earlier studies on excretion of khat alkaloids in humans showed that both cathine and norephedrine are slowly absorbed and metabolised (Maitai and Mugera, 1975). In another study, four healthy non-drug using volunteers chewed four portions of 0.6 g khat leaves per kilogram body weight for 1 hr and plasma concentration-time data analysed using a two-compartment model with two-segment absorption (Toennes \textit{et al.}, 2003). It was found that the mucosa of the oral cavity is the first absorption segment where the major proportion of the alkaloids is absorbed. According to the findings of World Health Organization (1985), khat is rapidly absorbed after mastication, metabolized in the liver with only a small fraction appearing in urine (Kalix and Braenden, 1985). The more rapid and intense action of
cathinone compared to cathine, is explained by its higher lipid solubility, facilitating quicker access into the central nervous system (Zelger et al., 1980).

The pharmacokinetic parameters of cathinone and other constituents of khat leaves have been determined over 8 h, with peak plasma levels attained after 1 – 3.5 h (Halket et al., 1995; Brenneisen et al., 1990). Similarly, maximal plasma concentration (t_{max}) of cathinone, cathine and norephedrine are reported to be reached at 2.3, 2.6 and 2.8 h, respectively (Toennes et al., 2003). After ingestion of 0.8 mg/kg cathinone, it was reported that total amount of cathinone absorbed in the body after 9 h was 25 ± 13 µg min ml^{-1} and terminal elimination half-life was 260 ± 102 min (Widler et al., 1994). There is rapid metabolism of S (-)-cathinone to norephedrine and cathine following its administration to humans (Brenneisen et al., 1990; Nencini and Ahmed, 1989). In four human subjects who chewed khat leaves for 1 h, then spat out the residues and subsequently had their urine samples tested for the presence of cathinone and its metabolites, cathinone was detected for up to approximately 26 h, while cathine and norephedrine were detected for at least 80 h (Toennes and Kauert, 2002). Only 7% or less of absorbed (-)-cathinone is excreted in the form of norephedrine and cathine (Toennes and Kauert, 2002; Kalix et al., 1990; Brenneisen and Geisshusler, 1987). The amount of norephedrine excreted in urine is much higher than that ingested suggesting that (-)-cathinone is also metabolized to R, S (-)-norephedrine (Toennes and Kauert, 2002).

1.4.4 DOPAMINERGIC SYSTEM

Neurons of the ventral mesencephalon also referred to as A8, A9 and A10 cell groups, have been collectively designated as the mesotelencephalic dopamine (DA) system (Dahlström and
Fuxe, 1964). These investigators actually identified twelve groups of catecholamine cells (designated A1- A12) distributed from the medulla oblongata to the hypothalamus. Initially, the mesolimbic DA system was thought to originate from the A10 cells of the ventral tegmental area (VTA) that then project to structures closely associated with the limbic system. This system was considered separate from the nigrostriatal DA system originating from the more lateral substantia nigra (SN; A9 cell group) (White, 1996; Kalivas, 1993; Roth et al., 1987; Bannon and Roth, 1983). Five additional cell groups A13-A17 are located in the diencephalon. Olfactory bulb and retine as well as 3 adrenaline-containing cell groups C1 – C3 were later added (Hökfelt et al., 1984). The DA neurons (A12) in the arcuate nucleus of the hypothalamus have been shown to co-localize with various neuropeptides including growth hormone releasing hormone, neurotensin, galanin, enkephalin and dynorphin, suggesting a broader neuroendocrine role of these neuron subtypes (Lookingland and Moore, 2005).

The mesolimbic and mesocortical DA systems are important in modulation of functions such as motivation, control of emotions and cognition sub-served by prefrontal cortex and limbic regions (LeMoal and Simon, 1991). Substantial evidence indicates that the mesolimbic pathway, particularly in DA cells innervating nucleus accumbens, is implicated in the pleasurable reward following psycho-stimulation by use of natural or drug enforcers (Koob, 1992; Di Chiara and Imperato, 1988). Lesion to DA terminals in nucleus accumbens induces hypo-exploration, enhanced latency in initiation of motor responses, disturbances in organizing complex behaviours and inability to switch from one behavioural activity to another (LeMoal and Simon, 1991). Therefore, the mesolimbic DA system is deemed
important for acquisition and regulation of goal-directed behaviours, established and maintained by natural or drug reinforcers (Kiyatkin, 1995; LeMoal and Simon, 1991).

The nigro-striatal DA system originates from the substantia nigra (SN) (A9 cell group). It is important due to its involvement in the pathogenesis of Parkinson’s disease (PD) (Grace and Bunney, 1985). In mammals, the SN is a heterogeneous structure that includes two distinct compartments: the substantia nigra pars compacta (SNC) and the substantia nigra pars reticulata (SNR). The SNC represents the major source of striatal DA and, as already mentioned, its degeneration causes PD. On the contrary, the SNR mainly contains g-amino-n-butyric acid (GABA)-ergic neurons which constitute one of the major efferences of the basal ganglia (Grace and Bunney, 1985).

**1.4.5 SEROTONERGIC SYSTEM**

Serotonin is a neuromodulator whose detailed properties are as yet much more mysterious than dopamine, but which is implicated in a wealth of important phenomena, ranging from analgesia (LeBars, 1988; Sawynok, 1988), hallucinations (Aghajanian and Marek, 1999) to a variety of mood disorders such as anxiety and depression (Westenberg et al., 1996). Virtually all parts of the central nervous system (CNS) receive innervation from serotonergic fibres arising from cell bodies located in two trunks of the midbrain serotonergic nuclei: the dorsal raphe (DR) and the median raphe (MR) (Moukhles et al., 1997; Van Bockstaele et al., 1994). Serotonin-containing bodies of the raphe nuclei project to dopaminergic cells in the VTA and SN, as well as nucleus accumbens, prefrontal cortex and striatum (Moukhles et al., 1997; Van Bockstaele et al., 1994; Hervé et al., 1987) (See Fig. 1-4). There are also serotonergic
projections from the raphe to the periaqueductal gray, which is involved in the control of defensive and aversively motivated behaviours (see Graeff, 2003, for a review).

**Figure 1-4.** Schematic representation of serotonin–dopamine interaction in the mesocorticolimbic and nigrostriatal DA-ergic system. Serotonin-containing cell bodies of the raphe nuclei send projections to dopaminergic cells in both the ventral tegmental area (VTA, A10) and the substantia nigra (SN, A9), and to their terminal fields in the nucleus accumbens, prefrontal cortex and striatum. [Adapted from Di Giovanni et al. (2008)].

At electron microscopy there is presence of synaptic contacts of $^3$H5-HT-labelled terminals with both dopaminergic and non-dopaminergic dendrites in all sub-nuclei of the VTA, and in the SNC and SNr (Moukhles et al., 1997; Kalivas, 1993; Hervé et al., 1987). There is
differential distribution of 5-HT receptor subtypes within the dopaminergic systems (Hoyer et al., 1994; Barnes and Sharp, 1999) that have led to the insight of dopamine-serotonin systems interaction in the brain (Fig. 1-5)

![Diagram of the rat brain with serotonin-immunoreactive cell bodies.](image)

**Figure 1-5.** Mid-saggital view of the rat brain with serotonin-immunoreactive cell bodies. The blue and red ovals encompass the two major subdivisions of the brain serotonergic system. The major functions of 5-HT and the brain areas where they occur are also shown. Abbreviations: DRN, dorsal raphe nucleus; MRN, medial raphe nucleus; NRM; nucleus raphe magnus; NRO, nucleus raphe obscurus; SNc, substantia nigra pars compacta; VTA, ventral tegmental area. [Adapted from Di Giovanni et al. (2008)].

### 1.4.6 NORADRENALINE

Noradrenaline, 3, 4-dihydroxyphenylethanolamine, is released from terminals of noradrenergic neurons in the brain from most postganglionic sympathetic neurons and from
chromaffin cells in the adrenal medulla. The cell bodies of central noradrenergic neurons are all clustered within two bilateral groups of nuclei (A1 – A7) in the brain stem (Fig. 1-6). These comprise the locus coeruleus (LC) complex and the lateral tegmental nuclei. The locus coeruleus proper (nucleus A6) accounts for approximately 45% of all the noradrenergic neurons in the brain (Stanford, 2001). The activity of noradrenergic neurons within locus coeruleus is governed by GABAergic (inhibitory) projection from nucleus prepositus hyperglossi and glutamatergic (excitatory) input from the nucleus paragingantocellularis (Aston-Jones et al., 1991).

**Figure 1-6.** The distribution of noradrenergic neurons in the brain. The cell bodies are clustered in nuclei (A1 – A7) in the pons/medulla regions of the brainstem and their axons project both rostrally and caudally to most regions of the neuraxis. The major nucleus is the locus coeruleus (A6). (Adapted from Stanford, 2001)

Many brain areas are innervated by neurons from LC and lateral tegmental area (LTA). The frontal cortex, hippocampus and olfactory bulb appear to be innervated by neurons originating
from LC whereas most of hypothalamic nuclei are almost exclusively innervated by neurons from LTA (Fig. 1-7).

Figure 1-7. Brain areas receiving a prominent noradrenergic innervation. Most brain regions are innervated by neurons projecting from both the locus coeruleus and tegmental system. (Adapted from Stanford, 2001).

Synthesis of dopamine, adrenaline and noradrenaline share a common pathway. The amino acid L-tyrosine (precursor substrate) undergoes hydroxylation in the presence of tyrosine hydroxylase (TH) to form L-dihydroxyphenylalanine (L-DOPA) followed by decarboxylation by DOPA decarboxylase to form dopamine (Fig. 1-8). This occurs in the cytoplasm of catecholamine-releasing neurons. Dopamine is transported to the storage vesicles where dopamine β-hydroxylase converts it to noradrenaline.
Noradrenaline neurons influence arousal behaviours such as sleep/wakefulness, depression and anxiety (Stanford, 2001). The precise features of environmental stimuli that provoke increased noradrenergic transmission are unclear. Previous studies showed neither ‘novelty’ nor ‘aversiveness’ of the stimulus alone is responsible (McQuade and Stanford, 2000). Increased noradrenergic transmission in the brain mediates changes in selective attention. Another concept is that noradrenergic transmission influence emotional impact of a given stimulus. It is possible that the role and consequences of central noradrenergic transmission depends on type or severity of stimulus or individual differences in neurobiological coding.
behaviour. Overall, it is extremely unlikely that noradrenergic transmission is the sole factor determining behavioural response to even simple emotional stimuli.

1.4.7 EFFECTS OF CATHINONE ON THE REWARD CIRCUIT OF THE BRAIN

Research on behavioural and cognitive function in human khat users has been focussed mostly on observational and single-case studies, but little information has been posted in literature on interventional studies (Hoffman and al’Absi, 2010). The general understanding on these findings is that the effects observed following khat consumption are generally of central stimulation and include euphoria, excitation, anorexia, increased respiration, hyperthermia, logorrhoea, analgesia and increased sensory stimulation (Nencini and Ahmed, 1989). Khat chewers believe that they reason more clearly and are more alert, although their concentration and judgement of issues are objectively impaired (Pantelis et al., 1989). In view of its potency and high lipid solubility (Hassan et al., 2007; Kalix and Braenden, 1985), facilitating access into the central nervous system (Zelger et al., 1980), it can be assumed that khat-induced psychostimulation is predominantly due to cathinone content of the leaves (Kalix, 1990). This is substantiated by the brief stimulation after khat chewing (Kalix et al., 1990), which is in agreement with the finding that cathinone is metabolized rapidly in the body (Brenneisen and Geisshüsler, 1987). Cathine and norephedrine possess weaker central stimulant properties since they are less lipophilic (Nencini and Ahmed, 1989).

There is enough evidence in existing literature suggesting that khat and cathinone induce psychostimulation via meso-striato-corticolimbic dopaminergic pathway (Kalix, 1990). The dependence-producing potential, analgesia and anorexic effects of khat and cathinone are
believed to be partly mediated via this pathway (Gosnell et al., 1996). Hypothalamo-hypophyseo-adrenocortical axis has also been demonstrated to be susceptible to drug abuse via dopaminergic transmission (Steckler and Holsboer, 1999; Sillaber et al., 1988). Activation of the hypothalamo-hypophyseo-adrenocortical axis and ascending catecholaminergic neurons play a critical role in metabolic and behavioural adaptation to various forms of stress (Ishikawa et al., 2007). High levels of glucocorticoids have been shown to contribute to development, maintenance and outcome of substance abuse disorders (Karlsgodt et al., 2003; King et al., 2003). In another related study, it was shown that psychostimulants increase corticosterone levels (Mello and Mendelson, 1997). On the other hand, suppression of glucocorticoids by adrenalectomy reduces extracellular concentrations of dopamine in nucleus accumbens in response to psychostimulants (Barrot et al., 2000; Piazza et al., 1996). Together, these findings indicate a relationship between pleasurable effects of the drug (that influences neural behaviours in drug ‘addicts’) with activation of stress system in the body.

In general, khat consumers show a range of experiences and manifestations from minor reactions to development of psychoses. Some of these include: logorrhoea, hyperactivity, irritability, loss of concentration, anxiety, dizziness, agitation and aggression. These reactions occur after low to moderate exposure to khat (Cox and Rampes, 2003; Griffiths et al., 1997). A number of studies have reported on psychiatric disorders with features of manic-like psychosis following khat use (Gough and Cookson, 1984; Giannini and Castellani, 1982), schizophreniform psychosis (Yousef et al., 1995; McLaren, 1987; Luqman and Danowski, 1976), paranoid psychosis (Nielen et al., 2004; Alem and Shibre, 1997; Critchlow and Seifert, 1987) and depression (Pantelis et al., 1989).
1.4.8 EFFECTS OF KHAT, CATHINONE AND OTHER PSYCHOSTIMULANTS ON CARDIOVASCULAR SYSTEM AND BODY TEMPERATURE

Several studies on khat effects in humans have shown elevated blood pressure and increase in heart rate (Hassan et al., 2005; Hassan et al., 2000; Gugelmann et al., 1985). It was postulated that this effect was mediated by beta adrenergic receptors (Hassan et al., 2005). Increase in blood pressure, heart rate and cardiac contractile force in anaesthetized dogs (Kohli and Goldberg, 1982) and positive ionotropic and chronotropic actions in isolated atria (Gugelmann et al., 1985) were reported after the administration of the active ingredient, (-)-cathinone. Incidences of acute myocardial infarction among regular khat chewers in Yemen have recently been reported (Al-Motarreb et al., 2005). Findings on khat-induced coronary spasm (Al-Motarreb et al., 2004) were supported by the amphetamine, cocaine and 3, 4-methylenedioxymethamphetamine (MDMA), which is structurally and functionally similar to cathinone and both are believed to increase risk of acute coronary syndrome (Ragland et al., 1993).

Khat has been shown to cause central nervous system stimulation, presumably as a consequence of the sympathomimetic effects of (-)-cathinone (Balint and Balint, 1994). Central thermoregulatory disturbances from sympathomimetics may arise from complex interactions between dopamine and 5-HT in the brain stem and hypothalamus (Callaway and Clark, 1994; Yamawaki et al., 1983; Ricuarte et al., 1980). There are several reports linking khat to increase in body temperature (Kalix, 1991; Luqman and Danowski, 1976). Previous studies on rats have shown that a non-selective dopamine receptor agonist, such as apomorphine, when given in the presence of a D₂ dopamine receptor antagonist, such as haloperidol, can elicit an effect on 5-HT receptors resulting in hyperthermia (Yamawaki et al.,
Hyperthermia also commonly follows increased heat production from physical activity. It has been shown by earlier investigators that khat at high doses causes, among other effects, hyperactivity (Kalix, 1991; Pantelis et al., 1989), which may cause changes in core body temperature. Both lack of central dopaminergic activity and serotonergic hyperstimulation have been associated with altered hypothalamic thermoregulatory control as well as abnormal central sympathetic and motor activity that may influence peripheral body heat production and dissipation (Parada et al., 1995; Schwartz et al., 1995; Nimmo et al., 1993; Lee et al., 1985). Dopamine injection into the preoptic nuclei of anterior hypothalamus decreases core body temperature in animal models, suggesting that decreased dopaminergic activity at this level may precipitate hyperthermia (Yamawaki et al., 1983). An earlier study investigating the role of (-)-cathinone in the brown fat tissue thermogenesis (uncoupling oxidative phosphorylation in brown fat tissue cells) obtained evidence suggesting that beta-adrenergic receptors might be involved in the responses obtained (Tariq et al., 1989).

Another proposed mechanism of sympathomimetic-induced hyperthermia suggests that these agents may initiate neuronal damage and increase hypothalamic concentrations of interleukin-1 beta commonly known as the cytokine of fever (Albers and Sonsall, 1995). Peripherally, sympathomimetics such as 3, 4-methylenedioxymphetamine have been shown to raise metabolism by up to 118% while elevating core body temperature (Gordon et al., 1991). Seizures, hyperkinetic muscle action and motor excitability may also contribute to a rise in core body temperature.

Khat contains sympathomimetic compounds that cause vasoconstriction of peripheral vessels in the rabbit (Kalix, 1991) and cardiac effects (Balint and Balint, 1994) thereby impairing heat
dissipation. Although the exact mechanism remains unknown, clinical data and animal studies indicate that ambient temperature, motor activity, metabolic regulation, autonomic vascular changes and central disturbances in thermoregulation all contribute to profound elevations in core body temperature.

1.4.9 CORRELATION OF BEHAVIOUR AND PERIPHERAL BIOCHEMICAL MARKERS OF MONOAMINERGIC ACTIVITY.

Biological correlates of human behaviour that rely heavily on measurements of various parameters in peripheral tissues have been used in biomedical research to reflect some aspect of central nervous system function. Since sympathomimetic drugs produce their effects by increasing synaptic levels of biogenic amines: dopamine, noradrenaline and serotonin, through multiple mechanisms (Fischman and Madras, 2005), the concentrations of these amines can be used to correlate specific behaviours manifested by an experimental animal model or in controlled clinical trials. Some of the peripheral variables that have been suggested to measure aspects of central serotonergic function are blood serotonin (Rao et al., 1998), plasma free tryptophan (Møller et al., 1980), and total plasma tryptophan (Fernstrom et al., 1976). Plasma free and total plasma tryptophan have been shown to correlate positively with some neural behaviours (approach, heterogroom and eat, and inversely with ‘avoid’ and ‘be solitary’) in studies done in the vervet monkeys. Whole blood serotonin correlated inversely with “avoid” and “be solitary” (Raleigh et al., 1981). In these findings, it was suggested that in normal, drug naive monkeys, plasma free and total plasma tryptophan are better correlates of the central serotonergic activity influencing behaviour than is whole blood serotonin.
Studies have shown that the biological correlates of central noradrenergic system activity measured in cerebrospinal fluid/blood increased in relation to enhanced pharmacological activity of Locus coeruleus (LC)- a noradrenergic locus (Stanford, 2001). These measures included activity of tyrosine hydroxylase in the LC and noradrenaline release in LC projection areas. An earlier study conducted in patients with psychiatric disorders demonstrated that an increase in peripheral norepinephrine results from activation of sympathetic nervous system and reflects, rather than causes anxiety (Starkman et al., 1990). Further investigations on amphetamine have shown that plasma levels of glucocorticoids and adrenocorticotrophic hormone (ACTH) are increased by its acute administration in rodents and humans (Smith et al., 2004; Swerdlow et al., 1993; Jacobs et al., 1989; Halbreich et al., 1981).

1.5.0 EFFECTS OF KHAT, CATHINONE AND OTHER PSYCHOSTIMULANTS ON HYPOTHALAMO-HYPOPHYSEAL-ADRENOCORTICAL AXIS

Hypothalamo-hypophyseo-adrenocortical axis and pre-striatal dopamine (mesolimbic) system may be susceptible to drug abuse, since most of these drugs have an affinity for dopaminergic transmission (Steckler and Holsboer, 1999). Activation of the hypothalamo-hypophyseo-adrenocortical axis and ascending catecholaminergic neurons play a critical role in metabolic and behavioural adaptation to various forms of stress (Ishikawa et al., 2007). Discrepancies on findings regarding effects of psychostimulants on hypothalamo-hypophyseo-adrenocortical axis have been observed. Preliminary studies showed that psychostimulants increase corticosterone levels (Mello and Mendelson, 1997) and these increased glucocorticoids contribute to substance abuse disorders (Karlsgodt et al., 2003; King et al., 2003). On the other hand, suppression of glucocorticoids reduces extracellular concentrations of dopamine in nucleus accumbens in response to psychostimulants (Barrot et al., 2000;
Piazza et al., 1996). Studies on khat have demonstrated increases in adrenocorticotropic hormone in humans (Nencini et al., 1984) and plasma cortisol in rabbits (Nyongesa et al., 2008). However, in baboons, khat decreased plasma cortisol and prolactin (Mwenda et al., 2006). High levels of glucocorticoids have also been shown to increase rates of response to psychostimulant drugs (Goeders and Guerin, 1996 b; Piazza et al., 1991) though mechanism modulating the rise in plasma cortisol remains speculative. This is supported by the finding that adrenolectomy attenuate psychostimulant self-administration (Deroche et al., 1997; Goeders and Guerin, 1996 a) and reduction in the amount of alcohol intake in alcohol-preferring rats (Fahlke et al., 1994).

In several other investigations designed to examine the relationship between cortisol levels, mesolimbic dopamine release and subjective drug response to amphetamine in healthy volunteers, increase in cortisol levels was associated with greater amphetamine-induced dopamine release in left ventral striatum (LVS), left dorsal putamen (LDP) and right dorsal putamen (RDP) (Oswald et al., 2005). These findings paralleled those implicating interaction of stress with alteration of mesocorticolimbic dopamine neurotransmission (Marinelli and Piazza, 2002). Glucocorticoids are not only activated by stress but their secretion also precedes many goal-seeking behaviours such as food-seeking (Bassareo and Di Chiara, 1999; Taber and Fibiger, 1997; Wilson et al., 1995; Salamone et al., 1994; Westerink et al., 1994) and drug-seeking behaviour (Cadoni et al., 2003; Marinelli and Piazza, 2002).

There is also growing evidence demonstrating interaction of stress with serotonergic systems. Functional interactions of corticotrophin-releasing hormone (CRH) with serotonergic neurons in the midline raphe have been shown in experimental animals (Linthorst et al., 1997).
Findings have also demonstrated a precise overlap of processes containing CRH and serotonin in the dorso-lateral sub-nucleus (Ruggiero et al., 1999). Corticotropin-releasing hormone subtype 2 receptors were expressed in the brainstem raphe indicating a neuro-peptidergic interaction with serotonin (Chalmers et al., 1995). An identical locus is enriched in CRH receptors in the macaque (Millan et al., 1986) and interconnects with limbic and visceral reflex centres (Fulwiler and Saper, 1984).

1.5.1 HYPOTHALAMO-HYPOPHYSEAL-GONADAL AXIS IN THE MALE

Of the three components of the male reproductive system, the hypothalamus and the anterior pituitary gland have solely regulatory functions, which are mediated by the hormones secreted from these two organs. The third component, the testes, also produces key hormones controlling male sexual characteristics and behaviours, the most important of which is testosterone. In addition, the testes are responsible for sperm production.

The hypothalamus, which is located at the base of the brain, is often called the master control unit of the reproductive systems of both men and women. Among other hormones, the hypothalamus produces gonadotropin releasing hormone (GnRH) which is secreted in pulses into a system of blood vessels that connect the hypothalamus to the anterior pituitary gland located just beneath the hypothalamus.

In response to the GnRH stimulus, the anterior pituitary gland produces two hormones that control reproductive functions: luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and releases them into the general circulation. These two hormones have different functions in males and females. In the male, LH stimulates the testes to produce testosterone,
whereas FSH plays an important role in sperm maturation. In addition, the anterior pituitary gland produces prolactin. In the male, elevated prolactin levels play a role in reproduction by indirectly suppressing testosterone levels in the body.

The testes consist primarily of the seminiferous tubules, the site of sperm cell formation and maturation. Interspersed among those tubules are interstitial cells, including Leydig cells, which produce testosterone. In addition to its function in reproduction, testosterone helps regulate many diverse body functions, including bone and muscle development, red blood cell turnover, and development and maintenance of male secondary sexual characteristics, such as sexual drive, growth of facial and body hair, and deepening of the voice during puberty in boys. Consequently, an insult to the hormonal system controlling testosterone production can result not only in infertility but also in other deleterious consequences, such as accelerated bone loss, decreased muscle function, and lower than normal numbers of red blood cells.

Another important testicular cell type is the Sertoli cell. These cells play a critical role in sperm development by supporting and nourishing the germ cells during their development. The three components of the male reproductive system: the hypothalamus, anterior pituitary, and testes form a finely tuned system called the hypothalamo-pituitary-gonadal (HPG) axis, which is controlled through a classic negative feedback mechanism. As testosterone levels in the blood rise, the anterior pituitary becomes less responsive to stimulation by GnRH, resulting in reduced LH and FSH secretion. Because LH induces testosterone production, reduced LH secretion results in lowered testosterone levels. Conversely, if testosterone levels in the blood decline, such as during injury to the testes, the anterior pituitary’s responsiveness to GnRH increases and more LH and FSH are secreted, thereby promoting testosterone
production by Leydig cells. Following a classical negative feedback loop high testosterone levels inhibit further secretion of GnRH.

In addition to this feedback loop, several hormones produced within and outside the testes regulate testosterone production. One such hormone is β-endorphin, a molecule similar to morphine produced within the testis. It suppresses testicular testosterone production and/or release. Furthermore, β-endorphin released in the hypothalamus results in decreased GnRH levels. Lower GnRH levels, in turn, lead to reduced LH and FSH secretion from the anterior pituitary and reduced testosterone production by the Leydig cells. (More information on the hypothalamo-pituitary-gondal axis function is found in article by Hiller-Sturmhöfel and Bartke, 1998 pp. 153–164).

1.5.2 KHAT AND CATHINONE AND THE HYPOTHALAMO-HYPOPHYSEAL-GONADAL AXIS

Numerous and contradictory findings have been reported about khat and (-)-cathinone on reproductive function. These reports are all convincingly supported by enough evidence, leading to a dilemma as to whether or not khat is a boon or bane to humanity. Studies reported khat as an aphrodisiac (for review see Feyissa and Kelly, 2008; Bentur et al., 2008), with its cathine and norephedrine alkaloids being shown to stimulate the final stages of sperm maturation and inhibition of acrosomal loss (Adeoya-Osiguwa and Fraser, 2005). Similar findings showed that rabbits fed on freeze-dried leaves of khat for three months had an increased rate of spermatogenesis (Al Mamary et al., 2002). Furthermore, cathine and caffeine were shown to increase sexual motivation when given separately or in combination
(Taha et al., 1995). Similarly, studies in olive baboons showed enhanced plasma testosterone and decreased prolactin and cortisol levels following khat treatment (Mwenda et al., 2006).

In a World Health Organization bulletin, reports of khat on impairment of sexuality, inability to sustain erection, loss of libido and spermatorrhoea (WHO, 1980), consistent with those of Pantelis et al. (1989) have been highlighted. Other studies reported a significant increase in number of abnormal sperm cells following (-) cathinone exposure in mice (Qureshi et al., 1988), rats (Islam et al., 1990) and accompanying degenerative changes of testicular tissue with decrease in plasma testosterone levels in rats (Islam et al., 1990). Mutagenicity of germ cells in albino mice has also been demonstrated following (-) cathinone treatment (Tariq et al., 1990). Studies in humans showed that long-term khat consumption caused deleterious effects on sperm parameters and steroidogenesis (El-Shoura et al., 1995). The biggest question now is; which way forward? On the whole, however, a greater percentage of the studies show solid proof that khat impairs rather than boosts sexuality.

There are about four possible mechanisms through which khat and (-) cathinone can influence plasma testosterone levels. Through:

- Inhibition of release of luteinizing hormone-releasing hormone from the hypothalamus resulting in a series of secondary effects on the pituitary and testes.
- Blockage of the biosynthesis of the sex steroid hormones at the level of the testes.
- Suppression of the synthesis and/or release of LH from the pituitary and thereby inhibit LH-dependent testicular steroidogenesis.
- Enhancement of metabolism of testosterone by the liver.
The present study considered the first three hypotheses: that is, determination of the integrity of the hypothalamus, reproductive hormone profiles and examination of the sex steroid hormone biosynthetic pathway and testicular ultrastructure.

1.5.3 SEX STEROID HORMONE METABOLISM

Steroid hormones are derived from acetate with cholesterol as an intermediate product. It is well known that smooth endoplasmic reticulum (SER) is involved in endogenous synthesis of cholesterol from acetate and glucose (Christensen, 1975) and that cholesterol is the main product stored in lipid droplets (Moses et al., 1969). Cholesterol is metabolized into pregnenolone, which in the presence of 17 α- hydroxylase and 3 β- hydroxysteroid dehydrogenase (3 β-HSD) can be converted into 17 α- hydroxyprogrenolone and progesterone, respectively. The entire biosynthetic pathway gives rise to four major classes of steroid hormones: the progestagens, estrogens, androgens and corticosteroids. Progestagens lead to formation of androstenedione, which is converted into testosterone in the presence of 17 β –hydroxysteroid dehydrogenase (17 β-HSD). In the presence of 17, 20 desmolase, 17 α- hydroxyprogrenolone is converted into dehydroepiandrosterone, which is in turn converted into androstenediol in the presence of 17 β-HSD. Androstenediol is then converted into testosterone under the influence of 3 β-HSD. Steroid hormones are synthesized by gonads, adrenal gland, ovaries and placenta. The figure below shows the role of different enzymes in the synthesis of steroid hormones in gonads and adrenal cortex (Fig. 1-9).
Figure 1-9. Biosynthesis of steroid hormones in adrenal cortex and gonads. Individual enzymes are highlighted and showing different isoforms of cytochrome P450s (CYP) and hydroxysteroid dehydrogenases (HSD). (Adapted from Paynes and Hales, 2004).

Hydroxysteroid dehydrogenases (HSDs) play an important role in the biosynthesis and inactivation of all steroid hormones. They exist in multiple isoforms, which show tissue specificity in expression and this, coupled with properties of each isoform (reductase or dehydrogenase), can determine the role of enzyme in steroid hormone action. In testis, 3β-HSD and 17 β-HSD play an essential role in testicular steroidogenesis (Andersson et al., 1995). Several isozymes of 17 β-HSD have been identified in human steroidogenic tissues. The isozymes type I, II and III of 17 β-HSD (Andersson et al., 1995) and type IV (Adamski et al., 1995) are found in microsomes of testis where they reduce androstenedione to testosterone.
1.5.4 VERVET MONKEY BIOLOGY AND USE IN BIOMEDICAL RESEARCH

1.5.4.1 Reproduction

Vervets are seasonal breeders. Among captive vervets in Kenya, the mating season lasts from July to September and births are grouped between November and January (Eley et al., 1986). In wild African populations, the breeding season lasts from April to June and the majority of births are concentrated from October to December (Baldellou and Adan, 1997). In the West Indies, births occur throughout the year but most are concentrated from April to July (Horrocks, 1986). Seasonal breeding in vervet monkeys evolved due to the need to reproduce during times of food abundance. After the rainy season, food resources are much more plentiful compared to other times of the year. Females nursing their young ones, together with their young ones are not as likely to be nutritionally stressed during this period, thereby increasing their chances of survival (Baldellou and Adan, 1997).

In the wild, females attain sexual maturity at about four years of age and give birth at around five years old (Cheney et al., 1988). However, in captivity vervets are not affected by seasonal pattern of breeding but rather readily reproduce throughout the year (Seier, 2005). Here, females mature fairly fast such that they can give birth at the age of two years (Fairbanks and McGuire, 1985). Female vervets do not exhibit external signs of oestrus (Cheney and Seyfarth, 1990; Eley et al., 1989). In captivity the ovarian cycle lasts about 32.5 days, and is characterized by menstruation. Peak sexual receptivity occurs around the 13th day of the cycle (Eley et al., 1989; Else et al., 1986). Female vervets undergo ovarian cycles throughout the year, but the cycles are irregular during non-breeding seasons (Else et al., 1986). Males reach sexual maturity at around five years of age, but do not achieve full adult weight until after six years of age. This seems to limit the opportunity for mating until then.
The gestation period in vervets is between 163 to 165 days (Andelman, 1987; Eley et al., 1986) while the inter-birth interval varies from 1 to 2 years, depending on presence or absence of a surviving offspring of the previous breeding season. In the West Indies, the inter-birth interval is slightly less than 1 year (Horrocks, 1986).

1.5.4.2 Use in neuro-behavioural and reproductive health research

The vervet monkey (Chlorocebus aethiops, formerly Cercopithecus aethiops) has long been among the most important non-human primate (NHP) models for biomedical research, other than the rhesus macaque and much greater than that of any other NHP (Baulu et al., 2002). The dramatic recent growth in the use of vervet as a model derives, in part, from its recognized utility as an old world monkey alternative to the rhesus macaque, of which there is now a critical shortage for biomedical research. In addition, Human Immunodeficiency Virus (HIV) research also has an increasing focus on vervets because, in contrast to macaques, they are readily infected with simian immunodeficiency virus (SIV) but do not progress to disease.

Vervet as an alternative to rhesus:

The vervet is similar to rhesus in behaviour and physiology (Disotell, 2000; Ziegler, 1990) and is about equally closely related to humans (Raaum et al., 2005; Page, 2001). At the same time it is more readily accessible, less expensive, and may be accompanied by fewer health and safety risks (Gordon et al., 2005).

Investigation of neurobehavioural phenotypes:

Neuro-behavioural phenotypes form a central focus of vervet monkey investigation. Behavioural observations over several decades have identified heritable traits relating to aggression, maternal-infant interactions, anxiety, and novelty seeking and impulsivity
More recently, paradigms have been established that demonstrate the enormous value of NHPs as models intermediate between humans and rodents for elucidating cognitive processes such as working memory and response inhibition that are relevant to human diseases such as schizophrenia and attention deficit disorder respectively (James et al., 2007).

**Investigation of the immune system and infectious disease:**

The vervet is growing in importance as a model species for acquired immunodeficient syndrome (AIDS) research. African populations of the vervet are natural carriers of SIV and unlike Asian macaques, do not show signs of illness when infected. Caribbean-derived vervets are free of SIV and thus represent an excellent model for experimental study of the immune response on initial infection. Several laboratories are using this model to understand how the immune system prevents disease progression, with potential implications for the care and prevention of HIV-related diseases in humans (Goldstein et al., 2006).
CHAPTER 2

2.0 GENERAL MATERIALS AND METHODS

2.1 ANIMALS AND HOUSING

Fourteen adult vervet monkeys were captured in the wild and put under quarantine at the Institute of Primate Research (IPR) for three months during which time they were tuberculin-tested. The animals were housed in individual standard monkey cages (0.6 m x 0.66 m x 0.8 m) for the duration of the study. Daily supply of monkey chow, vegetables, fruits and commercial multivitamins was given around 0900 h, while water was provided ad libitum. Lighting conditions with approximately 12 h: 12 h (light: dark cycle) and an average room temperature of 23 °C with a relative humidity of approximately 60% was provided in the animal house. Cage cleaning and regular change of beddings was also observed. Complete animal health care and supervision was provided to the animals throughout course of study and only healthy animals were selected for investigation. All study procedures followed accepted veterinary protocols for analgesia and anaesthesia that were approved by the Institutional Review Committee (IRC) at IPR. These protocols were guided by International Guiding Principles for Biomedical Research involving animals, and developed by the Council for International Organizations of Medical Sciences.

2.2 CHEMICALS

Goserelin acetate (Zoladex)- a Luteinizing Hormone Releasing Hormone (Sigma Ltd St. Louis, MO, USA), immunohistochemistry antibodies of LH, prolactin and Adrenocorticotropic
hormone (DakoCytomation, Denmark), Luteinizing hormone, androstenedione, progesterone, cortisol, prolactin and testosterone enzyme immunoassay kits. Polymerase chain reaction reagents included: ZR Tissue & Insect RNA Microprep Kit, RevertAid Premium First Strand cDNA Synthetic Kit, DreamTaq DNA Polymerase, dNTP Mix, TopVision LE GQ Agarose, GR Green Nucleic Acid Stain, Maxima Probe/ROX qPCR Master Mix ((Nova Tec Immundiagnostica GMBH, Germany). Khat samples were obtained from Meru district in Kenya.

2.3 EXTRACTION OF CATHINONE FROM KHAT

Fresh leaves and shoots of khat (ghiza variety) were weighed (500 g) and crushed with mortar and pestle into very small pieces (< 5 mm) and dissolved in 250 ml methanol in a conical flask. The extraction protocol followed that previously described by Lee (1995). In brief, the mixture was shielded from light and sonicated at room temperature for 15 min with intermittent shaking and stirring followed by filtration through cotton gauze and then grade 1 Whatman filter paper to get rid of fine particles. The non-filtered plant material was re-extracted in 250 ml fresh methanol and sonicated for 24 h. The mixture was then filtered and admixed with initial methanol-extracted khat material and condensed to near dryness (< 1ml) using a stream of air. A very dilute solution of sulphuric acid (approximately 0.02 N) was used to re-suspend and acidify the residue. This was followed by chloroform extraction to remove neutral organic compounds as well as the remaining plant solids. A small amount of saturated sodium bicarbonate solution was added to the aqueous solution to basify the extracts followed by methylene chloride to extract cathinone and cathine. A stream of air was again used to reduce the extract to a minimal amount. The resultant solution was then vacuum-dried.
at 337 millibar in a Rotar Vump for 4 to 5 hours into an oily paste. The dry weight of the extracts was determined and thin layer chromatography used to confirm the presence of alkaloids. The plant extracts were spotted directly onto a pre-coated 5 by 10 cm silica gel 60 plate. The plate was then developed in ethyl acetate: methanol: aqueous ammonia (85:10:5), and viewed under an ultraviolet lamp. The spots were visualized using a 0.5% ninhydrin solution, and the plate heated using a heat gun. Colour development was used to localize the alkaloids (purple for cathine and burnt orange for cathinone) among the moving spots. The $R_f$ values for cathinone and cathine were also calculated. The vapour phase infrared spectra was obtained on a Hewlett-Packard Model 5890 Series II Gas Chromatograph, using a 12 m by 0.32 mm HP-5 (0.52 µm loading) capillary column and a temperature program of 70 $^\circ$C for 1 min, 15 $^\circ$C/min to 270 $^\circ$C, with a final temperature hold for 5 min, equipped with a Hewlett-Packard Model 5965B Infrared Detector. About 300 g of total cathinone was extracted and it was approximately 97% pure.

2.4 EXPERIMENTAL DESIGN

The fourteen adult vervet monkeys (eight males and six females) were randomly assigned to five groups. Group I animals were controls and comprised of two animals while group II, III, IV and V were test animals and comprised of three animals each. The animal identification by group and sex was as follows: GI (2148_1 [male], 2149_1 [female]), GII (2107_1, 2107_2 [males], 2107_3 [female]), GIII (2169_1, [male], 2169_3, 2169_4 [females]), GIV (2158_1, 2158_2 [males], 2158_3 [female]) and GV (2155_1, 2155_2 [males], 2155_3 [female]). Group III had one male and two females while control group had one male and one female. Group II, III, IV and V animals were administered (-)-cathinone at 0.8 mg/kg, 1.6 mg/kg, 3.2
mg/kg and 6.4 mg/kg body weight, respectively via intragastric tube. Controls were administered normal saline. The study was carried out in two phases: Pre-treatment and treatment phases.

2.4.1 IN VIVO STUDIES

2.4.1.1 Pre-treatment phase (40 days)

All animals were habituated to presence of observers and handling for 4 weeks before commencement of experiments constituting pre-treatment phase (1 month). Caution was taken to avoid stress to the animals. A detailed description of protocol on cage enrichment and animal acclimatization to presence of observer is given in chapter 3 of this thesis.

The femoral vein of each animal was cannulated using a 22 G blood vessel cannula after shaving and swabbing the area with 70% alcohol. Heparinised saline was introduced into the cannula to prevent blood clotting inside the lumen. The adhesive tape was used to anchor the cannula in situ to prevent it from dropping. Blood samples (3 ml) were then collected from the femoral vein at 5-days interval for cortisol, prolactin, luteinizing hormone, progesterone and testosterone analysis.

2.4.1.2 Treatment phase (4 months)

Control animals were administered 10 ml normal saline (0.9% sodium chloride) via oral gavage three times a week (Mondays, Wednesdays and Fridays) for 4 months (Fig. 3-1). Control and test animals were anaesthetized with ketamine hydrochloride intramuscularly (initial dose of 10
mg/kg body weight followed by 2.5 mg/kg body weight at 30 min intervals) at 0930 h before administration of normal saline and (-)-cathinone at 1000 h, respectively. The dose of ketamine used was not enough to induce narcosis but only sufficient to immobilize the animals. Test animals, grouped into four groups were treated with respective doses (0.8, 1.6, 3.2 and 6.4 mg/kg body weight) of (-)-cathinone via oral gavage three times a week (Monday, Wednesday and Friday) for 4 months. The doses were chosen based on previous studies in humans (Kalix, 1984 b) and rats (Mohammed and Engidawork, 2011) which indicated optimum effect of (-)-cathinone on various body parameters to be within this dose range. Specific experimental protocols following treatment are discussed in the following chapters.
CHAPTER 3

ACUTE AND SUB-CHRONIC EFFECTS OF PURIFIED CATHINONE FROM KHAT (CATHA EDULIS) ON BEHAVIOUR PROFILES IN VERVET MONKEYS (CHLOROCEBUS AETHIOPS)

3.0 INTRODUCTION

Khat is customarily consumed by millions of inhabitants in the south-west part of Arabian Peninsula and East African countries between Sudan and Madagascar namely: Djibouti, Ethiopia, Somalia, Kenya, Tanzania and Uganda. Fresh leaves and shoots are chewed for their euphoriant and stimulatory effects (Al-Bekairi et al., 1991). Cathinone is the principal ingredient of khat, and is found naturally as (-)-enantiomer. The (+)-enantiomer is not found (Kalix and Braenden, 1985). The (-)-cathinone resembles (+)-amphetamine in chemical structure and biological activity (Zelger et al., 1980).

(-)-Cathinone is a lipophilic alkaloid and readily crosses the blood-brain barrier to reach the primary sites of action in the central nervous system (Cox and Rampes, 2003). Although behavioural consequences associated with khat and cathinone exposure in humans and experimental animals have been extensively documented, the corresponding magnitude of these psychological behavioural changes remain unclear due to the paucity of objective and,
therefore, relevant human data. The framework for postulating khat/cathinone associated behavioural changes in sleep patterns, mood, attention, aggression, anxiety, locomotor activity, and affiliative behaviour has been extrapolated from results of khat studies predominantly in mice (Kimani and Nyongesa, 2008), rats (Banjaw et al., 2005; Kalix et al., 1995; Islam et al., 1994), monkeys (Schuster and Johanson, 1979) and humans (Pantelis et al., 1989). For example, long-term khat chewing has been shown to induce a depressive mood, irritability, anorexia and insomnia (Al-Motarreb et al., 2002; Nencini et al., 1989). Reports on studies on khat and cathinone in non-human primates are scarce. Earlier studies lack clarity on behavioural observation over an extended period of time. There is also scanty literature on effects of khat and cathinone on appetite. Most available literature is on rodents where khat reduced food consumption, maternal weight gain and lowered food efficiency index in pregnant rats (Islam et al., 1994).

In this study, we administered (−)-cathinone to adult male and female vervet monkeys housed in single cages. Individual caging was considered since it allowed for individual behavioural assessment independent of influence from other mates. Such measurements are highly relevant for behavioural models of human psychological conditions and since the study was designed to determine the effect of (−)-cathinone doses in presence/absence of cage enrichment, the primary consideration was individual observations and whether or not cage enrichment had any effect on specific behaviours. Cage enrichment is regarded as a reward since animals are conditioned to differentiate between a reward and a no reward situation. In the present study, cage enrichment aimed at determining whether behavioural changes observed could be altered by a reward or was due to a reward. Behavioural studies on khat and (−)-cathinone in primates is scarce. The available literature highlights on subjective effects
of khat in humans such as induction of mood changes, irritability, anorexia and insomnia following long-term khat use (Al-Motarreb et al., 2002; Nencini et al., 1989). Similarly, earlier studies do not indicate clarity on experimental design and behavioural observations over an extended period of time. This has brought about generalization of characterizations for interpreting (-)-cathinone effects in human khat consumers hence conflicting reports. There is also scanty literature on effects of khat and (-)-cathinone on consummatory behaviour in humans and non-human primates. Most available literature is on appetitive behaviour in rats (Eisenberg et al., 1987; Foltin et al., 1983), guinea pigs (Jansson et al., 1988) and humans (Murray et al., 2008; Kalix and Braenden, 1985). Appetitive behaviour basically concerns ability or failure to seek food while consummatory behaviour refers to eating according to the Pavlovian homeostatic model.

Khat use is a human habit; it is not consumed by other animals. However, controlled and invasive studies in humans are limited by ethical constraints. This study aimed at determining effects of acute and sub-chronic exposure to (-)-cathinone on specific types of behaviour in presence and absence of cage enrichment. It was hypothesized that long-term administration of (-)-cathinone, and cage enrichment influence behavioural alterations regardless of (-)-cathinone dose. Vervet monkeys were used since they are a good model for investigating neuro-behavioural phenotypes relating to aggression, maternal-infant interactions, novelty seeking and impulsivity (Fairbanks et al., 2004; Fairbanks et al., 2001 b; Fairbanks et al., 2001 a; Fairbanks et al., 1999) as well as elucidation of cognitive process (James et al., 2007).
3.1 MATERIALS AND METHODS

3.1.1 ANIMALS AND HOUSING

This is the same as was described previously in section 2.1 of general materials and methods.

3.1.2 ANIMAL HANDLING AND EXPERIMENTAL DESIGN

This is same as was described previously in section 2.4 of general materials and methods. Briefly, a total of fourteen adult vervet monkeys (eight males and six females) were randomly divided into four groups of 3 test animals each and a fifth group with two animals (one male and one female) serving as controls. Group III had one male and two females while other test groups had two males and one female. The summary is indicated in Table 3-1 below. The study was carried out in two phases:

3.1.2.1 Pre-treatment phase

Pre-treatment period comprised of 2 months. However, the first month served to habituate the animals to the presence of the observer as well as handling while during the second month of pre-treatment phase, blood sampling together with individual behavioural observations were done. Caution was taken to avoid stress to the animals.

Environmental (cage) enrichment during pre-treatment phase

Experimental animals did not have prior experience with the plastic toys used for cage enrichment (Fig. 3-1). Cage enrichment was achieved with plastic toys that were hollow and bright-coloured according to the protocol of Bayne (1989). Briefly, focal subjects were observed on 3 days of each week: Monday, Wednesday and Friday. Day 1: no toys (control)
observed for 1 h; Day 2: first presentation of toy and observed for 1 h; Day 3: presentation of same toy for 20 min followed by filling with bananas or carrots and removal of toy without any food enrichment. Three observers standing at strategic positions in full view of focal subjects made the behavioural scoring. This was done during acclimatization for 4 weeks followed by 1 month of pre-treatment phase. The aim of behavioural observations during pre-treatment phase was to establish baseline values of specific behaviours of interest.

![Figure 3-1. Cage enrichment with a coloured toy](image)

### 3.1.2.2 Treatment phase

Treatment given to the animals is as was described in general materials and methods under section 2.4.1.2.
i). Environmental (cage) enrichment during treatment phase

The protocol was similar as that employed during pre-treatment phase. Similar toys were used but with holes made on the sides that could be filled with food on some days. Focal subjects were observed on 3 days of each week: Monday, Wednesday and Friday as for pre-treatment.

ii). Behavioural studies

Behavioural definitions

Fourteen individual behavioural categories were condensed into 5 composite behaviour scores: aggression, anxiety, abnormal behaviour, withdrawal and appetitive/consummatory behaviour using a one-zero sampling technique (Melega et al., 2008; Martin and Bateson, 1993). The aggression composite score was defined by yawn, engage in stare, jaw thrust, bouncing off cages, shaking cage walls and head jerk (Melega et al., 2008). The anxiety composite score combined 3 (pacing, scratch and self-directed) behaviours that have been validated as indicators of anxiety in non-human primates (Melega et al., 2008; Castles et al., 1999; Aureli, 1997; Maestripieri et al., 1992). The description of abnormal behaviour combined measures of “responses independent of appropriate stimuli”, “fine motor” and “whole body stereotypy” (Castner and Goldman-Rakic, 1999). Withdrawal behaviour was defined by duration of time taken by a focal subject on the platform or at the corner of the cage while appetitive/consummatory behaviour was defined by the latency of time to respond to food introduced in the cage (Rapkin et al., 1995). Table 3-2 gives a summary of behavioural definitions that were followed.

Behavioural scoring
All behaviours were observed from 1100 h to 1300 h of each observation day. Animals were administered (-)-cathinone via intra-gastric tube (Fig. 3-2) at 1000 h three times a week (Monday, Wednesday and Friday) as described under general materials and methods and cage enrichment followed the protocol described by Bayne (1989). Focal subjects were observed for their interest with empty toys and toys with food. A video camera (Type-Image video camera CAM) connected to a video camera recorder (VCP-C10, Toshiba PTE Ltd., Singapore) was placed at a strategic position within the animal house and three different observers who had been habituated to the animals scored individual behavioural scores. Inter-rator reliability, which was obtained by dividing the number of times behaviour was scored by the observers by the number of times each of the observers scored the behaviour, was 90%. Behavioural observations were collected on all focal subjects. Behaviour was scored if it occurred one or more times during a 3- min session. Three x -3 minute sessions were done for each of the frequency behaviours (aggression, anxiety and abnormal behaviour) per experimental day. For durational behaviours of withdrawal and appetitive/consummatory behaviour, three x 8- min sessions were done per each observation day.
**Table 3-1.** Animal grouping by number, sex, age and treatment dose

<table>
<thead>
<tr>
<th>Animal group</th>
<th>No. of animals</th>
<th>Sex</th>
<th>(-)-Cathinone dose (mg/kg)</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2</td>
<td>1M, 1F</td>
<td>0</td>
<td>A</td>
</tr>
<tr>
<td>II</td>
<td>3</td>
<td>2M, 1F</td>
<td>0.8</td>
<td>A</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>1M, 2F</td>
<td>1.6</td>
<td>A</td>
</tr>
<tr>
<td>IV</td>
<td>3</td>
<td>2M, 1F</td>
<td>3.2</td>
<td>A</td>
</tr>
<tr>
<td>V</td>
<td>3</td>
<td>2M, 1F</td>
<td>6.4</td>
<td>A</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>8M, 6F</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3-2. Summary of composite behaviour definitions. [Adapted from Melega et al. (2008)].

<table>
<thead>
<tr>
<th>COMPOSITE BEHAVIOR</th>
<th>BEHAVIOURAL CATEGORY</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Frequency Behaviours</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggression</td>
<td>Yawn</td>
<td>Yawning with teeth showing</td>
</tr>
<tr>
<td></td>
<td>Engage in a stare</td>
<td>Open mouth, ears back and eyelids up</td>
</tr>
<tr>
<td></td>
<td>Jaw thrust</td>
<td>Grinding teeth</td>
</tr>
<tr>
<td></td>
<td>Bouncing off cage walls</td>
<td>Focal subjects jumping off cage walls within cages</td>
</tr>
<tr>
<td></td>
<td>Vigorous shaking of cage walls</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Head jerk</td>
<td>Rapid sideways jerking of the head</td>
</tr>
<tr>
<td>Anxiety</td>
<td>Pacing</td>
<td>Any repetitive, stereotypic locomotor behaviour at least 3 times in succession. (Melega et al., 2008).</td>
</tr>
<tr>
<td></td>
<td>Scratch</td>
<td>Focal subjects scratching themselves</td>
</tr>
<tr>
<td></td>
<td>Self-directed</td>
<td>Engaged in a brief touch to face or body, hand or foot rubbing, licking hand, body, hair picking, masturbation, shrug. (Melega et al., 2008).</td>
</tr>
<tr>
<td>Abnormal behavior</td>
<td>Responses independent of stimuli</td>
<td>Engaged in abnormal attention or orienting responses made in absence of apparent stimulus e.g. grasp at air, stare off into space (Castner and Goldman-Rakic, 1999).</td>
</tr>
<tr>
<td></td>
<td>Fine motor stereotypy</td>
<td>Engaged in abnormal focussed repetitive motor responses directed towards objects or self-directed e.g. repetitive grasping at face, and repetitive movements of hand or foot. (Melega et al., 2008).</td>
</tr>
<tr>
<td></td>
<td>Whole body stereotypy</td>
<td>Engaged in abnormal repetitive whole body movements, including circling, side-to-side, somersaulting - other than pacing. (Melega et al., 2008).</td>
</tr>
<tr>
<td><strong>Durational behaviors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Withdrawal</td>
<td>Withdrawn/isolated</td>
<td>Focal subject withdraws to a corner or platform within the cage.</td>
</tr>
<tr>
<td>Eat</td>
<td>Latency to food response</td>
<td>Time taken for the focal subject to place food in the mouth, chew, forages through food or carries food in mouth or drinks water. (Rapkin et al., 1995).</td>
</tr>
</tbody>
</table>
Figure 3-2. Intra-gastric administration of (-)-cathinone to the vervet monkey.
3.1.3 STATISTICS

Behavioural data for pre-treatment and treatment phases were analysed jointly. The mean scores for each dependent variable were calculated for each subject across each of the independent variables when it was deemed applicable. The mean scores were then used as raw data for all ANOVAs. All behavioural observations were done during morning hours of each observational day. For each dependent behavioural measure (aggression, anxiety, abnormal behaviour, withdrawal and appetite), a 5 treatment group (cathinone dose: 0.8, 1.6, 3.2 and 6.4 mg/kg body weight, and control) x 25 observational days univariate repeated measures ANOVA was performed, using treatment group as a between-subject factor and observational day as within-subject factor. The Huynh-Feldt correction was applied for those tests and Bonferroni adjustments used for post hoc multiple comparisons. Statistical significance was set at 5% level.
3.2 RESULTS

3.2.1 BEHAVIOURAL OBSERVATIONS

3.2.1.1 Behavioural scores of aggression

The results show a significant effect of (-)-cathinone on ‘yawn’, ‘bouncing off cage walls’ and ‘head jerk’ as indicators of aggression. The individual scores for ‘yawn’ were more observable during absence of cage enrichment as indicated by a significant main effect of days ($F_{(16, 144)} = 26.6, P < 0.001$). Groups with (-)-cathinone treatment showed greater scores than the control group as indicated by a significant main effect of group ($F_{(4, 9)} = 12.9, P < 0.01$) on this measure. There was a significant group by day interaction ($[F_{(64, 144)} = 2.45, P < 0.001]$; Fig. 3-3 a) for this score. The individual behavioural scores of ‘engage in a stare’ ($F_{(16, 144)} = 10.2, P < 0.001$; Fig. 3-3 b) and ‘jaw thrust’ ($F_{(10, 94)} = 10.2, P < 0.001$; Fig. 3-3 c) differed over days. Changes in scores for ‘bouncing off cage walls’ differed by group as indicated by a significant group by day interaction ($F_{(68, 154)} = 1.74, P < 0.01$; Fig. 3-3 d). Changes in scores on ‘vigorous shaking of cage walls’ were moderated by (-)-cathinone groups as indicated by a significant group by day effect ($F_{(44, 101)} = 1.58, P < 0.05$; Fig. 3-3 e). (-)-Cathinone treatment group showed greater ‘head jerk’ than the control animals (Group effect: $F_{(4, 9)} = 48.0, P < 0.001$), and patterns of changes in this score differed among groups (group by day effect: $F_{(42, 94)} = 2.18, P < 0.01$; Fig. 3-3 f). This increase of individual behavioural scores was more pronounced in absence of cage enrichment. During pre-treatment period individual scores for ‘engage in a stare’ were observable in both presence and absence of cage enrichment. However, there was a fixed order of observations during cage enrichment, which could be regarded as a limitation of the study.
Figure 3-3 a. A significant increase in ‘yawn’ for group by day interactions (P < 0.001), between subject groups and over days (P < 0.01) in (-)-cathinone subjects compared to controls (n = 14). Note that the increase was more pronounced in absence of cage enrichment.
Figure 3-3 b. Individual scores for ‘engage in a stare’ in (-)-cathinone subjects did not differ significantly from controls for group by day interactions and between subject groups but significant (P < 0.001) over experimental period (n = 14).
Figure 3-3 c. Behavioural scores for ‘jaw thrust’. There was no significant difference for group by day and between subject groups on this measure. However, the increase was significant over experimental period (P < 0.001). Note that the effect was more pronounced in absence of cage enrichment among (-)-cathinone subjects compared to controls (n = 14).
Figure 3-3 d. A significant increase in ‘bouncing off cage walls’ for group by day (P < 0.01), between (-)-cathinone subject groups (P = 0.07) and over days (P < 0.001). The increase was more pronounced during absence of cage enrichment in (-)-cathinone subjects compared to controls (n = 14).
**Figure 3-3 e.** A significant increase in ‘vigorous shaking of cage walls’ for group by day ($P < 0.05$), group ($P = 0.07$) and days ($P < 0.001$) in (-)-cathinone subjects compared to controls ($n = 14$). The behaviours were more observable during absence of cage enrichment.
Figure 3-3 f. Increase in ‘head jerk’ for group by day (P<0.01), group (P<0.001) and days (P<0.001) in (-)-cathinone-treated animals. The increase was more observable during absence of cage enrichment in (-)-cathinone subjects compared to controls (n = 14).
### 3.2.1.2 Behavioural scores of anxiety

Scores for ‘pacing’ changed over days \((F_{(19, 174)} = 14.0 , P < 0.001)\), and were greater among \((-\)-cathinone groups relative to controls \((F_{(4,9)} = 11.7, P < 0.01)\). However, these findings were qualified by a group by day interaction \((F_{(77, 174)} = 1.78, P < 0.01\); Fig. 3-4 a). Scores for ‘scratch’ differed by days \((F_{(12, 115)} = 33.9 , P < 0.001)\), and by group with greater number of observations in \((-\)-cathinone groups as compared with the control group \((F_{(4, 9)} = 26.4, P < 0.001)\), and there was a group by day interaction \((F_{(51, 115)} = 3.44, P < 0.001\); Fig. 3-4 b). There was a group \((F_{(4, 9)} = 44.9, P < 0.001)\) and group by day interaction \((F_{(90, 203)} = 4.67 , P < 0.001\) interaction on ‘self-directed’ behavioural scores (Fig. 3-4 c). The effect of \((-\)-cathinone on these measures of anxiety was dose-dependent.
Figure 3-4 a. A dose-dependent increase in ‘pacing’ for group by day (P < 0.01), group (P < 0.01) and day (P < 0.001) in (-)-cathinone subjects compared to controls (n = 14). There was no significant difference on this measure both in presence and in absence of cage enrichment.
Figure 3-4 b. A significant increase in ‘scratch’ for group by day, between groups and over days ($P < 0.001$) in (-)-cathinone subjects compared to controls ($n = 14$).
**Figure 3-4 c.** A dose-dependent increase in ‘self directed’ behaviour for group by day, between subject groups and over days (P<0.05) in (-)-cathinone-treated animals compared to controls. The increment was more elaborate during cage enrichment. (n = 14).
### 3.2.1.3 Abnormal behaviour

Results show a dose-dependent increase in stereotypical responses in (-)-cathinone subjects compared to controls. Although there was a general increase in ‘response independent of stimuli’ among all subjects over experimental period ($F_{(22, 204)} = 69.8$, $P < 0.001$) these changes tended to be pronounced with increase in (-)-cathinone dose (group by day interaction: $F_{(90, 204)} = 6.91$, $P < 0.001$; Fig. 3-5 a). Individual scores for ‘fine motor stereotypy’ showed a significant increase across observational days ($F_{(18, 166)} = 87.8$, $P < 0.001$), and there was also a group by day interaction ($F_{(73, 166)} = 8.13$, $P < 0.001$; Fig. 3-5 b). Similarly, there were group versus observational day interaction ($F_{(76, 171)} = 8.06$, $P < 0.001$), as well as group ($F_{(4,9)} = 74.5$, $P < 0.001$) and days effects ($F_{(19, 171)} = 94.7$, $P < 0.001$) in ‘whole body stereotypy’ (Fig. 3-5 c). The increase on these measures was more pronounced for dose 6.4 mg/kg body weight compared to other doses (0.8, 1.6 and 3.2 mg/kg body weight) of (-)-cathinone. Overall, (-)-cathinone subjects, at all doses, showed high levels of abnormal behaviour compared to controls.
**Abnormal - response independent of stimuli**

Group x day: p<.001  
Group: p<.001 (con<others; 6.4>0.8, 1.6)  
day: p<.001

Figure 3-5 a. A dose-dependent increase in ‘response independent of stimuli’ for group by day, group and over days (P < 0.001) in (-)-cathinone subjects compared to controls (n = 14).
Figure 3-5 b. Behavioural scores for ‘fine motor stereotypy’ showing a significant increase for group by day interactions, between subject groups and over days (P < 0.001) in (-)-cathinone subjects compared to controls (n = 14).
Figure 3-5 c. A significant increase in ‘whole body stereotypy’ for group by day interactions, between subject groups and over experimental period (P < 0.001). The increase was more significant for dose 6.4 mg/kg bwt of (-)-cathinone compared to other doses and controls (n = 14).
3.2.1.4 Appetitive behaviour

The results show a significant increase in latency of response to introduction of food over observational period ($F_{(15, 139)} = 1261, P < 0.001$), and on average the 6.4 mg/kg had greater latency than other groups ($F_{(4,9)} = 2248, P < 0.001$). These findings were qualified by a significant group by observational day interactions ($F_{(62, 139)} = 80, P < 0.001$) which indicate dose-dependent relationship between (-)-cathinone dose and latency of response to food across experimental period (Fig. 3-6). The increase was more elaborate among (-)-cathinone subjects during cage enrichment compared to absence of cage enrichment. From these results it is possible that cage enrichment, to some extent, affected the focus of focal subjects to the introduction of food leading to a fixed order in the observations. This is regarded as a limitation of the study methodology on environmental enrichment.
**Figure 3-6.** A significant increase (P < 0.001) in latency of response to food among (-)-cathinone subjects compared to controls (n = 14). Note a more elaborate dose-dependent increase during cage enrichment.
3.2.1.5 Withdrawal behavioural score

Duration of time spent by focal subjects in withdrawal to the corner of the cage increased over observational period ($F_{(11, 102)} = 2006$, $P < 0.001$; Fig. 3-7) and (-)-cathinone groups showed longer duration than the control group ($F_{(4,9)} = 1052$, $P < 0.001$). These findings were qualified by a group by observational day interactions ($F_{(45, 102)} = 245$, $P < 0.001$) suggesting dose-dependent increase in withdrawal score. This increase in withdrawal period of (-)-cathinone subjects was significant following cage enrichment compared to period in absence of cage enrichment. During pre-treatment phase there was no observable withdrawal of focal subjects among all subject groups indicating that (-)-cathinone contributed greatly to this behavioural observation.
Figure 3-7. A significant increase (P < 0.001) in time spent by focal subjects withdrawn to corner of cage for group by day, between groups and over days. Note a dose-dependent significant increase during cage enrichment compared to absence of cage enrichment (n = 14).
3.3 DISCUSSION

The aim of this study was to establish, in single-caged vervet monkeys, a profile of behavioural alterations resulting from acute- and sub-chronic exposure to (-)-cathinone in presence and/or absence of cage enrichment over a period of 5 months. However, the first one month was pre-treatment period, where animals were observed in order to establish baseline levels on behaviour patterns under investigation. (-)-Cathinone was administered on alternate days as described in earlier chapter. This took into consideration the plasma half-life of (-)-cathinone in the body of vervets. Studies in humans who chewed khat for 1 h showed that (-)-cathinone was detected in urine for up to approximately 26 h while cathine and norephedrine were detected for at least 80 h (Toennes and Kauert, 2002). The regimen also took into account the likely development of tolerance to (-)-cathinone by the animals. It is possible that after multiple repeated exposures to khat and (-)-cathinone, tolerance develops. Tolerance and cross-tolerance between (-)-cathinone and cathine have been reported in animal studies (Foltin et al., 1983).

The results have shown a dose-dependent increase in frequencies of individual behavioural scores that measure aggression, anxiety and abnormal-related behaviours. Durational behaviours of withdrawal/isolation and eat/appetite also showed a dose-dependent increase over observational period of time. The frequency of behavioural scores on aggression, anxiety, abnormal responses, withdrawal and appetitive behaviours were not affected by cage enrichment.
3.3.1 EFFECTS ON BEHAVIOUR

3.3.1.1 Aggression

During pre-treatment phase neither presence nor absence of cage enrichment influenced behavioural patterns. However, following (-)-cathinone treatment behavioural changes became apparent with increasing dose levels and over time period. All individual behavioural categories defining aggression increased in both presence and absence of cage enrichment but significantly so in absence of cage enrichment. The significant effect during absence of cage enrichment might have been due to the novelty effect of (-)-cathinone. There were low mean frequencies of ‘yawn’, ‘engage in a stare’, ‘jaw thrust’, ‘bouncing off cage walls’, ‘vigorous shaking of cage walls’ and ‘head jerk’ during cage enrichment compared to absence of cage enrichment. It is also apparent that mean frequency scores of ‘engage in a stare’ and ‘jaw thrust’ did not differ significantly for group and group by day effects. This is unexpected and may be attributed to limitation in methodology used. However, since ‘yawn’ was the only behaviour observed during pre-treatment period while other observational categories defining aggression were absent, it is likely that (-)-cathinone primarily influenced aggressive behaviour in these animals.

Earlier studies on khat extracts and (-)-cathinone reported stereotyped behaviour, self-administration and anorectic effects in animal species (Gordon et al., 1993, Calcagnetti and Schechter, 1992) similar to that evoked by [S-(+)-amphetamine] (Goudie, 1985). Furthermore, both khat extract and (-)-cathinone have been shown to enhance baseline aggressive behaviour in isolated rats (Banjaw et al., 2005). These behaviours are partly influenced by disorders in the hypothalamic dopaminergic system (Ishikawa et al., 2007) and
partly due to dopaminergic and serotonergic activity in the mesolimbic system (Eisch and Harburg, 2006, Jones and Bonci, 2005). Further evidence has shown involvement of both khat extract and (-)-cathinone in depletion of serotonin and its metabolite 5-hydroxyindole-acetic acid in both anterior and posterior striatum (Banjaw et al., 2005). The present study simulated the use of (-)-cathinone in vervet monkey model as it appears in human khat ‘addicts’. The results show a dose-dependent increase in aggressive behaviour which is in agreement with earlier findings where chronicity of use or multiple acute high doses of (-)-cathinone exposure in humans showed a wide range of dose-dependent behavioural changes including aggression, hyperactivity, various forms of psychotic illness, anxiety, paranoia and social withdrawal (Berman et al., 2009).

3.3.1.2 Anxiety

The result of the present study showed that anxiety scores for ‘pacing’, ‘scratch’ and ‘self-directed’ steadily increased in a dose-dependent manner in absence and presence of cage enrichment and over observational period of time. (-)-Cathinone subjects exhibited more anxiety levels during cage enrichment. Stereotypical behaviours have been demonstrated in vervet monkeys following maternal separation and these measures are reminiscent of anxiety symptoms in humans (Marais et al., 2006).

Studies on (-)-cathinone and amphetamine in animals have shown their sympathomimetic effects on dopaminergic (Pehek et al., 1990) and serotonergic (Kalix, 1984 b) synapses as well as peripherally via noradrenergic storage sites (Kalix, 1983 b). Centrally acting neurotransmitters such as dopamine and serotonin have been shown to have modulatory effects on specific behaviours (Spoont, 1992). The receptors for both dopamine and serotonin
appear overlapping in the limbic and cortical regions of the brain (Goldman-Rakic et al., 1990), and this explains the inhibitory effect of serotonin on facilitatory effects of dopamine. Noradrenaline has also been shown to influence specific behaviours when interacting with other biogenic amines in the brain of different animal models and in human case studies. For example, reduced levels of serotonin are found in patients with anxiety disorders (Charney et al., 1990) and reduction in both serotonin and noradrenaline is associated with major depression (Sulser, 1989; Meltzer and Lowy, 1987). Recent studies showed that anxiolytic mechanism may include some interaction of noradrenaline and serotonin, rather than increasing synaptic levels of serotonin (Marais et al., 2006). Studies on (-)-cathinone have demonstrated maintenance of drug-seeking behaviour in rats habituated to amphetamine and monkeys trained to lever-press for cocaine injection (Yanagita, 1979). Similar studies in humans by use of amphetamine showed drug-related effects of insomnia, irritability, anxiety, sadness and nightmares (Efron et al., 1997). (-)-Cathinone causes these effects via its effect on central dopaminergic (Pehek et al., 1990) and serotonergic (Kalix, 1984 b) and peripherally at the noradrenergic storage sites (Kalix, 1983 b).

3.3.1.3 Abnormal behaviour

Abnormal behavioural scores observed in the present study showed a steady increase in (-)-cathinone subjects in a dose-dependent manner and over experimental period. These behaviours were more apparent following cage enrichment. A study by Zelger et al. (1980) reported stereotypical behaviour and hyper-locomotion in rats treated with (-)-cathinone, and these paradigms characterize abnormal behaviour (Melega et al., 2008). The results of the present study on scores for ‘fine motor’ and whole body stereotypy’ confirm these earlier
findings in other animals and, probably, a similar mechanism of action of these drugs is involved in influencing these behaviours.

3.3.1.4 Withdrawal/Isolation and appetitive behaviour

The durational behaviours of withdrawal and appetite loss in (-)-cathinone–treated animals showed an increase in a dose-dependent manner over the experimental period. This increase was again more pronounced following cage enrichment compared to observations in absence of cage enrichment but the difference in both set-ups was not significant. Cage enrichment may have potentiated the effect on these measures. However, similar studies in humans and experimental animals have reported changes in affiliative and anorectic behaviours following khat use (Gordon et al., 1993, Pantelis et al., 1989). Studies have shown social withdrawal as a common side effect of [S-(+)-amphetamine] in children with attention deficiency disorder (Clinical Practice Guideline, 2001). On the other hand, anorectic behaviour is characterized by development of tolerance (Zelger and Carlini, 1980). It is, therefore, possible that while cage enrichment appeared to have influenced the observed behaviours, the effect may have been primarily due to (-)-cathinone exposure as it showed a dose-dependent response.

Methamphetamine is a derivative of amphetamine and, like amphetamine, has been shown to cause alterations in the human striatal dopamine system effects (Johanson et al., 2006, Wang et al., 2004). Studies on effects of methamphetamine on the dopaminergic system showed dopamine receptor density alterations following multiple exposures (Melega et al., 2008). Although not tested, the findings of the present study are a possible pointer to the association between behavioural changes and receptor density alterations or levels of catecholamines at the periphery. This is explained by the fact that amphetamines and (-)-cathinone influence
release of catecholamines via a similar mechanism (Kalix, 1996). For example, studies have shown that (-)-cathinone increases levels of dopamine, serotonin and noradrenaline in the brain via catecholaminergic synapses (Calcagnetti and Schechter, 1993; Kalix, 1991). Serotonin (5-Hydroxytryptamine [5HT]) is a short-acting widespread neurotransmitter which acts on a number of receptor subtypes found at high density in the limbic system and raphe nuclei as well as in the hypothalamus (Blundell, 1984). Agonists at the 5-HT$_{2c}$ receptor show the most consistent inhibition of food intake and the 5-HT$_{2c}$-knockout mouse is hyperphagic and obese (Tecott et al., 1995). Another study investigating involvement of 5-HT$_{2C}$ receptors in the regulation of food intake in the Siberian hamsters treated with fenfluramine demonstrated that fenfluramine exerted a potent hypophagic effect (Schuhler et al., 2005). Because fenfluramine is known to increase 5-HT release and inhibit its reuptake, these observations demonstrated the involvement of the serotonergic system in the regulation of food intake in the hamster. In general, agonists at the 5-HT receptors and drugs that inhibit the uptake of serotonin reduce feeding. Additionally, 5-HT stimulates noradrenaline release and modifies behaviour and mood (Neary et al., 2004). However, at high doses, the massive release of 5-HT not only gives rise to acute psychotic symptoms but also causes chemical damage to the cells that release it (Kalant, 2001).

The observed effects of (-)-cathinone on appetitive/consummatory behaviour in the present study are consistent with findings of Nyongesa et al. (unpublished data) which showed a dose-dependent decrease in food intake and body weight gain of rabbits following exposure to khat extract. The arcuate nucleus of the hypothalamus is involved in central appetite regulation by use of Agouti-related protein (AgRP) that increases food intake through antagonism of melanocortin MC3 and MC4 receptors and thus blockade of inhibition of the
anorexigenic agonist α-melanocytic stimulating hormone (α-MSH) (Neary et al., 2004). Virtually all AgRP neurones co-secret neuropeptide Y (NPY), (Goldstone et al., 2002), which have Y2R - a presynaptic inhibitory autoreceptor. The arcuate nucleus also contain galanin-like peptide (GALP)-containing neurones, which plays a critical role in the regulation of mammalian energy balance and reproduction (Gottsch et al., 2004), and several discrete populations of neurones in this nucleus are targets for the regulatory hormone leptin (McMinn et al., 2000). In rodents and primates, arcuate nucleus contains mRNA of neuropeptides involved in feeding and/or reproduction including neuropeptide Y (NPY), α-melanocyte stimulating hormone (α-MSH), agouti-related protein (AgRP), and galanin (Cone et al., 2001). GALP infusions have been shown to stimulate luteinizing hormone (LH) release in a dose-related manner in male rats, mice and macaques (Cunningham et al., 2004; Krasnow et al., 2004) and this LH release is likely mediated via GnRH-1-dependent pathways (Cunningham et al., 2004; Matsumoto et al., 2001). Previous studies, however, showed that khat suppresses appetite independent of ghrelin and peptide YY (PYY) secretion (Murray et al., 2008). From these observations, it is possible that alterations in consummatory behaviour of the vervet monkeys following (-)-cathinone treatment may have been due to its effect on the arcuate nucleus or as a result of increased levels of 5-HT in the brain. It is, therefore, possible that (-)-cathinone caused dopamine and serotonin receptor density alterations that influenced changes in feeding behaviour.

The study had its own limitations. The methodology was aimed at modelling an experimental animal to emulate the situation in human khat chewers. Khat is chewed over a long period of time while the juice is extracted. Toennes et al. (2003) reported a major role buccal mucosa plays in the absorption of (-)-cathinone, cathine and norephedrine alkaloids extracted into
saliva following chewing. In the present study, it was not possible to achieve this route of administration in monkeys, instead (-)-cathinone was given as a bolus via gastric gavage. Khat chewing is a daily habit in addicts and so the observed effects in khat chewers may be as a result of these multiple exposures. This regimen was hampered by the mode of administration where animals were anaesthetized during each time of (-)-cathinone administration due to difficulty in achieving intubation in awake monkeys. One possibility is to identify sweetener that can be mixed with khat leaves so that experimental animals are able to chew on them rather than administration via other routes. It was also unethical to keep animals under anaesthesia daily for purposes of drug administration. Moreover, since anaesthesia eliminates cardiovascular measures that are indicative of (-)-cathinone effects, this is regarded as a limitation of the study. On the other hand, it is well understood that vervet monkeys live in relatively stable multi-male, multi-female social groups (Iseb et al., 1991). Isolation into individual cages done in this study may have partly influenced their behavioural manifestations even though they were adequately habituated. There was also some individual variation in behavioural parameters of vervet monkeys suggesting that larger sample size is required when studying behaviour in this species. These are gaps for future consideration.

**Conclusion**

The findings of the present study have shown that, indeed, (-)-cathinone exposure caused behavioural alterations in a dose-dependent manner. The effects were pronounced following long-term use. There was continual increase in some scores of aggression in a dose-dependent manner in (-)-cathinone-treated animals while the same scores decreased in controls over time. Time-dependent changes in frequency behaviours of aggression, anxiety and abnormal responses as well as durational behaviours of appetite and withdrawal demonstrated how
long-term drug use influences different behavioural profiles. Cage enrichment did not have an effect on measures of aggression and anxiety while those measures defining abnormal behaviour, withdrawal and appetite/consummatory behaviour showed a significant effect on following (-)-cathinone exposure. However, the study did not consider counter-balancing between cage enrichment and its absence on (-)-cathinone effects to different behaviours leading to a fixed order in observations of different behaviours following (-)-cathinone administration.
CHAPTER 4

SERUM HORMONAL PROFILES FOLLOWING ACUTE AND SUB-CHRONIC CATHINONE EXPOSURE IN VERVET MONKEYS (CHLOROCEBUS AETHIOPS)

4.0 INTRODUCTION

Khat has been used as a culturally sanctioned stimulant not only in many countries of eastern Africa but also in the Middle East. (-)-Cathinone [S-(-)-alpha aminopropiophenone] is the primary psychoactive alkaloid of khat (Kalix and Braenden, 1985; Kalix, 1984 a). The general semblance between biochemical effects of (-)-cathinone and those of amphetamine as well as their chemical structure points to the fact that both substances have similar mechanism of action (Graziani et al., 2008; Houghton, 2004; Cox and Rampes, 2003), with equal potential for abuse (Kalix, 1984 a). Acute and chronic exposure of khat and (-) cathinone in consumers have been shown to cause a wide range of effects from mental, respiratory, digestive to reproductive dysfunction. Research findings in humans and experimental animals have reported changes in sleep patterns, mood, attention, aggression, anxiety, locomotor activity, and affiliative behaviours (Pantelis et al., 1989; Kalix, 1994), learning and memory (Kimani and Nyongesa, 2008) and sexual behaviour (Tariq et al., 1990). There is growing evidence in literature indicating that khat and (-)-cathinone induce psychostimulation primarily via meso-striato-corticolimbic dopaminergic pathway (Kalix, 1990), although there may be other systems involved. The addiction potential, analgesia and anorexic effects of khat and (-)-
cathinone are believed to be partly mediated via this pathway (Gosnell et al., 1996). This is consistent with studies demonstrating the involvement of hypothalamo-hypophyseal-adrenocortical axis in psychostimulation via dopaminergic transmission (Steckler and Holsboer, 1999). Preliminary studies showed increased corticosterone levels following use of psychostimulants (Mello and Mendelson, 1997) and these high levels were shown to contribute to substance abuse disorders (King et al., 2003). Studies on khat showed increase in adrenocorticotropic hormone in humans (Nencini et al., 1984), consistent with cortisol measurement in rabbits following khat extract exposure (Nyongesa et al., 2008) but different from findings in baboons where decreased plasma cortisol was observed (Mwenda et al., 2006). These conflicting findings especially with respect to cortisol may be explained partly by the time of exposure to khat/(-)-cathinone and possibly species difference. The aforementioned investigations on khat covered an acute phase of exposure, in which elevated cortisol measurements, independent of treatment, are expected as a result of lack of adequate habituation of animals to handling and manipulations. Similarly, acute exposure is not quite a reflection of effects in a regular khat chewer. Species difference may also have contributed to the variance in results. The present study considered both acute and sub-chronic exposure of (-)-cathinone to the vervet monkeys.

Prolonged elevations of glucocorticoid concentrations have been shown to reduce the response of luteinizing hormone (LH) to gonadotropin releasing hormone (GnRH) (Wang, 1986). The concentrations of circulating gonadotropins in turn depend on the frequency and amplitude of GnRH pulses discharged into the pituitary portal vessels, which in turn depend on endocrine status (Dierschke et al., 1970). Previous studies have shown that (-)-cathinone is an inhibitor of several anterior pituitary hormones (Wagner et al., 1982). This was indeed the
findings in rabbits where LH levels were suppressed in a dose-dependent manner following crude khat extract administration (Nyongesa et al., 2008).

Several investigations have similarly shown that khat and (-)-cathinone have effects on circulating levels of testosterone although these reports are contradictory. For example, (-)-cathinone has been shown to have cytotoxic effect on gonads leading to androgenic deficiency in mice (Tariq et al., 1987). Khat and (-)-cathinone have also been reported to interfere with mitosis in body cells (Al-Meshal, 1987) leading to a decrease in plasma testosterone in humans (El-Shoura et al., 1995; Balint et al., 1991; Kalix and Braenden, 1985) and rats (Islam et al., 1990). On the contrary, reports have cited khat as an aphrodisiac (Krikorian, 1984; Margetts, 1967), a medicament for premature ejaculation (Pantelis et al., 1989; Luqman and Danowski, 1976) and a libido booster that increases sexual desire (Elmi, 1983) and sperm power booster (Adeoya-Osiguwa and Fraser, 2005). These findings are similar to those in baboons (Mwenda et al., 2006) which showed that khat causes an increase in plasma testosterone levels. This may explain the increase in aggressive behaviour presented in chapter 3 of this thesis where increasing doses of (-)-cathinone caused aggression in a dose- and time-dependent manner. Khat and (-)-cathinone have also been shown to have biphasic effect on reproductive function. For instance, in vitro studies on effect of khat extract on isolated mouse interstitial cells showed that high concentrations (30 mg/ml and 60 mg/ml) of khat extract adversely affected cell viability by about 50% while low concentrations (0.06 mg/ml, 0.6 mg/ml and 6 mg/ml) caused minimal effect (Nyongesa et al., 2007). These findings showed a similar pattern to those of 6-hydroxydopamine on mouse Leydig cells that showed increasing doses being stimulatory then inhibitory to testosterone production (Wango et al., 1995).
Previous studies focused on acute effects of (-)-cathinone; however, no study has systematically examined sub-chronic effects of (-)-cathinone treatment. The present study was designed to investigate the effects of (-)-cathinone on hypothalamo-hypophyseal-adrenocortical and gonadal axes. We hypothesized that sub-chronic to chronic administration of (-)-cathinone would alter neurobiological functions in the hypothalamo-hypophyseal-adrenocortical and gonadal systems, leading to changes in production of cortisol, prolactin, LH, testosterone and progesterone hormones. Progesterone measurements were done for comparison with those in a parallel ex vivo study designed to determine effects of (-)-cathinone on testosterone biosynthetic pathway described in chapter 6 of this thesis.
4.1 MATERIALS AND METHODS

4.1.1 ANIMALS AND HOUSING

The same animals described under general materials and methods were used for this study.

4.1.2 EXPERIMENTAL DESIGN

Animal identification and grouping is same as was described previously in section 2.4 of general materials and methods.

4.1.2.1 Pre-treatment phase

This was done according to the previous description under section 3.1.2.1 of chapter 3. The femoral vein of each animal was cannulated using a 22 G blood vessel cannula after shaving and swabbing the area with 70% alcohol. Heparinised saline was introduced into the cannula to prevent blood clotting inside the lumen. The adhesive tape was used to anchor the cannula in situ to prevent it from dropping off and blood collection done. Blood samples (2 ml) were then collected from femoral vein at 5-days interval for cortisol, prolactin, LH, progesterone and testosterone analysis.

4.1.2.2 Treatment phase

i). Drug administration

(-)-Cathinone administration followed the previous description under section 3.1.2.2 of treatment phase. At the end of this 1st treatment phase that lasted 4 months, group II and V animals were co- treated with GnRH agonist goserelin acetate (ZOLADEX) in the presence of
low and high dose (0.8 and 6.4 mg/kg body weight) of (-)-cathinone for additional 2 weeks during which blood samples were collected at 5-day intervals and processed for hormone analysis. ZOLADEX (3.6 mg) was prepared in 10% DMSO in PBS and and an equivalent of 50 μg/kg body weight/injection was administered subcutaneously.

ii) Blood sampling

Food was restricted prior to use of anaesthetics. Blood samples were collected from femoral vein of animals following anaesthesia with 10 mg/kg body weight of ketamine hydrochloride intramuscular. Femoral vein of each animal was cannulated as described under 4.1.2.1 above and, thereafter, 2 ml blood samples collected into heparinized LP3 tubes at 20 min-interval for 2.5 h starting at 10th min following (-)-cathinone administration. Sampling was done at 1000 h on Mondays and Wednesdays of every week. Serum was stored at -20°C until assayed for cortisol, prolactin, LH, progesterone and testosterone. Dopamine concentrations were estimated from serum prolactin based on inverse proportionality. This was based on findings that spontaneous secretion of prolactin is inhibited by dopamine (Birge et al., 1970; MacLeod et al., 1970) and that dopamine receptors are found on lactotroph membranes (Goldsmith et al., 1979). Other studies showed that dopamine is found in hypophysial stalk plasma (Plotsky et al., 1978; Ben-Jonathan et al., 1977) in amounts sufficient to inhibit prolactin release (Gibbs and Neill, 1978).

iii) Hormonal analysis

Hormonal assays for serum cortisol, prolactin, progesterone, LH and testosterone were done by use of enzyme immunoassay technique using the kits from Nova Tec Immundiagnostica GMBH, Germany. The technique uses the principle of competition of hormone in sample with enzyme conjugated hormone for limited binding sites on the specific antibody.
Validation of the assay method for use in the vervet monkey followed that of Eley et al., (1989). In all hormone assay procedures, assays were done in triplicate. The optical density of specimen was measured using HumaReader HS (Gessellschaft für Biochem und Diagnostica, mbH, Germany).

*Cortisol enzyme immunoassay*

The assay procedure of the protocol supplied in the kit was followed

*Prolactin enzyme immunoassay*

This followed the assay procedure stipulated in the protocol supplied with the kit.

*Progesterone enzyme immunoassay*

The assay procedure within the protocol of the kit was followed

*Testosterone enzyme immunoassay*

The assay procedure within the protocol of the kit was followed

*LH bioassay*

Male animals were anaesthetised with 10 mg/kg ketamine hydrochloride intramuscularly and both testes harvested. The animals were later sacrificed and samples collected for other experiments. The testes were decapsulated and fat trimmed off, then rinsed in ice-cold 10 ml Modified Eagle Medium (MEM) in plastic Petri dish. The media was aerated with medical gas (95% O₂: 5% CO₂) in ice-cold water before use. Testes were minced with a pair of scissors and cells dispersed mechanically using a Fin pipette by sucking in and out several
times. The minced tissue was transferred to a 50 ml conical flask containing 100 ml media, and mixed gently for 15 min at 4 °C. The suspension was then filtered through fine cotton gauze to obtain interstitial cells which were transferred into 100 ml conical flask and incubated for 15 min in 5% carbon-dioxide water bath at 4 °C. The cell suspension was then aliquoted into 6 plastic test tubes and subjected to percoll density gradient centrifugation three times and the pellets pooled into a conical flask. The pooled pellets were re-suspended in medium and Leydig cells isolated by sieving the suspension through cotton gauze, diluted with the incubation media and purified by 3 beta hydroxysteroid dehydrogenase (3β-HSD) staining method. The purified Leydig cells were then counted using a haemocytometer to obtain a working concentration of approximately 2 x 10⁵ cells/ml. Luteinizing hormone levels were determined by assaying testosterone levels in the suspension medium following the enzyme immunoassay (EIA) protocol.

4.1.3 STATISTICS

Results are expressed as mean ± SEM. Hormonal data for pre-treatment and treatment phases for cortisol, prolactin, progesterone, testosterone and LH were analysed jointly. For each hormonal measure, a 5 treatment group (0, 0.8, 1.6, 3.2 and 6.4 mg/kg body weight) x 20 experimental weeks (4 weeks of pre-treatment phase and 16 weeks of treatment phase) x 7 sampling times (at 20, 40, 60, 80, 100, 120 and 140 min period) repeated measures ANCOVA was conducted. Treatment group was used as a between-subject factor, week and sampling time as within-subject factors and sex as a covariate. Huynh-Feldt correction was applied for the analysis and Bonferroni adjustments performed for post hoc multiple comparison tests. Statistical significance was set at 5% level.
4.2 RESULTS

The results on effect of GnRH agonist on LH are not reported here due to the small sample size used for group II and V animals that were involved. There were no controls for comparison. However, effect of GnRH agonist on immunolocalization of gonadotrophs is reported in chapter 5 of this thesis. The results of all hormones reported below showed no significant effect of cage enrichment on these parameters over the experimental period, suggesting that the effects observed were primarily due to (-) cathinone treatment.

4.2.1 Effect of (-)cathinone on serum cortisol

The results show a significant group effect [F (4, 8) = 218, P < 0.001] indicating dose-dependent decrease in cortisol levels (Fig. 4-1). Cortisol also changed across the 20-week period [F (18, 142) = 21.7, P < 0.001] with levels during the treatment phase (from week 5 to week 20) being generally lower than those during the 4 week pre-treatment phase (Fig. 4-2). There was also variation in cortisol levels over sampling time [F (6, 48) = 7.98, P < 0.001] (Fig. 4-3). Multiple comparison tests indicated that levels at the first sampling time of the pre-treatment phase was greater than the last two sampling times (P < 0.05) suggesting habituation. There was also a significant group x week interaction [F (71, 142) = 4.86, P < 0.001], which indicated that reduction in cortisol levels over study weeks was pronounced as (-)-cathinone dose increased (Fig. 4-4). Decrease in cortisol levels over sampling period tended to be pronounced during the treatment phase relative to pre-treatment phase as indexed by group x time interaction [F (24, 48) = 2.06, P < 0.05] (Fig. 4-5).
4.2.2 Effect of (-)-cathinone on serum prolactin

Higher (-)-cathinone- induced decrease in overall prolactin concentrations as indicated by a significant group effect \[F_{(4, 8)} = 267, P < 0.001\] (Fig. 4-6) was observed. Prolactin levels observed during treatment phase were lower than pre-treatment phase showing main effect of week: \[F_{(15, 118)} = 129, P < 0.001\] on this measure (Fig. 4-7). Prolactin changed over sampling period \[F_{(6, 48)} = 19.5, P \leq 0.001\] with lower levels in the last four sampling periods relative to the first sampling period (\(P \leq 0.001\)) (Fig. 4-8). In addition, a significant group x week interaction \[F_{(59, 118)} = 13.03, P < 0.001\] indicated dose- and time-dependent effects of (-)-cathinone on serum prolactin levels (Fig. 4-9). Decrease in prolactin across sampling period tended to be pronounced during the treatment phase as indicated by group x time interaction \[F_{(24, 48)} = 3.65, P < 0.001\] (Fig. 4-10).

4.2.3 Effect of (-)-cathinone on serum Lutenizing hormone

The result also show that high doses of (-)-cathinone increased LH concentrations as indicated by a significant group effect \[F_{(4, 8)} = 14612.4, P < 0.001\] (Fig. 4-11). There was a significant group by week interaction with the two high dose \(F_{(4, 76)} = 333.8, P < 0.001\] indicating dose- and time-dependent effects of (-)-cathinone on LH secretion (Fig. 4-12). Luteinizing hormone levels observed during treatment phase were higher than pre-treatment phase showing main effect of week: \[F_{(1, 19)} = 1185, P < 0.001; \text{multiple comparisons: } P \leq 0.001\] on this measure. There was significant effect of (-)-cathinone on LH levels over sampling period \[F_{(4, 24)} = 344.5, P < 0.001\] indicating a group by sampling period interaction (Fig. 4-13). Overall, the results show that (-)-cathinone had a strong effect on hypothalamo-hypophyseal axis as a function of dose, sampling period and experimental time as shown by week x sampling period x group interaction \[F_{(4, 24)} = 7.4, P < 0.001\].
4.2.4 Effect of (-)-cathinone on serum testosterone

The changes in serum testosterone in (-)-cathinone subjects and controls are presented in Figures 4-14 and 4-15. The results show a significant group effect \( F(4, 8) = 28.85, P < 0.001 \) based on linearly independent pair-wise comparisons among estimated marginal means. There was also a significant difference in means across 20-week period \( F(1, 19) = 10.25, P < 0.001 \) at highest dose (6.4 mg/kg) compared to moderate doses and controls with levels during the treatment phase (5 - 20 weeks) being generally lower than those during the 4 week pre-treatment phase. The results show a biphasic effect of (-)-cathinone on this parameter. At dose 3.2 mg/kg body weight, serum testosterone increased then decreased as shown by group x week interaction \( F(4, 76) = 7.14, P < 0.001 \) (Fig. 4-14). Low and medium doses generally increased serum levels of testosterone while highest dose significantly decreased testosterone levels as indicated by group by period interaction \( F(4, 24) = 3.19, P < 0.001 \) (Fig. 4-15).

4.2.5 Effect of (-)-cathinone on serum progesterone

The results show a general pattern of decrease in serum progesterone in dose-and time-dependent manner as shown by group and week interaction \( F(4, 76) = 3.72, P < 0.001 \) (Fig. 4-16). There was also a significant group effect \( F(4, 8) = 114.53, P < 0.001 \) based on linearly independent pair-wise comparisons among estimated marginal means. Serum progesterone levels observed during treatment phase were lower than pre-treatment phase as shown by effect of week: \( F(1, 19) = 38.66, P < 0.001 \) as well as for controls.

Correlational analysis was conducted to examine associations between cortisol and prolactin. There were no correlation between the two hormones during the pre-treatment phase except one inverse relationship \( r = -0.57, P < 0.05 \) observed at 40 min of sampling period in the
third week. In contrast, there were many positive correlations between the two hormones during the treatment phase. For example, during the fifth week of treatment, there were positive correlations at all sampling periods (r > 0.53, Ps < 0.05). Similar patterns of positive relationships were found during week 6, 7 and 8. In the latter half of the treatment phase (i.e., from the eleventh to twentieth week), positive correlations of cortisol and prolactin were found mostly from 100 min, 120 min and 140 min of sampling period (P < 0.05) (Fig. 4-17).
Figure 4-1. Changes in cortisol levels across groups. Entries show mean and standard error of the mean. There was a significant dose-dependent decrease in serum cortisol in treatment groups compared to controls. * P < 0.05, ** P < 0.01, *** P < 0.001. * means statistically significant. (n = 14).
Figure 4-2. Changes in cortisol levels across study weeks. Entries show mean and standard error of the mean. Serum cortisol levels during the treatment phase were lower than during pre-treatment phase. (n = 14).
Figure 4-3. Pattern of cortisol release over sampling periods. Note that cortisol levels at the last two periods were significantly lower than the cortisol levels at 20th to 80th minute of sampling indicating maximum plasma concentration of (−)-cathinone at these periods. * means statistically significant at P < 0.05, ** means statistically significant at P < 0.01, (n = 14).
Figure 4-4. Group differences in patterns of changes in serum cortisol across experimental period. There was a significant ($P < 0.001$) group by week interaction among treatment groups compared to controls. ($n = 14$).
Figure 4-5. Group differences in patterns of change in serum cortisol levels across sampling periods. Note a group x sampling period interaction with dose group 3.2 and 6.4 mg/kg body weight of (-)-cathinone showing a significant (P<0.05) decrease compared to low dose groups and controls. (n = 14).
Figure 4-6. Changes in serum prolactin levels across treatment groups. Entries show mean and standard error of the mean. Note a significant decrease in serum prolactin at (-)-cathinone dose 6.4 mg/kg body weight compared to other treatment groups and controls. ** P < 0.01, *** P < 0.001. * - statistically significant. (n = 14).
Figure 4-7. Pattern of serum prolactin levels during pre-treatment and treatment weeks. Entries show mean and standard error of the mean. Levels during the treatment phase were lower than the levels during the pre-treatment phase. (n = 14).
Figure 4-8. Changes in prolactin levels over sampling period. Entries show mean and standard error of the mean. Levels at the last four periods were lower than the levels at the first period. ** P < 0.01, *** P < 0.001. * - statistically significant. (n = 14).
Figure 4-9. Group differences in patterns of changes in serum prolactin across study weeks showing a strong group by week interaction. There was a significant group by week interaction with treatment group 3.2 and 6.4 mg/kg body weight of (-)-cathinone compared to that of (-)-cathinone dose 0.8 mg/kg and 1.6 mg/kg body weight. (n = 14).
Figure 4-10. Group differences in patterns of change in serum prolactin levels across sampling periods. There was a significant group by sampling period interaction compared to controls. (n = 14).
Figure 4-11. Changes in serum LH levels across groups. Entries show mean ± standard error of the mean. Note a significant increase (P<0.001) in LH levels for dose 3.2 and 6.4 mg/kg body weight of (-)-cathinone compared to low doses and controls (n = 14).
Figure 4-12. Changes in serum LH levels across study weeks. Entries show mean ± standard error of the mean. At dose 3.2 mg/kg and 6.4 mg/kg body weight of (-)-cathinone from week 5 to week 20 LH levels were significantly higher (P<0.001) than at dose 0.8 mg/kg and 1.6 mg/kg body weight of (-)-cathinone as well as controls.
Figure 4-13. Group differences in patterns of change in serum LH levels across sampling periods. Note a significant (P<0.001) group x sampling period interaction with (-)-cathinone-treated animals at dose 3.2 and 6.4 mg/kg body weight showing a steady increase compared to dose 0.8 mg/kg and 1.6 mg/kg body weight and controls (n = 14).
Figure 4-14. Group differences in patterns of changes in testosterone across study weeks. Note a significant increase in testosterone at dose 3.2 mg/kg and a significant decrease at dose 6.4 mg/kg body weight of (-)-cathinone compared to controls. (n=14).
**Figure 4-15.** Group differences in patterns of change in serum testosterone levels across sampling periods (a significant group x sampling period interaction). Note the biphasic effect with two high doses (3.2 mg/kg increased while 6.4 mg/kg decreased testosterone levels over time) of (-)-cathinone, (n=14).
Figure 4-16. Group differences in patterns of changes in serum progesterone across study weeks. There was a dose- and time-dependent decrease in serum progesterone over study period in treatment groups compared to controls, (n=14).
Figure 4-17. Correlations between serum cortisol and prolactin during week 1 of treatment phase. Note a strong positive correlation $r^2 = 0.53$ between serum prolactin and cortisol.
4.3 DISCUSSION

4.3.1 Effect of (-)-cathinone on cortisol and prolactin.

The results of the present study show that administration of (-)-cathinone produced a dose- and time-dependent decrease in serum cortisol and prolactin. The effects are suggestive of pleasurable responses from reward centres in the brain that necessitate psychic dependence on khat use (Halbach, 1972). Recent findings showed a decrease in serum cortisol levels in Sprague Dawley rats treated with 5 mg/kg body weight of (-)-cathinone (Mohammed and Engidawork, 2011). These results are consistent with those of other findings in baboons (Mwenda et al., 2006) indicating that khat causes a decrease in plasma cortisol and prolactin levels.

Although the present study indicated a decrease in serum cortisol and prolactin, most previous studies concur with findings on prolactin but not cortisol measurements. This is supported by earlier reports implicating (-)-cathinone in inhibition of release of several anterior pituitary hormones (Wagner et al., 1982). Previous studies have shown that (-)-cathinone causes an increase in levels of extracellular dopamine but decreases dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA) in vivo (Pehek et al., 1990). Findings on earlier in vitro studies and of tissue content (reviewed in Kalix and Braenden, 1985) indicating that (-)-cathinone, like amphetamine, causes release of dopamine and inhibits uptake of dopamine in vivo points to a similar mechanism. It appears that the dose-dependent inhibitory effect of (-)-cathinone on prolactin in the present study shared a similar mechanism of action. Studies on behavioural effects of khat and (-)-cathinone have shown strong evidence to suggest involvement of dopaminergic system (Kalix and Braenden, 1985; Zelger et al., 1980). It is
very likely, therefore, that (−)-cathinone influenced behaviours reported in chapter 3 of this thesis via observed low serum prolactin levels in treated animals.

Previous studies reported varying findings on use of psychostimulants and their effects on the hypothalamo-hypophyseal-adrenocortical system. For example, high levels of glucocorticoids were shown to contribute to development, maintenance and outcome of substance abuse disorders (King et al., 2003). Other studies on khat have reported increase in plasma cortisol in rabbits (Nyongesa et al., 2008) and adrenocorticotropic hormone in humans (Nencini et al., 1984). These findings are at variance with those in baboons (Mwenda et al., 2006), rats (Mohammed and Engidawork, 2011) and those of the present study. Studies have shown that stress stimulates adrenocorticotropic hormone production and subsequent adrenal steroid secretion (Czeisler et al., 1976), and this response in adrenocortical secretion correlates positively with the intensity of stressful stimuli (Murton et al., 1998). From the aforementioned, animal handling and manipulations are likely to exaggerate cortisol measurements in response to a given drug exposure. This has been explicitly explained by Dettmer et al. (1996) in a study on behavioural and cortisol responses to repeated capture and venipuncture in Cebus apella. In this study, it was shown that animals that were behaviourally habituated to handling and blood collection procedures had low cortisol levels compared to non-habituated naive subjects. In the present study, all animals were habituated to handling for four weeks before experimentation and during blood sampling both controls and test animals were handled the same way. Furthermore, serial blood sampling was done at constant time (1000 h) of every sampling day of the week to avoid influence of time of day on cortisol levels as glucocorticoid hormone release is characterized by a circadian cycle (Akana et al., 1996). The observed pattern of cortisol hormone release in the present study is likely a
function of (-)-cathinone treatment. The (-)-cathinone doses used in the present study were based on knowledge of previous studies in humans and experimental animals (Mohammed and Engidawork, 2011; Widler et al., 1994; Pehek et al., 1990; Brenneisen et al., 1990). Most of these studies did interventional studies with similar protocol as that employed in the present study. From the results of the present study, it is apparent that behavioural scores reported in chapter 3 were not influenced by cortisol secretion. Earlier studies reported locomotor responses induced by injection of psychostimulants in nucleus accumbens (Delfes et al., 1990) and decrease in locomotor responses occur by suppression of corticosterone and re-established by restoring basal levels of the hormone (Marinelli et al., 1994). In another separate study glucocorticoid secretion was shown to precede many goal-seeking behaviours such as food-seeking (Bassareo and Di Chiara, 1999; Wilson et al., 1995) and drug-seeking behaviour (Cadoni et al., 2003). In the present study, however, the appetitive/consummatory behaviour was impaired in (-)-cathinone subjects indicating little role of cortisol on the observed behaviours.

The findings of the present study also show a strong positive correlation between cortisol and prolactin during treatment phase suggesting that the mode of action of (-)-cathinone on the two systems producing these hormones may be similar and appear to influence each other. Studies have shown that activation of the hypothalamo-hypophyseo-adrenocortical axis and ascending catecholaminergic neurons play an important role in metabolic and behavioural adaptation to stress (Ishikawa et al., 2007). Similarly, a precise overlap of processes containing corticotrophin-releasing hormone (CRH) and serotonin in the dorso-lateral sub-nucleus have been demonstrated (Ruggiero et al., 1999). Since cortisol is synthesized in specific cells of the adrenal glands, released in the peripheral blood flow and its production
controlled centrally by classical negative feedback system of the hypothalamo-hypophyseal-adrenocortical axis (McEwen, 2000), its low levels observed in the present study may partly be explained by reduced synthesis of CRH and/or release at the hypothalamus or reduced responsiveness of its receptors at the pituitary and adrenal gland as a function of (-)-cathinone exposure.

Another hypothesis that attempts to explain the observed low serum cortisol levels in the present study is the possible involvement of the extra-hypothalamic CRH systems that have been implicated in behavioural arousal (Landgraf, 2005) and depression (Mitchell, 1998). Studies in humans showed an association between CRH innervations and serotonergic neurons in the midline raphe (Ruggiero et al., 1999), consistent with the findings in rats that demonstrated localization of the CRH neuropeptide and its receptor binding sites (De Souza, 1995) as well as mRNA expression of subtype 2 to the midline raphe (Chalmers et al., 1995). Serotonin-containing bodies of the raphe nuclei project to dopaminergic cells in the ventral tegmental area, substantia nigra, nucleus accumbens, prefrontal cortex and striatum (Moukhles et al., 1997; Van Bockstaele et al., 1994). This association may explain, in part, the behavioural output following a given stimulus to the hypophyseo-adrenocortical axis. Studies in humans, for example, showed that pregnant mothers undergoing depression produced decreased salivary cortisol and increased serotonin and dopamine (Field et al., 2005), consistent with a similar study on postpartum depressive mothers that showed behavioural and stress hormone changes including decrease in anxiety levels and salivary cortisol following massage therapy (Field et al., 1996). It appears that the rewarding system in the brain of humans including non human primates consists of a network of connections between serotonergic and dopaminergic projections in the meso-corticolimbic system and
hypothalamic CRH projections, and that these interconnections may be unique in these species different from other animals. The present study did not consider investigation in this direction. Further research may prove essential to the understanding of pharmacokinetics of psychostimulants to the reward centres of the brain. However, the serotonergic system has been reported to be involved in cathinone-induced stereotyped movement, aggression and sexual arousal (Abdulwaheb et al., 2007; Banjaw et al., 2006; Connor et al., 2002). Aggression and stereotypy that characterize abnormal behaviour have been reported in chapter 3 of this thesis. It is possible (-)-cathinone caused the observed behaviours via its action on the serotonergic system.

The similarity in results on hormonal profiles in non-human primates as seen in the present study and that of Mwenda et al. (2006) but different from humans (Nencini et al., 1984), raises important unanswered questions: Is there a threshold of (-)-cathinone exposure above which persistent hormonal changes in hypophyseal-adrenocortical system are induced? What factors influence differences in vulnerability to persistent neuro-endocrine changes following cathinone exposure? Does the cumulative exposure as a result of daily khat use produce persistent dopaminergic and hormonal changes associated with behavioural manifestations observed in khat ‘addicts’? Is there a species difference in the reward centres of the brain connecting the hypothalamo-hypophyseal-adrenocortical axis? Does the choice of dose of drug matter when dealing with different species of animals under experimentation? These questions underscore the need to determine which animal species, dose range and/or experimental paradigms best simulate daily human khat consumption. The mechanisms underlying changes in hypothalamo-hypophyseal-adrenocortical axis and dopaminergic system following khat use remain speculative. However, some evidence demonstrate marked
species differences in vulnerability to simulate induced hormonal changes in rabbits (Nyongesa et al., 2008), baboons (Mwenda et al., 2006) and rats (Mohammed and Engidawork, 2011). It is also possible that after multiple repeated exposures to khat, tolerance develops and this may have contributed to variability in effects. Tolerance and cross-tolerance between (-)-cathinone and cathine have been reported in animal studies (Foltin et al., 1983) and these effects are probably mediated by pre-synaptic dopamine release (Schechter, 1990). Similar reports indicate that after chronic exposure to amphetamines, animals exhibit either tolerance or sensitization during subsequent drug administration, indicating adaptations in the neurobiological substrates of these behaviours (Berman et al., 2009).

In the present study, a dose- and time-dependent effect of (-)-cathinone on cortisol and prolactin hormonal profiles in vervet monkeys are presented. The pattern of dose and time as functions of khat/(-)-cathinone stimulatory effects has been demonstrated in several studies. Neuropharmacological evaluation of effects of khat in rats showed that different concentrations caused psycho-stimulation followed by depression, characterized by a stimulatory electro-encephalogram (EEG) pattern with lower doses (50 – 100 mg/kg) while higher doses (400 mg/kg) caused initial activation followed by EEG depression (Saleh et al., 1988). These EEG patterns were observed to resemble the progression of psychostimulation and excitation to that of sedation, anxiety and depression with continued khat chewing (Hassan et al., 2002), indicating a dose and time effect to neurobiological manifestations in the khat chewer. Similarly, acute and sub-chronic administration of Catha edulis leaves or S (-)-cathinone showed increased locomotor activity in rats (Banjaw and Schmidt, 2006). Similarities between (-)-cathinone and amphetamine have also been demonstrated with regard to induction of stereotyped behaviour in rats (Banjaw and Schmidt, 2005) and mice (Al-
Meshal et al., 1991) at high doses. Earlier reports showed that cathinone induces tremor at low doses and seizure at high doses (Berardelli et al., 1980). Recently, studies showed a dose- and time-dependent effect of khat extract on spatial learning and memory in mice (Kimani and Nyongesa, 2008). In this study it was observed that low doses of khat extract had no significant effect on learning but impaired memory while high doses impaired learning but improved memory. Increase in aggression, anxiety and abnormal behaviours as well as withdrawal and impaired consummatory/appetitive behaviour following (-)-cathinone administration to vervet monkeys have been described in chapter 3 of this thesis. These differences on neuro-behavioural manifestations indicate the effect of dose of khat/(-)-cathinone and duration of exposure on different neurobiological systems of the brain.

4.3.2 Effect of (-)-cathinone on Luteinizing hormone, progesterone and testosterone.

The results of the present study show a dose- and time-dependent increase in LH with the significant increase at high doses of (-)-cathinone. Conversely, serum progesterone levels decreased in a dose- and time-dependent manner while the effect of (-)-cathinone on testosterone were biphasic, with low and moderate doses increasing while high dose decreasing its production. From the results, it appears (-)-cathinone played a stimulatory role either at the level of the hypothalamus or directly on pituitary gland to cause the observed increase in LH. There is prevailing evidence that gonadotrophic releasing hormone (GnRH) produced by the hypothalamic neurosecretory cells is involved in regulation of both LH and Follicular stimulating hormone (FSH) secretion (Hadley, 1988; Bardin and Paulsen, 1981). The increase in testosterone following increasing LH in the present study is interesting. Following the classical negative feedback loop, it is expected that increasing levels of testosterone could have inhibited further production of LH although this depends on whether
or not elevated testosterone levels persisted for long. However, earlier studies showed evidence of a differential control of LH and FSH secretion (Burger, 1989; Hall et al., 1988). Norepinephrine has been shown to play a principal role in promoting LH release via GnRH while serotonin inhibits LH release (Knol, 1991). Since khat and (-)-cathinone are sympathomimetic in that they produce their effects by increasing synaptic levels of biogenic amines: dopamine, noradrenaline and serotonin, through multiple mechanisms (Fischman and Madras, 2005), it is possible the observed effects in the present study were due to levels of these neurotransmitters in circulation.

The biphasic effects of khat and (-)-cathinone have been reported by several investigators. For example, previous studies showed that low dose of khat was stimulatory while high dose was inhibitory to sexual motivation and performance in male rats (Abdulwaheb et al., 2007), consistent with testosterone measurement in mice isolated interstitial cells in vitro (Nyongesa et al., 2007). Quite recently, a study in rats demonstrated that mild doses of khat improved sexual motivation but did not interfere with performance while high doses impaired both motivation and performance (Mohammed and Engidawork, 2011). It was suggested that alteration of both dopamine (at low dose) and serotonin (at high dose) levels in the central nervous system could explain the biphasic sexual behaviour of rats after khat administration (Abdulwaheb et al., 2007; Taha et al., 1995). However, the role of testosterone on this measure can not be ignored. The effect of (-)-cathinone on stereodogenic structures, particularly smooth endoplasmic reticulum and mitochondria cristae, as well as dehydroxysteroid dehydrogenase enzymes (3β- HSD and 17β- HSD) have been reported in chapter 5 and 6 of this thesis, all of which point to the observed testosterone measurements. The increase in testosterone following (-)-cathinone treatment may in part explain increases in
indicators of aggression (yawn, engage in a stare, bouncing off cages walls, vigorous shaking of cages and head jerk) reported in chapter 3 of this thesis. In primates, the correlation between aggression and increase in circulating testosterone levels has been reported (Alberts et al., 1992; Bernstein et al., 1979).

The decrease in serum progesterone in (-)-cathinone subjects may be explained independently or in relation to the observed testosterone secretion pattern. Firstly, progesterone is one of the intermediate hormones that are produced in the steroid biosynthetic pathway that ultimately ends up with testosterone and estrogen production (Christensen, 1975). The decreasing levels of progesterone may, therefore, be explained by its enzymatic breakdown by hydroxysteroid dehydrogenases into formation of androstenedione and subsequently testosterone (Andersson et al., 1995). Secondly, high levels of LH and testosterone in the present study may explain decreasing levels of progesterone. Earlier studies have shown that endogenous corticosteroid and progesterone are negatively correlated to LH and testosterone (Welsh et al., 1981). The observed increase in LH and testosterone in the present study may have influenced the secretion of progesterone hormones.

It is worth noting that vervet monkeys are social animals that live under social complex interactions and so studies on sexual function under captive conditions must be interpreted with caution. However, previous studies have shown that vervet monkeys readily adapt to captivity and reproduce throughout the year (Seier, 2005) and so the observed hormone profiles may not be due to seasonality in reproduction. Owing to the necessity of obtaining blood samples at regular intervals as well as repeated (-)-cathinone administration as per stringent schedule, it would be a stressful undertaking for group-housed vervets.
Conclusively, the findings of the present study have shown that (-)-cathinone exposure caused hormonal alterations in a dose- and time-dependent manner. These results confirm and extend previous findings, in demonstrating effects of (-)-cathinone on hypothalamic-hypophyseal-adrenocortical and gonadal axes as well meso-corticolimbic systems.
CHAPTER 5

IMMUNOHISTOCHEMICAL LOCALIZATION OF ANTERIOR PITUITARY CELL TYPES OF VERVET MONKEYS (CHLOROCEBUS AETHIOPS) FOLLOWING SUB-CHRONIC CATHINONE EXPOSURE

5.0 INTRODUCTION

The cells within the pituitary gland respond to continuously changing central nervous system as well as adrenocortical and gonadal signals and provide appropriate responses. As a result, changes in pituitary cell function serve to integrate a number of complex mechanisms associated with response to various stressful stimuli or successful reproduction. It would, therefore, be expected that natural products that are routinely consumed or used by human population may affect aspects of reproductive capacity or elicit stressful responses via alteration of pituitary function. However, studies on use of psychotropic drugs often ignore measures of hypothalamo-pituitary or pituitary function in response to various degrees of exposure to these drugs.

Many drugs of abuse exert an inhibitory or stimulatory action on pituitary function by modifying hypothalamic control of pituitary hormone secretion (Steckler and Holsboer, 1999; Sillaber et al., 1988). They do so by altering neurotransmitter and neuropeptide activity (Cooper et al., 1986). Previous studies have shown that amphetamine, which resembles (-)-cathinone in chemical structure and function (Graziani et al., 2008; Houghton, 2004; Cox and
Rampes, 2003) and with similar potential for abuse (Kalix, 1984 a), causes increase in gonadotrophic cells following long-term use (Tsai et al., 1996). In a similar study long-term use of amphetamine caused an increase in mixed cell follicles in the adenohypophysis and reduction in number of lactotrophs in the immunohistochemical sections (Ishikawa et al., 2007).

Previous studies on khat and (-)-cathinone have shown varying effects on the pituitary gland. For example, earlier studies showed that (-)-cathinone is an inhibitor of several anterior pituitary hormones (Wagner et al., 1982). Similar studies on effects of escalating doses of khat extracts suppressed plasma LH levels in rabbits (Nyongesa et al., 2008). In other studies there was increase in adrenocorticotrophic hormone in humans (Nencini et al., 1984), consistent with cortisol measurement in rabbits following khat extract exposure (Nyongesa et al., 2008) but different from findings in baboons where decreased plasma cortisol was observed (Mwenda et al., 2006). The levels of these pituitary hormones correspond to the activity of the specific cell types that synthesize them that is: gonadotrophs, corticotrophs and lactotrophs. It is this scanty and contradictory information that led to the hypothesis of the present study. It was hypothesized that cathinone variously affects the expression of gonadotrophs, corticotrophs and lactotrophs and consequently their hormonal secretions or release of releasing factors from hypothalamus. This study investigated effect of (-)-cathinone on hypothalamo-pituitary- adrenocortical and gonadal axes. The hypothalamic function was tested by use of GnRH agonist goserelin acetate (ZOLADEX) followed by immunolocalization of gonadotrophs on anterior pituitary sections while pituitary function was evaluated by direct immunolocalization of lactotrophs and corticotrophs.
5.1 MATERIALS AND METHODS

5.1.1 ANIMALS AND HOUSING

See section 2.1 of general materials and methods.

5.1.2 EXPERIMENTAL DESIGN

The experimental design for this study is the same as was described previously in section 2.4 of general materials and methods. At the end of the 4 month (-)-cathinone alone treatment, animals on low and high doses (0.8 and 6.4 mg/kg body weight) of (-)-cathinone were administered ZOLADEX at 50 μg/kg body weight single subcutaneous injection for a further 2 weeks while continuing with (-)-cathinone treatment. ZOLADEX was prepared at 3.6 mg in 10% DMSO in PBS. At the end of treatment period, all animals were euthanized with a high dose of ketamine and anterior pituitary gland harvested for immunohistochemical evaluation.

5.1.3 IMMUNOHISTOCHEMISTRY

The anterior pituitary glands of all the animals were harvested and fixed with 4% formaldehyde in phosphate-buffered saline (pH 7.2) for 1 h then embedded in paraffin wax. Serial sections (5 μ thick) were carefully cut and labelled with animal number and the stain to be used. Immunostaining was done by avidin-biotin complex method and colour development done using 3, 3’ diaminobenzidine. The slides were placed on Dako Autostainer Slide Racks (code S3704) and inserted in an oven at 100 °C for 15 minutes and allowed to cool before proceeding with antigen retrieval. Appropriate labels of slides were generated from a computer connected to Dako Link 48 instrument, fixed carefully on slides to avoid damage to the barcode. Thereafter, slides were inserted into the Dako target retrieval solution in PT Link
tanks (code PT100/PT101), which incorporated pre-heat temperature, antigen retrieval temperature and time as well as cool down settings at 97°C for 20 minutes. The Dako target retrieval solution was prepared (3:1) according to specifications and used for both deparaffinization and antigen retrieval. Each autostainer slide rack was removed from PT Link tanks and immediately dipped into PT Link rinse solution (code PT109) containing diluted room temperature Dako Wash buffer (10x) (code S3006) and left for 1 – 5 minutes. The slides were then removed and washed gently with diluted room temperature wash buffer to remove any debris on slides before insertion into Autostainer instrument. Thereafter, slides were placed on a Dako autostainer instrument containing respective antibodies. Primary antibodies were monoclonal mouse anti-human adrenocorticotrophic hormone, monoclonal mouse anti-human luteinizing hormone, polyclonal rabbit anti-human prolactin and polyclonal rabbit anti-human S100 (Dako). Detection was performed using anti-mouse or anti-rabbit Dako Envision system (biotinylated secondary antibodies for mouse and rabbit) as appropriate. Peroxidase activity was revealed by diamino 3, 3’-benzidine Substrate Chromogen System (Dako) for LH, prolactin, ACTH and S100. Hematoxylin and eosin were used for assessing normal morphology of cells of the pituitary gland.

A summary of type of antigen, antibody, species, source as well as dilution of the antibodies for staining are given (Table 5-1).

The Autostainer machine runs at an automated duration of time, which appears on the screen during the entire staining period. After completion of staining, the slides were carefully removed from the autostainer and drained of excess distilled water and arranged in a staining rack followed by dehydration, clearing and counterstaining with hematoxylin and cover slipped for microscopic examination.
5.1.4 HISTOLOGICAL EVALUATION

The sections were examined and desired fields photographed using a Zeiss Axioskop 40 plus microscope (Carl Zeiss MicroImaging GmbH, Germany) and a ProgRes CT5 USB C plus digital camera (Jena, Germany) with ProgRes Capture Pro 2.8 software (JENOPTIK/Optical Systems). Immunohistochemistry results for antibodies of interest were classified on presence or absence of staining as well as average number of immunostained cells per three randomly selected high magnification fields (x 40 objective). Slides that were stained with hematoxylin and eosin were used for standard histology.

5.1.5 MORPHOMETRIC ANALYSIS

Quantification of immunostained cells for ACTH, prolactin and LH on anterior pituitary sections was done by direct manual counting technique previously applied in measuring the mitotic rates in the endometrium of primates (Brenner et al., 2003). Immunostained cells were observed under light microscopy and three non-overlapping fields at high power magnification (x 400) randomly selected with the help of an ocular grid and counted using a mechanical tabulator. The labelling index (LI) was used to express the number of stained cells as a percentage of the entire population of cells on a section of the slide. The mean values of LI were then used for comparison among different treatment groups. In order to calculate the LI, the following formula was used:

\[ \text{LI} = \frac{(\text{PiC}+)/\text{PiCT}) \times 100 }{ } \]

Where,
PiCT = total number of cells for each stain (ACTH, prolactin, LH) that was counted in a given field (positive and negative cells for anterior pituitary),

PiC+ = number of immunostained cells (positive cells) for a given stain counted on the same area,

LI = labelling index, usually expressed as a percentage
5.2 RESULTS

Pituitary sections of all the animals (n = 14) from all the four (\textasciitilde\textasciitilde)-cathinone dose groups were generally histologically similar compared to controls. The sections were characterized by clusters and islands of cells separated by thin sinusoidal spaces and slightly larger vascular profiles. The anterior pituitary parenchyma consisted of predominantly acidophils with rare numbers of basophils and chromophobes. Acidophils appeared uniform in size, stained deeply eosinophilic, and laden with cytoplasmic granules. Some of the acidophils exhibited degranulation. Basophils stained slightly basophilic and chromophobes showed pale cytoplasm. The sinusoids were lined by single layer of endothelium and appeared slightly widened in some areas. Fairly dilated sinusoids were distinguished as an effect of fixation and processing but not of any morphological significance. Some eosinophilic cells with lobulated nuclei were also observed to be a common occurrence in the vervet monkey since these features were seen in sections of controls and treated groups. There were no other significant morphological changes observed. Histology of the sections showed physiologically active glands (Fig. 5-1).

Since immunochemical staining with all antibodies did not show significant difference in pituitary sections of animals treated with 0.8 mg/kg and 1.6 mg/kg body weight of (\textasciitilde\textasciitilde)-cathinone, the immunohistochemical pictures in the latter case are not presented here but the former are presented for data comparison since it is the lowest (\textasciitilde\textasciitilde)-cathinone dose used in the study. Immunohistochemistry for staining of basophils showed no obvious difference in the intensity of cytoplasmic staining across treatment groups and controls. In sections stained with mouse anti-human ACTH antibody, two cell populations were identified with the predominant proportion of mostly acidophils that appeared negative for ACTH stain.
Positively staining cells, interpreted as corticotrophs, revealed uniform intracytoplasmic staining and appeared arranged individually and occasionally in clusters of 2 - 3 cells (Fig. 5-2 a-d). A random selection of three fields at x 40 objective showed a dose-dependent decrease in LI of cells. Mean LI of corticotrophs were: 48 ± 4, 36 ± 5, 35 ± 5.6, 22 ± 6 and 14 ± 3.5 for doses 0, 0.8, 1.6, 3.2 and 6.4 mg/kg body weight of (-)-cathinone (Fig. 5-3).

The sections stained with mouse anti-human Luteinizing Hormone showed a similar pattern obtained with ACTH staining, with two cell populations under light microscopy. The larger percentage of cells was composed mostly of acidophils that were negative for LH stain. The staining pattern was cytoplasmic and positive cells, interpreted as gonadotrophs, were mostly scattered throughout the field and occasionally in groups of 2 – 3 cells. These cells maintained a dispersed pattern and intensity of staining was similar for within- and between- treatment groups. However, the number of stained cells when taken at three randomly selected high power fields (x 40 objective) was significantly different across treatment subjects as shown by their LI (Fig. 5-4). The mean LI for positive cells for low dose of (-)-cathinone co-treated with GnRH agonist was higher (46 ± 8) compared to doses 1.6 and 3.2 mg/kg body weight as well as controls. At doses 1.6 and 3.2 mg/kg body weight, mean values of 28 ± 5.4 and 43 ± 10, respectively, were counted indicating that GnRH agonist indeed boosted the expression of positive staining cells. At the highest (6.4 mg/kg body weight) dose of (-)-cathinone, there was a significantly higher (P < 0.05) number of positive cells (78 ± 14.8) compared to other doses and controls (20 ± 10). The results show a dose-dependent effect of (-)-cathinone on positive cell count and the synergistic effect of GnRH agonist (Fig. 5-5).
In sections stained with polyclonal rabbit anti-human prolactin, two cell populations were observed at light microscopy, most of which were negative for prolactin. Positive cells (lactotrophs) were scattered in the field occasionally in clusters of 2-4 cells. These positive cells maintained a dispersed pattern, and intensity of staining was similar for within- and between- treatment groups. However, the LI of stained cells when taken at three randomly selected high power fields (x 40 objective) was significantly different across treatment subjects (Fig. 5-6). Results show a dose-dependent decrease in number of positive cells counted in three randomly selected x 40 fields. At doses (0.8, 1.6, 3.2 and 6.4 mg/kg body weight) of (-)-cathinone, 61 ± 3, 58 ± 5, 36 ± 6.2 and 18 ± 5, respectively compared to controls (63 ± 4.5) were counted (Fig. 5-7).

Immunostaining of cells with polyclonal rabbit anti-human S100, a multifunctional marker of cellular degeneration, showed no apparent staining in both pituitary sections of (-)-cathinone subjects and controls (Fig. 5-8). In order to validate the specificity of the assay, positive control for S100 (skin melanoma known to be positive for this stain) was performed in parallel with test samples (Fig. 5-9). This means that the observed difference in labelling index of positive cells with different antibodies was due to the effect of (-)-cathinone on expression of the hormones in the positive cells and not due to cellular degeneration. The absence of staining with S100 in samples and staining for positive control shows that the assay worked but cellular degeneration was absent.

A summary of effect of escalating doses of (-)-cathinone on anterior pituitary cell types considered in this study are shown (Table 5-2).
Table 5-1. Immunohistochemistry summary table. B stands for antigen retrieval for 20 minutes at 97°C in Dako target retrieval solution.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody code</th>
<th>Species</th>
<th>Source</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>M3501</td>
<td>Monoclonal mouse anti-human</td>
<td>Dako</td>
<td>1:50</td>
<td>B</td>
</tr>
<tr>
<td>LH</td>
<td>M3502</td>
<td>Monoclonal mouse anti-human</td>
<td>Dako</td>
<td>1:50</td>
<td>B</td>
</tr>
<tr>
<td>Prolactin</td>
<td>A0569</td>
<td>Polyclonal rabbit anti-human</td>
<td>Dako</td>
<td>1:200</td>
<td>B</td>
</tr>
<tr>
<td>S100</td>
<td>IR504</td>
<td>Polyclonal rabbit anti-human</td>
<td>Dako</td>
<td>Ready-to-use</td>
<td>B</td>
</tr>
</tbody>
</table>
Table 5-2. Mean number of cells at high power magnification for each (-)-cathinone dose. LI means labelling index; a# in presence of GnRH agonist and significantly different (P<0.05); * - significantly different at (P<0.05). bwt means body weight.

<table>
<thead>
<tr>
<th>LI (% of positive cells)</th>
<th>(-)-Cathinone dose (mg/kg bwt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>LI for ACTH at x 400 magnification</td>
<td>48 ± 4</td>
</tr>
<tr>
<td>LI for LH at x 400 magnification</td>
<td>20±10</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>LI for PRL at x 400 magnification</td>
<td>63±4.5</td>
</tr>
</tbody>
</table>
Figure 5-1. Hematoxylin and eosin staining of adenohypophysis of vervet monkey. Similar morphological features between treatment groups and controls were observed. The gland shows presence of acidophils (a), basophils (b) and sinusoids (s) lined by epithelial cells (filled up arrow). Open arrow shows degranulation into sinusoids. Original magnification x 400.
Figure 5-2. Dose-dependent effect of (-)-cathinone on corticotrophs in anterior pituitary gland. The pictures show positive staining for corticotrophs (full arrows) in saline-treated controls (A), (-)-cathinone-treated animals at 0.8 mg/kg (B), 3.2 mg/kg (C) and 6.4 mg/kg (D). Original magnification x 400.
Figure 5-3. Mean labelling index (LI) indicating number of corticotrophs as a percentage of total cells in sections of control animals and (-)-cathinone-treated groups. Results show a dose-dependent decrease in mean LI of cells counted in three random x 40 fields with a significant decrease (P < 0.05) at dose 6.4 mg/kg body weight of (-)-cathinone, (n=14). ** means statistically significant at P <0.05, *** means statistically significant at P <0.01.
**Figure 5-4.** The figure shows gonadotrophs (open arrows) in anterior pituitary gland of controls vervet monkeys (A) and vervet monkeys exposed to (-)-cathinone doses of 0.8 mg/kg plus GnRH agonist (B), 3.2 mg/kg (C) and 6.4 mg/kg plus GnRH agonist (D). (-)-Cathinone increased expression of gonadotrophs in a dose-dependent manner. Original magnification x 400.
**Figure 5-5.** Mean LI expressing number of gonadotrophs as a percentage of total pituitary cells of control and (-)-cathinone-treated animals. Note that animals on low and high doses of (-)-cathinone and co-treated with GnRH agonist expressed significantly higher (P<0.05) number of cells compared to (-)-cathinone alone and controls (n =14) when counted on three random fields at x 400 magnification. ** means statistically significant at P < 0.05, *** means statistically significant at P < 0.01.
Figure 5-6. Immunostaining for lactotrophs (arrows) in anterior pituitary gland of controls vervet monkeys (A) and vervet monkeys treated with (-)-cathinone doses of 0.8 mg/kg (B), 3.2 mg/kg (C) and 6.4 mg/kg (D). Original magnification x 400
Figure 5-7. Mean LI expressing number of lactotrophs in controls and (-)-cathinone-treated groups. Results showed a dose-dependent decrease in number of cells counted in three random x 40 fields. The high doses (3.2 and 6.4 mg/kg body weight) of (-)-cathinone caused a significant decrease (P < 0.05) compared to other doses and controls (n =14). * means statistically significant at P<0.05, ** means statistically significant at P<0.01.
Figure 5-8. Immunostaining of adenohypophysis of vervet monkey with S100. The cells were negative for the stain. Original magnification x 400.
Figure 5-9. Figure showing a skin tissue with melanoma positive for S100. Note the melanoma around the hair follicle (bigger full arrow). Arrow head shows nuclei of muscle cells. Original magnification x 400
5.3 DISCUSSION

Psychotropic drugs cause varying effects in the body of the user based on type and degree of use. Most of these drugs influence the central nervous system function via disregulation of the hypothalamic dopaminergic system (Quan et al., 2005). Drugs such as methamphetamine have been shown to reduce long-term potentiation while increasing synaptic transmission in the hippocampus of mouse (Swant et al., 2010). It is on the understanding of these varying effects of psychotropic drugs on the central nervous system that the hypothesis of the present study was designed.

The present study provides insight into the immunolocalization of anterior pituitary gland cells specifically: corticotrophs, gonadotrophs and lactotrophs of vervet monkeys following exposure to escalating doses of (-)-cathinone. The results show that increasing doses of (-)-cathinone suppressed the labelling index of corticotrophs and lactotrophs in a dose-dependent manner. The significant effects were observed with high doses (3.2 and 6.4 mg/kg body weight) of (-)-cathinone. These results can explain the dose-dependent decrease in serum cortisol and prolactin levels reported in chapter 4 of this thesis. On the other hand, cell density of gonadotrophs increased in a dose-dependent manner. However, in the presence of GnRH agonist, low (0.8 mg/kg body weight) and high dose (6.4 mg/kg) of (-)-cathinone significantly increased the immunolocalization of gonadotrophs. Luteinizing hormone levels in (-)-cathinone subjects co-treated with GnRH agonist are not reported. Due to small sample size of animals there were no controls for this intervention. However, results on immunolocalization of pituitary cells in animals co-treated with GnRH agonist are reported here. This finding is significant in that it points to the hypothalamic and pituitary integrity following (-)-cathinone
exposure. However, previous studies reported the inhibition of gonadotropin release by dopamine binding to the receptors on basophils thereby activating a membrane G protein (Yen, 1991). Previously, studies in rabbits reported a decrease in luteinizing hormone production following khat extract administration (Nyongesa et al., 2008). Hormonal results of the present study in chapter 4 show that (-)-cathinone increased and then decreased serum testosterone levels in vervets. High dose of (-)-cathinone, however, increased LH as well as labelling index of gonadotrophs but decreased serum testosterone levels. It appears that (-)-cathinone at high dose acted through different mechanism of action leading to variability on these measures. One possible mechanism is the cytotoxic effect of (-)-cathinone on Leydig cells (Tariq et al., 1987). Earlier studies showed that luteinizing hormone binds to its receptors on Leydig cells (Bardin and Paulsen, 1981) and stimulates synthesis of testosterone (Castro et al., 2002). From the foregoing, it is hypothesized that effect of (-)-cathinone on hypothalamo-hypophyseal axis may be species specific and that it influences hypothalamo-hypophyseo-gonadal axis via different mechanisms. Studies on receptor binding are required to confirm the responsiveness of the anterior pituitary cells to hypophyseotrophic hormones from the hypothalamus.

In this thesis, it is reported that (-)-cathinone caused a dose-dependent decrease in number of lactotrophs, consistent within dose-dependent decrease in serum prolacin levels reported in chapter 4. This is expected, given the understanding that (-)-cathinone induces release of dopamine from several brain regions including striatum (Pehek et al., 1990; Kalix and Braenden, 1985; Kalix, 1983 a). Since dopamine is a well known inhibitory factor to prolactin release, its increase leads to a proportionate decrease in prolactin levels. This finding is consistent with another study that showed decrease in prolactin levels following
administration of khat extract to baboons (Mwenda et al., 2006). The findings explain behavioural manifestations reported in chapter 3 of this thesis. On the contrary, previous findings on long-term use of amphetamine in humans showed hyperprolactemia due to hyperfunction of prolactin cells (Ishikawa et al., 2007). This is unexpected since amphetamine and (-)-cathinone have been shown to have same mechanism of action (Graziani et al., 2008; Houghton, 2004; Cox and Rampes, 2003) at central dopaminergic (Kalix and Braenden, 1985) as well as other peripheral sites (Kalix and Braenden, 1985; Nencini et al., 1984; Knoll, 1979).

Results also showed a dose-dependent decrease in corticotrophs in anterior pituitary sections as shown by the labelling index. Corticotrophs synthesize adrenocorticotropic hormone (ACTH) that in turn stimulates adrenal cortex to produce glucocorticoids. Cortisol is one such glucocorticoid. The results on decreased immunolocalization of corticotrophs reflect dose-dependent decrease in cortisol levels reported in chapter 4 of this thesis. This finding is similar to that in baboons where khat extract caused a decrease in plasma cortisol (Mwenda et al., 2006) but different from studies in rabbits (Nyongesa et al., 2008). The difference in findings may be explained partly by the time of exposure to khat/(-)-cathinone, possibly species difference and animal handling. Glucocorticoid hormone release is characterized by a circadian cycle (Akana et al., 1996). In the present study, animals were habituated to handling and presence of observer for one month before blood collection, and control animals were handled in the same way as treatment groups.

This study investigated the possibility of cellular degeneration that led to variation in the labelling index of various pituitary cell types following exposure to escalating doses of (-)-
cathinone. (-)-Cathinone has been reported to be cytotoxic (Tariq et al., 1987) and mitodepressive (Al-Meshal et al., 1989; Al-Meshal, 1987; De Hondt et al., 1984). Earlier studies using S100 (a multifunctional marker of cell degeneration) demonstrated presence of clusterin-containing mixed follicles in long-term amphetamine abusers which corresponded to cellular degeneration (Ishikawa et al., 2007). In the present study, S100 was used in parallel with a known positive control (skin melanoma) to detect any possible cellular degeneration in the anterior pituitary gland. All cells of pituitary sections of (-)-cathinone subjects and negative controls were negative for the stain when compared to the positive control. The variation in labelling index in various sections, therefore, is not suggestive of cellular degeneration but rather the reflection in variation of respective antigens in the anterior pituitary cell types.

Overall, the results demonstrated immunolocalization of anterior pituitary cell types following (-)-cathinone exposure and that these numbers correspond to the concentration of hormones in circulation. The results indicate that (-)-cathinone at different degree of exposure, indeed, has an effect on the hypothalamo-pituitary-adrenocortical and gonadal axes. It is interesting to note that the effect on gonadotrophs is, however, opposite to that of other anterior pituitary cell types. The significance of this lies in the selective action of (-)-cathinone on different functional systems of the body.
CHAPTER 6

ACUTE AND SUB-CHRONIC EFFECTS OF CATHINONE ON SEX STEROID HORMONE METABOLISM IN ISOLATED INTERSTITIAL TESTICULAR CELLS OF VERVET MONKEYS (CHLOROCEBUS AETHIOPS)

6.0 INTRODUCTION

Sex steroids regulate numerous physiological processes by exerting their effects on target organs including reproductive, cardiovascular and musculoskeletal systems as well as liver and brain (Ihemelandu, 1981). These hormones are synthesized by smooth endoplasmic reticulum (SER) of Leydig cells of testis, cells of inner layer of adrenal cortex, granulosa and luteal cells of the ovaries as well as placenta from cholesterol as the precursor molecule (Christensen, 1975). Cholesterol is stored mainly in lipid droplets (Moses et al., 1969) and is metabolized into pregnenolone. In the presence of hydroxysteroid dehydrogenases, a series of chemical reactions take place that result into conversion of Δ5- 3β hydroxysteroids, pregnenolone, 17 hydroxypregnenolone and dehydroepiandrosterone (DHEA) to the Δ4- 3-ketosteroids, progesterone, 17α- hydroxyprogesterone and androstenedione, respectively (Payne and Hales, 2004). These hydroxysteroid dehydrogenases are of two types: 3 beta hydroxysteroid dehydrogenase (3β-HSD) and 17 beta hydroxysteroid dehydrogenase (17β-HSD) and both belong to the same phylogenetic protein family (Penning, 1997). They are
involved in reduction and oxidation of steroid hormones requiring NAD\(^+\)/NADP\(^+\) as acceptors and their reduced forms as donors of reducing equivalents.

The human steroidogenic tissue contains several isoforms for 3\(\beta\)-HSD (Payne and Hales, 2004) and isozymes of the 17\(\beta\)-HSDs (Adamski and Jakob, 2001; Adamski et al., 1995), and each is a product of a distinct gene. The number of these isoforms or isozymes varies in different species, tissue distribution, catalytic activity, substrate and co-factor specificity, subcellular localization and mechanism of regulation (Payne and Hales, 2004). Studies in the mouse showed classification of 3\(\beta\)-HSD into several isoforms: I, III and VI as classic dehydrogenases/isomerases while IV and V as 3-keto reductases and, therefore, not involved in active steroidogenesis (Abbaszade et al., 1995; Clarke et al., 1993). In humans two distinct isoforms of 3\(\beta\)-HSD (type I and II) have been identified (Payne and Hales, 2004). 3\(\beta\)-HSD I is expressed in placenta, skin, and breast while 3\(\beta\)-HSD II is expressed in adrenal cortex, ovary and testis (Simard et al., 1996; Rheaume et al., 1991). Mouse 3\(\beta\)-HSD I is expressed in testis, ovary and adrenal gland while 3\(\beta\)-HSD VI is expressed in placenta, skin and testis (Abbaszade et al., 1997). It is not known if this scenario in expression is similar in vervet monkey testis. On the other hand, 17\(\beta\)-HSDs are not involved in biosynthesis of adrenal steroids. In humans nine different isozymes have been identified (Adamski and Jakob, 2001), with types I, III, and VII participating in gonadal steroidogenesis (Payne and Hales, 2004). The hydroxysteroid dehydrogenase enzymes involved in sex steroid synthesis in human and mouse are highlighted below (Table 6-1) while substrates involved in testosterone biosynthesis are shown (Fig. 6-1).
Figure 6-1. Diagramatic presentation of steroid hormone metabolism
Table 6-1. Table showing hydroxysteroid dehydrogenases involved in active steroidogenesis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosomal location</th>
<th>Protein name (molecular mass)</th>
<th>Synonyms</th>
<th>Tissue specific expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Mouse</td>
<td>Human</td>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>17β hydroxysteroid dehydrogenase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSD17B1</td>
<td>Hsd11b2</td>
<td>17q11-q21</td>
<td>11 (60.25cM)</td>
<td>17HSD1 (35 kDa)</td>
</tr>
<tr>
<td>HSD17B3</td>
<td>Hsd11b3</td>
<td>9q22</td>
<td>13</td>
<td>17HSD3 (34.5 kDa)</td>
</tr>
<tr>
<td>HSD17B7</td>
<td>Hsd17b7</td>
<td>1q23</td>
<td>1</td>
<td>17HSD (37.3 kDa)</td>
</tr>
<tr>
<td>B</td>
<td>HSD3B1</td>
<td>Hsd3b6</td>
<td>1p 13.1</td>
<td>3 (49.1 cM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSD3B2</td>
<td>Hsd3b1</td>
<td>1p 13.1</td>
<td>3 (49.1 cM)</td>
<td>3β-HSDII human</td>
</tr>
</tbody>
</table>
Testosterone is synthesized through metabolism of pregnenolone and DHEA by 3β-HSD and 17β-HSD and subsequently aromatized in the presence of P₄₅₀ aromatase cytochrome into oestrogen (Aizawa et al., 2007). To date, it is not clear whether steroidogenesis-related enzymes are affected by psychostimulants such as (-)-cathinone. In the present study, it was hypothesized that vervet monkey testes contain steroidogenic enzymes of different isoforms or isozymes of hydroxysteroid dehydrogenases and that their biosynthetic functions are variably affected by (-)-cathinone exposure. In this study, gene (mRNA) expression of these enzymes from testicular tissue of controls and (-)-cathinone-treated vervet monkeys was determined and their concentrations quantified by real-time polymerase chain reaction (PCR).
6.1 MATERIALS AND METHODS

6.1.1 ANIMALS AND HOUSING

The animals and housing procedures are same as was described previously under section 2.1 of general materials and methods.

6.1.2 EXPERIMENTAL DESIGN

6.1.2.1 Animal grouping

The same pattern of animal grouping used here has been described under general materials and methods in chapter 2, section 2.4 of this thesis. Briefly 14 animals were divided into 5 groups (I, II, III, IV and V) and labelled as GI for controls while GII, III, IV and V as treatment groups with (-)-cathinone at 0.8, 1.6, 3.2 and 6.4 mg/kg body weight, respectively. Controls were treated with normal saline. However, group III animals are not discussed in this chapter since results of these animals in the pilot study were not significantly different from group II animals. Only male animals were used in this study for investigation of expression of steroidogenic enzymes by use of semi-quantitative and real-time PCR. However, animal 2169_1 was not used since its testicular samples were not properly preserved for this study but is described in chapter 7 of this thesis. Samples of animal 2148_1 were duplicated into 2148_2. These same animals were used for ex vivo studies for measurement of intracellular progesterone, androstenedione and testosterone levels following testicular harvest and culture of interstitial cells. The harvested cells were incubated in culture medium in test tubes and treated as described below.
6.1.2.2 Leydig cell function test

To assess for regulation of 3 β- hydroxysteroid dehydrogenase (HSD) type I and 17 β- HSD type I, both in vivo and ex vivo studies were required. This is because long-term primary culture of Leydig cells was necessary and these Leydig cells could not withstand long-term culture period. Similarly, during drug metabolism, there are possible inherent differences in the sensitivity of in vivo and ex vivo preparations. Therefore, both in vivo and ex vivo studies for assessment of enzymatic regulation of steroidogenesis were done for data comparison.

The harvested testes were decapsulated, fat trimmed off and rinsed in ice-cold 10 ml Minimal Essential Medium Eagle (modified) in plastic Petri dish to remove excess blood followed by homogenization (Fig. 6-2). Isolated Leydig cells from testes of all animals were cultured in 100 μl of incubation media in test tubes as follows: controls (2 tubes) and test cells (10 tubes). Test cells were grouped and treated as follows: 2 tubes (50 μl of LH alone), 2 tubes (50 μl of LH + 100 μl of 0.8 mg/kg of (-)-cathinone [low dose]), 2 tubes (50 μl of LH+ 100 μl of 6.4 mg/kg of (-)-cathinone [high dose]), 2 tubes (low dose alone) and 2 tubes (high dose alone). The cells were incubated for 1 hr 15 min at 37°C. The control cells contained killed cells (by boiling in water for 5 min according to Oduma et al. (2006). At the end of the incubation period, the cell suspensions were centrifuged at 1500 x g, the supernatant decanted and analysed for progesterone, androstenedione and testosterone by enzyme immunoassay while pellets collected and stored at -80°C for mRNA expression of steroidogenic enzymes (3 β-HSD type I and 17 β-HSD type I).
6.1.2.3. Expression of steroidogenic enzymes in isolated Leydig cells co-incubated with (-)-cathinone

The cells collected above were stored at -80°C in RNALater for stabilization and protection of RNA from degradation and later analyzed for mRNA expression of 3 β-HSD type I and 17 β-HSD type I by semi-quantitative RT-PCR and real-time quantitative PCR analysis.

Reverse transcriptase polymerase chain reaction (RT-PCR) for mRNA expression of 3 β-HSD type I isoform and 17 β-HSD type I isozyme of hydroxysteroid dehydrogenase.

i) Total RNA extraction

RNA was extracted from tissues using TRIzol® Reagent (Invitrogen, Burlington, Ontario, Canada), a monophasic mixture of phenol and guanidine isothiocyanate. The reagent is capable of maintaining the integrity of the RNA due to highly effective inhibition of RNase activity while disrupting cells and dissolving cell components during sample homogenization. After homogenizing the sample with TRIzol® Reagent, chloroform was added, and the homogenate allowed to separate into a clear upper aqueous layer (containing RNA), an interphase and a red lower organic layer (containing the DNA and proteins, respectively). RNA was precipitated from the aqueous layer using isopropanol, followed by a subsequent wash and re-suspension into TRIzol® Reagent for use as a template for first strand cDNA synthesis. Complete protocol for RNA extraction is found at:  [http://tools.invitrogen.com/content/sfs/manuals/trizol_reagent.pdf](http://tools.invitrogen.com/content/sfs/manuals/trizol_reagent.pdf)

ii) RNA characterization, purity and integrity

RNA yield was quantified using a NanoDrop® ND-1000 spectrophotometer (Inqaba Biotech, South Africa) based on the convention that one absorbance unit at 260 nm equals 40 μg/ml RNA (Sambrook et al., 1989). Purity of the RNA was judged on the basis of A260: A280
ratio and its integrity and overall quality judged by inspection of the 28S and 18S ribosomal RNA (rRNA) bands on agarose gel.

**iii) First strand cDNA synthesis**

First strand cDNA was synthesized from total RNA of each sample (5µg) using the SuperScript™ III System for RT-PCR (Invitrogen, Burlington, Ontario, Canada). SuperScript® III Reverse Transcriptase is widely used because of its ability to reduce RNase H activity and provide increased thermal stability (Gerard et al., 1986; Kotewicz, 1985). Moreover, the enzyme is not significantly inhibited by ribosomal and transfer RNA and can be used to synthesize cDNA from total RNA. A complete protocol is found at:

http://tools.invitrogen.com/content/sfs/manuals/superscriptIIIfirststrand_pps.pdf

**iv) Semi-quantitative PCR analysis for mRNA expression of 3 β-HSD type I isoform and 17 β-HSD type I isozyme.**

Semi-quantitative PCR was performed according to the method described by Iemitsu et al. (2000). Each PCR reaction contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, dNTP at 200 mM each, and gene-specific primers:

- HSD3B-F (5′-GGGAGGAATTTTCCAAGCTC-3′),
- HSD3B-R (5′-CCAGAGGCTCTTCTTGATG-3′),
- HSD17B-F (5′-TGGGATCCCTAGAGCGTGTG-3′),
- HSD17B-R (5′-GACTCTCGCATAAGCCCTTC-3′) at 0.5 µM each, and 0.025 U/µl ExTq polymerase. The 3 β-hydroxysteroid dehydrogenase type I (Accession number: XM_002801725.1) and 17 β-hydroxysteroid dehydrogenase type I (Accession number: NM_001047132.1) used for designing primers were from a closely related species, *Macaca mulata*, selected from the regions that were seen to be highly conserved in this species.
vervet monkey genome has not been sequenced. The genes were identified from NCBI and gene-specific primers designed using primer 3 software (http://primer3.wi.mit.edu/). The designed primers were able to detect a specific isoform or isozyme found in the testes (type I). The PCR procedure was carried out using a PCR thermal cycler (GeneAmp PCR System 9700, ABI). The cycle profile included denaturation for 30 seconds at 94 °C, annealing for 1 min at 58 °C and extension for 1 min at 72 °C. These steps were repeated 40 times. Amplification products were separated on 1 % agarose gels stained with ethidium bromide and photographed by ScionImage UVP under UV light. Samples without reverse transcriptase were used as internal controls while samples without reverse-transcribed cDNA or without Taq gene were used as negative controls.

v) Real-time quantitative PCR analysis of mRNA expression of 3 β-HSD type I isoform and 17 β-HSD type I isozyme.

a) Optimizing the PCR Reaction for Real-Time Analysis

Before running the sample on the real-time thermal cycler, it was worthwhile to verify the specificity of the reaction by optimization in a regular thermal cycler without real-time analysis capabilities and product analysis by gel electrophoresis using 3 µl of amplicon (PCR product).

b) Real-time detection of PCR products

Real-time PCR incorporates traditional PCR thermal cycler technology with integrated fluorimeters and detectors that provide the ability to both excite a fluorochrome and detect the emitted light. In the current implementation, the excitation and detection channels took advantage of SYBR Green whose fluorescence increases 100- to 200-fold when bound to the
minor groove of double stranded DNA. The analysis was used to measure relative mRNA expression.

Each PCR amplification was performed in triplicate by 1 cycle of 95°C for 10 minutes and 40 cycles of 94°C for 15 sec and 60°C for 1 min. The expression of β-actin mRNA was used as an internal control. The quantitative values of 3 β-HSD type I and 17 β-HSD type I were normalized by that of β-actin mRNA expression.

6.1.2.4 Enzyme immunoassay for intracellular progesterone, androstenedione and testosterone

Enzyme immunoassay technique was employed to assay for progesterone, androstenedione and testosterone using ELISA kits from Nova Tec Immundiagnostica GMBH, Germany and protocols of the manufacturer were followed.

Figure 6-2. Vervet testicular tissues in incubation media (Minimal Essential Media).
6.1.3 STATISTICS

Values are expressed as mean ± SEM. Hormonal analysis was done using one-way ANOVA followed by Tukey's post hoc multiple comparisons test. Statistical significance was set at 5% level.
6.2 RESULTS

The present study examined the existence of steroidogenic enzymes (3β- HSD type I and 17β- HSD type I) in vervet testis. Consistent with the hypothesis, mRNA expression of 3β- HSD type I and 17β- HSD type I was detected in testis of vervet monkeys. The RNA that was extracted was of good yield based on the convention that one absorbance unit at 260 nm equals 40 μg/ml RNA (Sambrook et al., 1989). The purity of RNA for all samples was good following A260: A280 ratio and found to be in the range of 1.5 - 2 while the integrity and overall quality was judged by inspection of the 28S and 18S rRNA bands on agarose gel (Fig. 6-3). In this picture the 28S and 18S RNA bands appear prominent in animals 2148_1 and 2148_2 (controls). Animals at low dose (0.8 mg/kg body weight) of (-)-cathinone: 2107_1 showed a strong 28S and weak 18S while for animal 2107_2 both 28S and 18S RNA bands appeared strong. RNA of animal 2158_1 shows only 18S band while 2158_2 showed strong 28S and weak 18S. Animals treated with high dose (6.4 mg/kg) of (-)-cathinone (2155_1 and 2155_2) showed that both 28S and 18S RNA bands appeared strong (Fig 6-3). It is not very clear the cause of variance in RNA bands but it may be attributed to storage. Tissues of each animal were stored in separate vials in RNALater.

Optimization in a regular thermal cycler and product analysis by gel electrophoresis using 3 μl of amplicon showed good specificity of the reaction before performing real-time PCR (Fig. 6-4). Quantification of mRNA levels was done by real-time PCR. In order to detect the amplicon, the level of fluorescence signal was set sufficiently above background, that is, at cycle 16. This was informed by consideration of the detection limits of the equipment and background fluorescence due to the fluorescent chemistry used. The cycle at which the
threshold was met or exceeded, called the “cycle threshold” (CT), was used for making comparisons between samples. Standard curve analysis performed on the BioRad iQ5 real-time PCR instrument is shown (Fig. 6-5 a and b)

The results show that medium and high doses (3.2 and 6.4mg/kg body weight) of (-)-cathinone administered to animals (2158_1 and 2158_2; 2155_1 and 2155_2, respectively) significantly lowered (P < 0.05) while low dose (0.8 mg/kg body weight) of (-)-cathinone administered to animals (2107_1 and 2107_2) increased (P<0.05) mRNA levels of 3β- HSD type I enzyme expression (Fig. 6-6). Similarly, high doses of (-)-cathinone significantly suppressed (P < 0.05) mRNA expression of 17β- HSD type I while the effect of low dose of (-)-cathinone did not differ significantly from controls (Fig. 6-7). A clear comparison of cycle threshold of the expression of the two enzymes among different treatment groups and controls is shown (Table 6-2).

Finally, the study also sought to determine whether escalating doses of (-)-cathinone could affect hormone synthesis along the testosterone biosynthetic pathway. This was done by measuring levels of intracellular progesterone, androstenedione and testosterone in cultured interstitial cells of testes. Cultured interstitial cells exposed to luteinizing hormone (LH) standard showed increase in progesterone concentration while exposure to low dose (LD) and high dose (HD) of (-)-cathinone at 100 µl in the presence of LH showed a decrease in progesterone levels. This decrease in progesterone levels was significant with high dose of (-)-cathinone alone (Fig. 6-8). Animals exposed to low dose of (-)-cathinone in vivo showed increasing concentration of progesterone both in presence and absence of LH. Results also show a similar pattern for androstenedione release by cultured Leydig cells in presence of LH
alone, LH and LD and HD of (-)-cathinone and (-)-cathinone alone (Fig. 6-9). High doses of (-)-cathinone significantly suppressed (P<0.05) synthesis of testosterone in animals exposed to medium (2158_1) and high (2155_1) doses of (-)-cathinone of \textit{in vivo} studies. However, 2107_1 that was exposed to low dose of (-)-cathinone \textit{in vivo} showed increased testosterone levels following exposure to (-)-cathinone alone \textit{ex vivo} (Fig. 6-10).
**Figure 6-3.** Total RNA expression of steroidogenic enzymes in testis of vervet monkeys. Images on ethidium bromide-stained denaturing agarose gel showing integrity and quality of RNA extracted.

**Figure 6-4.** mRNA expression of steroidogenic enzymes in vervet testicular tissues. Representative images showing expression of 3β- HDS type I and 17β- HSD type I as revealed by RT-PCR. β-actin was used as an internal control.
Figure 6-5 a and b. mRNA expression of steroidogenic enzymes. Relative expression of 3β-HSD type I and 17β-HSD type I in vervet monkey testis (n = 8) as revealed by real-time quantitative PCR. Animals administered high doses of (−)-cathinone showed high cycle threshold for expression of genes for both enzymes compared to low dose of (−)-cathinone and controls. β–actin mRNA was used as an internal control for normalization.
Figure 6-6. mRNA expression of steroidogenic enzymes. Relative expression of 3β-HSD type I in testis of vervet monkeys (n = 8) as revealed by real-time quantitative PCR. β-actin mRNA was used as an internal control for normalization. Note a significant increase in mean cycle threshold in mRNA expression of 3β-HSD for medium and high dose (-)-cathinone subjects compared to controls and low dose (-)-cathinone subjects. (n = 8). ** means statistically significant at P<0.01, *** means statistically significant at P<0.001.
**Figure 6-7.** mRNA expression of steroidogenic enzymes. Relative expression of 17β- HSD type I in testis of vervet monkeys (n = 8) as revealed by real-time quantitative PCR. β-actin mRNA was used as an internal control for normalization. There was a significant suppression (P<0.05) of mRNA expression of 17β- HSD type I with medium and high dose of (-)-cathinone as shown by increase in mean cycle threshold on expression of this enzyme with the two doses. (n = 8). * means statistically significant at P<0.05, ** means statistically significant at P<0.01.
Table 6.2. Comparisons between samples based on “cycle threshold” (CT). √ means samples with low CT, X means samples with high CT. Note that most samples expressing 17β- HSD showed high CT compared to those of 3β- HSD indicating that mRNA expression of 17β-HSD was more suppressed by (-)-cathinone than was that of 3β- HSD.

<table>
<thead>
<tr>
<th>Sample</th>
<th>3B HSD</th>
<th>17BHSD</th>
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<tr>
<td>2158_1</td>
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<td>40</td>
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<tr>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>2158_2</td>
<td>27</td>
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<td>X</td>
<td>X</td>
</tr>
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Figure 6-8. Local steroidogenesis following addition of LH and (-)-cathinone to cultured interstitial cells. Results show that LH alone increased while LH and low or high dose of (-)-cathinone decreased progesterone secretion. The decrease was significant in presence of high dose of (-)-cathinone alone when compared to controls (n = 12). Control cells were incubated in media alone. Note that cells of animal exposed on low dose of (-)-cathinone (2107_1) showed relative increase in progesterone levels.
Figure 6-9. Local steroidogenesis following addition of LH and (-)-cathinone to cultured interstitial cells. Results show that LH alone increased while LH and low or high dose of (-)-cathinone decreased androstenedione secretion. The decrease was significant in presence of high dose of (-)-cathinone alone compared to controls (n = 12). Control cells were incubated in media alone. Note that cells of animal exposed to low dose of (-)-cathinone (2107_1) showed relative increase in androstenedione levels compared to high dose of (-)-cathinone.
Figure 6-10. Local steroidogenesis when LH and (-)-cathinone were added in cultured interstitial cells. Results show that LH alone increased while LH and low or high dose of (-)-cathinone decreased testosterone secretion. The decrease was significant in presence of high dose of (-)-cathinone alone compared to controls (n = 12). Control cells were treated with incubation medium alone. Note that cells of animal exposed on low dose of (-)-cathinone (2107_1) showed increase in testosterone levels in presence of LD and HD of (-)-cathinone.
6.3 DISCUSSION

The present study demonstrates, for the first time, the existence of 3β-HSD I and 17β-HSD I in vervet monkey testis and effect on their expression following (-)-cathinone exposure. Several previous studies have demonstrated expression of steroidogenic enzymes in various tissues. For example, 3β- HSD has been expressed in testis, ovary, adrenal gland and brain tissues (Zwain and Yen, 1999; Abbaszade et al., 1997) and 17β-HSD abundantly expressed in testis, ovary and brain (Payne and Hales, 2004; Zwain and Yen, 1999). Other studies also demonstrated existence of 3β-HSD, 17β-HSD and P450 aromatase cytochrome in rat skeletal muscle (Aizawa et al., 2007).

Results show that moderate and high doses of (-)-cathinone in animals (2158_1, 2158_2, 2155_1 and 2155_2) significantly suppressed (P<0.05) expression of mRNA of 3β-HSD type I in vervet testis while low doses of (-)-cathinone administered to animals (2107_1 and 2107_2) increased its expression. Previous studies showed that human 3β-HSD type II and mouse 3β-HSD type I and VI isoforms participate in active sex steroid biosynthesis in gonads (Abbaszade et al., 1997; Simard et al., 1996; Rheaume et al., 1991). These isoforms of 3β-HSD catalyze conversion of Δ5- 3β-hydroxysteroids, pregnenolone, 17α-hydroxypregnenolone and DHEA to the Δ4- 3-ketosteroids, progesterone, 17α-hydroxyprogesterone and androstenedione respectively, (Payne and Hales, 2004). Steroidogenic enzymes are characterized under multiple isoforms or isozymes and are expressed in cell and tissue-specific manner (Payne and Hales, 2004). The 3β-HSDs are membrane-bound enzymes distributed on mitochondria and microsomal membranes. From
the results of the present study it appears that 3β-HSD type I is expressed in the testis of the vervet monkeys and is homologous in amino acid sequence to that in humans (3β- HSD II) and mouse (3β- HSD I) and that all catalyze steroidogenesis. The results also showed that high dose of (-)-cathinone impair steroid biosynthesis and, therefore, by extension, reproductive capacity of an individual.

The results also showed expression of mRNA of 17β-HSD I in testicular tissue of the vervet monkeys and that the expression of this enzyme was suppressed by high dose of (-)-cathinone but not low dose. Previous studies in humans showed the exclusive involvement of 17β-HSD I, III and VII in the conversion of androstenedione to testosterone in gonads and not in adrenal glands (Payne and Hales, 2004; Andersson et al., 1995). These isozymes, however, differ in homology, tissue distribution, catalytic preferences, substrate specificity, subcellular localization and mechanism of regulation. From the foregoing, it appears the isozymes of 17β-HSD involved in human and vervet monkeys are similar. The 17β-HSDs convert inactive 17- ketosteroids into their active 17β- hydroxy forms. The finding on impairment of expression of mRNA of both 3β-HSD I and 17β-HSD I by high dose of (-)-cathinone is supported by the observed significant decrease in serum testosterone discussed in chapter 4 of this thesis even though testosterone hormone results did not show any disturbance in hypothalamo-pituitary integrity. This finding is supported by results on immunolocalization of pituitary trophs in chapter 5 of this thesis, where there was increase in labelling index of gonadotrophs with increasing doses of (-)-cathinone. To this end, interference along the hypothalamo-hypophyseal-gonadal axis following (-)-cathinone exposure seems to be localized at the gonadal level.
Escalating doses of (-)-cathinone in presence or absence of luteinizing hormone affected concentration of intracellular progesterone, androstenedione and testosterone differently. For instance, progesterone, androstenedione and testosterone levels were suppressed by high dose of (-)-cathinone while low dose of (-)-cathinone increased testosterone levels. Along the steroid biosynthetic pathway, pregnenolone synthesized from cholesterol is converted to progesterone in the presence of 3β-HSD. Similarly, dehydroepiandrosterone is converted to androstenedione in the presence of the same enzyme. Although not a subject of consideration in the present study, earlier studies by Gordon et al. (1980) provided evidence that alcohol inhibits Δ5- 3β-HSD via depletion of NAD⁺ during metabolism. In a similar study, alcohol and acetaldehyde significantly affected conversion of androstenedione to testosterone (Cicero and Bell, 1980). Quantification of 3β-HSD by real-time PCR performed in the present study indicate the suppression of this enzyme by high dose of (-)-cathinone and, therefore, confirms the observed decrease in progesterone and androstenedione measurements ex vivo. From the results intracellular testosterone levels were also affected by (-)-cathinone in cultured cells with low dose increasing while high dose suppressing its production. Overall, the quantification of the steroidogenic enzymes by real-time PCR correlated very well with data on hormone measurements ex vivo. In chapter 4 of this thesis it is shown that high dose of (-)-cathinone (6.4 mg/kg body weight) suppressed serum testosterone while low doses (0.8 and 1.6mg/kg body weight) and medium dose (3.2 mg/kg body weight) of (-)-cathinone increased its secretion consistent with the increase and then decrease in expression of 3β-HSD I as well as intracellular testosterone following low and high dose of (-)-cathinone. This biphasic effect of khat and (-)-cathinone on testosterone production was demonstrated in earlier studies in mice (Nyongesa et al., 2007) and rats (Mohammed and Engidawork, 2011). In another related study, Abdulwaheb et al. (2007) reported that low doses of khat extract (200 mg/kg/day)
enhanced sexual motivation while high doses of the extract (400 mg/kg/day) suppressed sexual motivation and performance in male rats. It was suggested that alteration of both dopamine (at low dose) and serotonin (at high dose) levels in central nervous system could explain the biphasic sexual behaviour of rats after khat administration (Abdulwaheb et al., 2007; Taha et al., 1995). From the foregoing, it appears (-)-cathinone directly affects conversion of pregnenolone to progesterone and androstenedione to testosterone _ex vivo_ and this effect is compounded _in vivo_ by the same mechanism.

Conclusively, the results of the present study strongly validate the assumption that changes in endocrine state are integrally involved in (-)-cathinone’s acute and chronic effects in many organ systems including reproductive system. The results have also shown that most of the reproductive complications reported in regular and long-term khat consumers are attributed to a greater extend to its localized effect on testicular function itself. These findings are significant since they are informative to the reproductive health implications in the chronic consumers of khat.
CHAPTER 7

MORPHOFUNCTIONAL EFFECTS OF CATHINONE ON VERVET MONKEY (CHLOROCEBUS AETHIOPS) TESTES FOLLOWING SUB-CHRONIC EXPOSURE

7.0 INTRODUCTION

The testicular parenchyma is broadly divided into numerous coiled seminiferous tubules and interstitium. The tubular epithelium consists of germ cells at various stages of development interspersed among Sertoli cells, and both cell populations depend on each other for the success of spermatogenesis. The testicular interstitium of primates, generally, comprise of wide areas of areolar connective tissue drained by large lymphatic vessels (Fawcett et al., 1973). The cellular elements of the interstitial tissue are many and varied; the most important and frequently encountered being the Leydig cell. Leydig cells of most species of animals studied are polygonal in shape with eccentric nuclei containing one or several nucleoli and numerous smooth and rough endoplasmic reticulum, abundant mitochondria, Golgi bodies and lipid droplets (Christensen, 1975). The ultrastructure and steroidogenic function of the Leydig cell have been described in several reviews (Ewing and Brown, 1977; Christensen, 1975; Hall, 1970; Christensen and Gillim, 1969). The steroidogenic enzymes responsible for converting cholesterol to testosterone are sequestered in mitochondria and smooth endoplasmic reticulum membranes of the Leydig cell (Zirkin et al., 1980). Any noxious
agents that interfere with these structures may impair the process of steroidogenesis and reproductive capacity of an animal or individual as a whole.

The process of spermatogenesis occurs within the seminiferous tubules of the testis. The germinal epithelia within these structures proliferate, differentiate and mature among Sertoli cells in a pattern that characterize the seminiferous cycle (Onyango et al., 2001). Premature germ cells undergo active mitotic division and exhibit the greatest dynamic transformation of chromatin. Within these forms of germ cells are abundant histones that are highly susceptible to noxious agents while maturation stages of sperm formation have highly condensed chromatin due to displacement of lysine-rich histones by small arginine and cysteine-rich protamines (Carlson, 1999). Insulting agents may, therefore, impair spermatogenesis by interfering with mitotic process of germ cells.

Khat and (-)-cathinone have been shown to have varying effects on the reproductive function in humans and experimental animals studied. Khat consumption in humans is most prevalent in males (Al Motarreb et al., 2002). It is due to this gender prevalence or bias of khat use that has necessitated most studies in males compared to females. Numerous reports on khat use show contradictory findings on male reproductive function. Impairment of sexuality (WHO, 1980; Halbach, 1972), inability to sustain erection (Elmi, 1983), loss of libido (Krikorian, 1984) and spermatorrhoea (Pantelis et al., 1989; Granek et al., 1988) have been reported. Khat extract and (-)-cathinone have also been shown to depress cell proliferation and inhibit RNA, DNA and protein synthesis in dividing cells leading to reduced spermatogenesis (Al-Meshal et al., 1989; De Hondt et al., 1984; Hammouda, 1971). In another separate study, feeding rabbits for 3 months with food containing different amounts of dried, ground khat
leaves stimulated spermatogenesis yet cauda epididymides and Leydig cells were unaffected when compared to controls (Al Mamary et al., 2002). This finding is similar to those in baboons where Leydig cells were unaffected by khat extract (Mwenda et al., 2006) but at variance with what was reported in the rat (Islam et al., 1990) where (-)-cathinone caused atrophy of Leydig and Sertoli cells as well as degeneration of spermatocytes and spermatids. Khat has also been reported to lower plasma testosterone levels in rabbits (Nyongesa et al., 2008). Similar studies by Nyongesa et al. (unpublished data) on effects of khat extract on rabbit testicular tissue revealed vacuolation in spermatogonia and zygotene spermatocytes while pachytene spermatocytes, spermatids and spermatozoa were unaffected. Vacuolation in seminiferous epithelium were reported in rats treated with heptachlor (Wango et al., 1997) and goats treated with ethane dimethanesulphonate (Onyango et al., 2001). From the foregoing, it appears khat and (-)-cathinone may have effects on testicular structure and function that affect reproductive performance. In the present study, it was hypothesized that (-)-cathinone causes structural alterations in the testis that influence changes in sexual responses and these variations may be species specific.

Species difference in testosterone secretion is attributed to some qualitative differences in Leydig cell structure and/or function (Ewing et al., 1979). The same investigators speculated that there might be a strong correlation between testosterone secretion and amount of membranous structures present in either the mitochondria and/or smooth endoplasmic reticulum (SER) of Leydig cells in different species. This view was confirmed by Zirkin et al. (1980) who reported a positive linear correlation between testosterone secretion and the volume density of SER in five mammalian species, including the rabbit. The present study was designed to investigate the effects of (-)-cathinone on volume density of membranous
structures present in mitochondria, SER, Golgi apparatus and lipid droplets as well as potential morphological changes in germinal epithelium affecting spermatogenesis. Understanding the function of testicular tissue requires reliable quantitative data describing the surface area and volume densities of cellular constituents of testis. Such data are needed for exact characterization of testicular function. Studies on effects of khat and (-)-cathinone on testicular ultrastructure and spermatogenesis in the vervet monkeys are scarce. This study was designed to elucidate through morphologic and morphometric means the hypothesis that seminiferous tubular epithelium and interstitial tissue of testis of vervet monkeys undergo transient changes following sub-chronic (-)-cathinone exposure thereby interfering with steroidogenesis and spermatogenesis.
7.1 MATERIALS AND METHODS

7.1.1 ANIMALS AND HOUSING

Eight males out of fourteen animals described under general materials and methods were used for this study. A detailed description is given in section 2.1 of general materials and methods.

7.1.2 EXPERIMENTAL DESIGN

This is same as was described previously in section 2.4 of general materials and methods. At the end of treatment phase 8 males were anaesthetized with 10 mg/kg body weight ketamine hydrochloride and aseptically castrated. One testis of each animal was used for this study (Fig. 7-1). The other testis was used for ex vivo studies discussed in the previous chapter 6.

7.1.3 TISSUE PROCESSING FOR TRANSMISSION ELECTRON MICROSCOPY

A little of 2.5% glutaraldehyde was injected beneath the tunica albuginea. The tissues were then immersed in sample bottles containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for electron microscopy. The testicular volumes were calculated using Scherle method (Scherle, 1970), also known as Water Immersion Volumetry. The whole testis of each animal was immersed in a jar containing phosphate buffer of known weight placed on a calibrated weighing balance (Fig. 7-2). The change in weight reading on the balance was taken to be the volume of the immersed testis (Fig. 7-3). This was based on the Archimede’s Principle that the buoyant force on a submerged object is equal to the weight of the fluid that is displaced by the object. The mean score on volume was taken after three successive readings of the same tissue. Testicular tissues were then trimmed into sufficiently small size (1 mm³) to permit proper fixation and processing (Fig. 4).
Standard techniques for electron microscopy processing were followed. Briefly, trimmed and fixed testicular samples were post-fixed with 4% Osmium tetroxide for about 2 h. Tissues were then rinsed in phosphate buffer for 15 min followed by dehydration in increasing concentrations of ethanol (20, 40, 50, 60, 70, 80, 90 and 100 [absolute]) three times every 15 min. Tissue blocks were then cleared using propylene oxide twice every 15 min followed by infiltration with epoxy resin mixture (EMS Sciences, UK) (13 ml Epon-812, 7 ml MNA, 8 ml DDSA and 16 drops of DMP-30) in the following proportions:

- Two parts of propylene oxide in 1 part of epoxy-resin mixture for 30 min
- One part of propylene oxide in 2 parts of epoxy-resin mixture for 30 min
- Infiltration in pure epoxy-resin mixture without accelerator (DPM-30) overnight.
- Finally, embedded in resin mixture as previously prepared, comprising of 100 ml resin mixture and 1.8 ml DMP-30 in plastic capsules and incubated in the oven at 60°C overnight for curing.

Gold sections (70 nm) were done using Sorvall® ultra microtome, placed on copper grids and left to dry. Grids were stained using lead citrate and uranyl acetate (EMS Sciences, UK) and viewed by means of transmission electron microscope. Selected areas were photographed and micrographs printed.

The volume densities (volume fraction) of mitochondria, smooth endoplasmic reticulum, Golgi apparatus and lipid droplets of Leydig cells were estimated from micrographs of central parts of testis photographed at x 6000 magnification. The volume density occupied by mitochondria, lipid droplets, Golgi apparatus and ‘membrane space’ occupied by smooth and rough endoplasmic reticulum membranes was evaluated by differential point counting as
earlier described (Kavoi et al., 2012; Weibel, 1979). Briefly, a transparent test grid with a square lattice of points was overlaid with random positioning on testicular electron micrographs projected on a computer monitor. The number of points hitting the structures of interest (mitochondria, smooth endoplasmic reticulum, lipid droplets and Golgi apparatus) and those falling on the projected field of testicular interstitial tissue were counted. Here, the volume density of component of interest \([V_v (l)]\) was calculated as a percentage using the following formula:

\[
V_v (l) = \left( \frac{\Sigma N_P l}{\Sigma N_P i} \right) \times 100,
\]

Where, \(V_v (l)\) is volume density of cell component, \(\Sigma N_P l\) is the number of test points hitting the image of the evaluated component of interest, and \(\Sigma N_P i\) is the number of all points falling on the cell image (interstitium).

By substituting in the equation above, volume densities of mitochondria, smooth endoplasmic reticulum, lipid droplets and Golgi apparatus used the following formulae, respectively:

\[
V_v (m) = \left( \frac{\Sigma N_P m}{\Sigma N_P i} \right) \times 100
\]

\[
V_v (s) = \left( \frac{\Sigma N_P s}{\Sigma N_P i} \right) \times 100
\]

\[
V_v (l) = \left( \frac{\Sigma N_P l}{\Sigma N_P i} \right) \times 100
\]

\[
V_v (g) = \left( \frac{\Sigma N_P g}{\Sigma N_P i} \right) \times 100
\]
Figure 7-1. Asceptic surgery of testes of the vervet.

Figure 7-2. Weight reading on the weighing balance before immersion of testis
**Figure 7-3.** Weight reading after immersion of testis
7.1.4 STATISTICAL ANALYSIS

Morphometric data from each animal was averaged per experimental group and standard error of means determined. Morphometric data was analysed using one way ANOVA and comparisons in means among groups performed using Tukey’s multiple comparisons post hoc test. Statistical significance was set at $P < 0.05$. 

Figure 7-4. Testicular tissue trimming for immersion in fixative
7.2 RESULTS

The interstitial tissue of testis comprised of Leydig cells, generally pleomorphic in shape but mostly polyhedral or polyangular in outline, among which were other cells including macrophages, fibroblasts and a few lymphatic vessels. In control animals, Leydig cell cytoplasm showed abundant sub-cellular organelles including numerous smooth and rough endoplasmic reticulum, spherical or elongate mitochondria, Golgi apparatus and a few lipid droplets (Fig. 7-5 A). Experimental animals on low dose (-)-cathinone (0.8 mg/kg and 1.6 mg/kg) presented similar findings with an increase in number of lipid droplets (Fig. 7-5 B). In experimental animals on medium (3.2mg/kg body weight) (Fig. 7-5 D) and high dose (6.4mg/kg body weight) of (-)-cathinone (Fig. 7-5 E and F), there were variations in these organelles. There was ballooning of mitochondria with evidence of disrupted mitochondria cristae and in some cases signs of degeneration of mitochondria were observed. Some mitochondria also appeared to be engulfing some material as shown by asterisk in Figure 7-5 F. Rough endoplasmic reticulum appeared disorganized as opposed to a regular pattern of arrangement in controls. At high dose of (-)-cathinone smooth endoplasmic reticulum and lipid droplets were rare. Table 7-1 shows a summary of the morphometric data on volume densities of various sub-cellular organelles that were evaluated in Leydig cells. These are mean values obtained from data scoring at three different fields on the same testicular section. The stereologic data showed that medium and high dose of (-)-cathinone significantly (P<0.05) altered the morphology of organelles involved in steroidogenesis, primarily: SER, RER, Golgi apparatus, mitochondria and lipid droplets (Fig. 7-6).

The seminiferous epithelium showed two populations of cells: Sertoli cells and germ cells at different stages of development. Spermatogonia were found along the basal lamina of
seminiferous tubules next to Sertoli cells (Fig. 7-7 A-D). (-)-Cathinone affected these cells differently. For instance, at high dose the nuclear membrane integrity of spermatogonia was disrupted (Fig. 7-7 D) compared to controls (Fig. 7-7 C). The chromatin material of spermatogonia at high (-)-cathinone dose appeared condensed, indicative of degeneration. Primary spermatocytes that were characterized by condensed chromatin in spherical nucleus were also affected by (-)-cathinone (Fig. 7-8 A-D). The results showed vacuolation in cytoplasmic organelles of spermatocytes at low (Fig. 7-8 B), medium (Fig. 7-8 C) and high (Fig. 7-8 D) dose of (-)-cathinone. The intensity of vacuolation was highest with high dose. Similarly, there was also evidence of decreased cytoplasmic mass in primary spermatocytes in (-)-cathinone- treated animals compared to controls. At high dose of (-)-cathinone the nuclear material appeared more condensed or disorganized. Similar vacuolations were observed in pachytene spermatocytes (Fig. 7-9). At low (Fig. 7-9 B), medium (Fig. 7-9 C) and high (Fig. 7-9 D) doses of (-)-cathinone, there were numerous vacuolations accompanied by displacement of the nucleus to the periphery of the cells. At the high dose most of the cytoplasmic organelles were displaced by an amorphous material and the nuclei shrunk. The cell membrane appeared irregular (Fig. 7-9 D).

The findings of this study also showed a marked effect on the process of spermiogenesis itself as shown (Fig. 7-10 A-D). The figures showed acrosomal phase spermatids characterized by presence of acrosome granule within the acrosome vesicle on one end of the nucleus of round spermatids similar to controls (Fig. 7-10 A). (-)-Cathinone interfered with spermiogenesis as evidenced by appearance of acrosome granule outside acrosome vesicle at low (-)-cathinone dose (Fig. 7-10 B), loss of the acrosome granule at medium dose (Fig. 7-10 C) and regression of the nucleus and disorganization of acrosome vesicle without granule at high (-)-cathinone
dose (Fig. 7-10 D). At these doses the cytoplasmic organelles appear disorganized and few while the general shape of the cells bear irregular outline. This is indicative of cellular degeneration. Finally, elongate spermatids appear to have been affected in a similar pattern like round spermatids. In controls (Fig. 7-11 A), elongate spermatids had a spear-shaped nucleus covered by the acrosomal membrane with flagella lined by a sheet of mitochondria. At low dose of (-)-cathinone (Fig. 7-11 B) similar features were observed. However, moderate (Fig. 7-11 C) and high dose (Fig. 7-11 D) of (-)-cathinone interfered with spermiogenesis. This was evidenced by oblong shapes of elongate spermatids with no signs of flagella formation. The spermatids appeared clumped together as a sign of possible phagocytosis by Sertoli cells.
Figure 7-5. Vervet monkey Leydig cell ultrastructure of control (A), (-)-cathinone-treated animals at 0.8mg/kg (B), 1.6mg/kg (C and D), 3.2mg/kg (E), 6.4 mg/kg (F). Note abundance of mitochondria (m), smooth endoplasmic reticulum (Se) and well arranged rough endoplasmic reticulum (arrow) in controls. At low dose (B), rough endoplasmic reticulum were few and disorganized. Lipid droplets (Ld), Golgi apparatus (arrow head) and membrane Whorls (w) were observed at medium dose (C and D). Mitochondria appeared to be losing integrity of inner cristae and in some areas degenerating (star) and engulfed by other mitochondria (D and E). Rough endoplasmic reticulum were few and appeared scattered. (n = 5). Bar = 0.5 µm in A-F.
**Figure 7-6.** A graph showing mean volume densities (%) of mitochondria, lipid droplets (LD), Golgi apparatus (GA), smooth (SER) and rough endoplasmic reticulum (RER) in Leydig cell of control and (-)-cathinone-treated vervet monkeys. Note a significant (P<0.05) decrease in volume densities of these organelles at dose 3.2 and 6.4 mg/kg body weight (n = 5).
Table 7-1. Mean volume densities of various organelles in Leydig cells of vervet monkey testis. SER - smooth endoplasmic reticulum, RER - rough endoplasmic reticulum, * - significant difference at 5% level, (n = 5).

<table>
<thead>
<tr>
<th>Mean volume densities (%)</th>
<th>(-)-Cathinone dose (mg/kg body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>11.96±1</td>
</tr>
<tr>
<td>Lipid droplets</td>
<td>4.12±0.4</td>
</tr>
<tr>
<td>SER</td>
<td>8±1.2</td>
</tr>
<tr>
<td>RER</td>
<td>9.47±0.7</td>
</tr>
<tr>
<td>Golgi apparatus</td>
<td>5.49±1.3</td>
</tr>
</tbody>
</table>
Figure 7-7. Vervet monkey Sertoli cell of controls (A) and test animals (B-D). There were no morphological differences in Sertoli cell among treatment groups compared to controls. However, (−)-cathinone caused morphological changes in early spermatocytes (Sp) at dose 3.2 mg/kg (C) and 6.4 mg/kg (D). Infiltration of material in cytoplasm of spermatocyte (arrow) interfered with integrity of nuclear membrane (n). Spermatogonia type A (Sa) are present. (n = 5). Bar = 0.5 µm in A-D
Figure 7-8. Spermatocytes of vervet monkeys showing controls (A) and (-)-cathinone-treated groups (B - D). Note cytoplasmic vacuolations (v) in treated animals. Some spermatocytes appear to lose most of the cytoplasmic content (C and D) at dose 3.2 and 6.4 mg/kg body weight, respectively. Chromatin material in the nucleus (n) of spermatocytes in C and D appear disorganized. (n = 5). Bar = 0.5 µm in A-D.
Figure 7-9. Spermatocytes of vervet monkeys showing controls (A) and (-)-cathinone-treated groups (B-D). Numerous cytoplasmic vacuolations (v) accompanied by disorganization of nuclear membrane in treated groups was observed. At high dose (6.4mg/kg) (D) nucleus (n) appear shrunken and cytoplasm is devoid of most of organelles with disrupted cell membrane. Chromatin material in the nucleus appeared more condensed indicative of degenerative changes. (n = 5). Bar = 0.5 µm in A-D.
Figure 7-10. Round spermatids of vervet monkeys in acrosomal phase of controls (A) and treated animals (B – D). (-)-Cathinone interfered with acrosome formation with low dose (B) showing acrosome granule (arrow) outside acrosome vesicle (V) while at medium (C) and high dose (D), there was complete granule loss and shape of acrosome vesicle appear distorted. The nucleus (n) appeared shrunken at high dose (D). (n = 5). Bar represents 0.5 µm in A - D.
Figure 7-11. Elongate spermatids of vervet monkeys. Controls (A) and low dose (0.8 mg/kg) of (-)-cathinone (B) shows spermatids with intact acrosome (arrow) and developing flagella with emerging mitochondrial sheet (m). At dose 3.2 mg/kg (C) and 6.4 mg/kg (D), normal sperm development is impaired. Note aggregation of spindle-shaped spermatids with few mitochondria next to disorganized round spermatid (Rs) in C. At medium and high doses of (-)-cathinone spermatids appear tailless with no signs of flagella formation. (n = 5). Bar = 0.5 µm in A-D.
7.3 DISCUSSION

The results of the present study show that high doses of (-)-cathinone at sub-chronic exposure to vervet monkeys resulted in alteration of sub-cellular organelles in Leydig cells implicated in steroidogenesis. This is principally due to decrease in SER, mitochondrial cristae, lipid droplets and Golgi apparatus. The decrease in SER may be a result of inhibition of formation of new SER membranes at high doses of (-)-cathinone following breakdown of old ones. Gonadotropins have been shown to play a crucial role in maintenance of protein synthesis (Rommerts and Brinkman, 1981). These findings are supported by those on hormonal studies reported in chapter 4 and immunohistochemical localization of pituitary cells reported in chapter 5 of this thesis. Hormonal results showed a dose- and time-dependent upregulation of LH while serum testosterone showed a biphasic pattern of secretion following (-)-cathinone exposure with low dose increasing while high dose decreased its production. Immunolocalization studies of pituitary cell types reported in chapter 5 showed an increase in gonadotrophs following administration of high dose of (-)-cathinone. The increase was significant in animals co-treated with GnRH agonist indicating that (-)-cathinone did not impair the integrity of hypothalamus or pituitary gland on this measure. In chapter 6 of this thesis, it is reported that at high dose of (-)-cathinone 3β- HSD I and 17β- HSD I were impaired. Since these enzymes play a crucial role in steroidogenesis (Payne and Hales, 2004), interference in their gene expression is indicative of impaired steroidogenesis. Further tests done on ex vivo measurement of intracellular testosterone of cultured interstitial cells showed decrease in testosterone levels. From these findings, it seems likely that (-)-cathinone interfered with steroidogenesis at the gonadal level. The stereological data concerning SER and mitochondria fit well with the results in chapter 6 which indicate adverse effects of high
dose and sub-chronic exposure of cathinone on steroidogenic enzymes. These enzymes are located on membranes of SER and mitochondrial cristae (Mazzocchi et al., 1982; Nussdorfer et al., 1980; Tamaoki, 1973). Therefore, decrease in number of SER and mitochondria coupled with ballooning effect of these mitochondria leading to disorganization of mitochondrial cristae may have contributed to the observed decrease in testosterone production at high dose of (-)-cathinone.

The results of the present study also showed decrease in lipid droplets following sub-chronic exposure to (-)-cathinone. This finding also points towards effect of (-)-cathinone on steroidogenesis. Cholesterol, a product stored in lipid droplets (Moses et al., 1969) is precursor of steroid hormones (Mazzocchi et al., 1982). On the same note, it is possible that the observed reduced serum testosterone levels in chapter 4 of this thesis was partly due to depletion of lipid droplets in Leydig cells following high dose (-)-cathinone exposure. The SER are involved in synthesis of cholesterol from acetate and glucose (Christensen, 1975). Similarly, reduction in the amount of Golgi apparatus in the present study is also a pointer towards impairment of steroidogenesis. A large body of evidence suggest that smooth endoplasmic reticulum and Golgi apparatus are integral in steroidogenesis (Mazzocchi et al., 1982; Christensen and Gillim, 1969). Overall, the findings of the present study on Leydig cell morphology are consistent with those in rats (Islam et al., 1990). However, it is difficult to make a logical comparison between findings of the present study and those in baboons (Mwenda et al., 2006) since in the latter case Leydig cell ultrastructure was not considered.
From the foregoing, it is reasonable to conclude that (−)-cathinone at high dose resulted in structural alterations of steroidogenic organelles that interfered with their function primarily at the enzyme level. These structural alterations of steroidogenic structures represent decreased steroidogenic enzyme expression and, therefore, testosterone synthesis at high dose of (−)-cathinone and following long-term exposure. The findings are consistent with those in earlier studies that reported cytotoxic effects of (−)-cathinone in relation to androgenic deficiency in mice (Tariq et al., 1987).

In the present study sub-chronic exposure to high doses of (−)-cathinone had adverse effects on developing germ cells in the seminiferous tubules of vervet monkeys, pointing towards impairment of spermatogenesis. The results show interference of germ cell proliferation, differentiation and maturation. The disorganized shape of spermatogonia as shown by cell membrane outline as well as vacuolation in primary and secondary spermatocytes, all point to degeneration in these cells. Previous studies by Nyongesa et al. (unpublished data) showed vacuolation in premature germ cells of rabbit testis following administration of 40.5 g/kg body weight of khat extract. It is argued that these premature germ cells are highly susceptible to noxious agents due to the abundance of histones in the chromatin material compared to mature forms that contain highly condensed chromatin due to argentine and cysteine-rich protamines (Carlson, 1999). Vacuoles in seminiferous epithelium are frequently encountered following impaired spermatogenesis. Similar vacuolations were reported in rats treated with heptachlor (Wango et al., 1997) and goats treated with ethane dimethanesulphonate (Onyango et al., 2001). There is also evidence of impairment in nuclear function as shown by irregular outline of nuclear membrane in primary and secondary spermatocytes. The volume of cytoplasm in these cells is generally reduced. Round spermatids also show peripheral
margination of chromatin material. All these occurrences are suggestive of impairment of cell division and development (Onyango et al., 2001). The findings of the present study compare favourably with those of previous investigators which showed depressed cell proliferation and inhibition of RNA, DNA and protein synthesis in actively dividing cells (Al-Meshal et al., 1989; De Hondt et al., 1984; Hammouda, 1971). Other studies reported effects of khat on impairment of spermatogenesis in roosters (Hammouda, 1978), mice (Qureshi et al., 1988), rat (Islam et al., 1990), and humans (El-Shoura et al., 1995).

The acrosomal phase of spermatids also showed defective development. Some (-)-cathinone subjects displayed displacement of acrosome granule from acrosome vesicles while in others the acrosome granule was absent altogether. At high dose the nucleus of these round spermatids was markedly reduced in size. This finding is supported by reports in rats where (-)-cathinone enantiomers caused degeneration of spermatocytes and spermatids (Islam et al., 1990). Results also showed that elongate spermatids of control animals had spear-shaped condensed nucleus and a flagellum with a mitochondrial sheet along mid and principal piece indicative of normal process of spermiogenesis (Johnson et al., 1970). Elongate spermatids of moderate and high (-)-cathinone dose groups appeared oblong in shape and showed tailless heads with no centrioles. The elongate spermatids appeared clumped together within sleeve-like pockets of Sertoli cells, possibly for subsequent phagocytosis.

The results of the present study show that the Sertoli cell ultrastructure was unaffected by (-)-cathinone administration to vervet monkeys. The ultrastructure of Sertoli cells revealed large multi-lobed (4 – 5) nuclei with prominent nucleoli in the basal region of the cytoplasm and dispersed heterochromatin and eurochromatin consistent with findings of Lebelo and Van der
Horst (2010). This is at variance with the findings of Islam et al. (1990) where (-)-cathinone caused degeneration in Sertoli and Leydig cells of rats. It is not clear whether the variance in findings is attributed to species differences or research protocol employed. Sertoli cells play a very crucial role in the process of spermatogenesis including: phagocytosis, spermiogenesis, formation of blood-testis barrier, spermiation and production of some regulatory proteins (Russell and Griswold, 1993; Johnson, 1991; Tindall et al., 1985). From the results of this study, the impaired spermatogenesis was a direct effect of (-)-cathinone on developing germ cells themselves and not as a secondary effect of Sertoli cells.

In conclusion, the findings of this study have shown that (-)-cathinone, at high dose and long-term exposure, has adverse effects on testicular function with particular reference to spermatogenesis and steroidogenesis. It appears from these findings that regular and long-term consumption of khat interferes with the reproductive capacity of an individual through direct effect on both processes, which seem to influence each other. It is, however, not clear whether these effects of (-)-cathinone on gonadal function are irreversible. Further research need to consider investigations on reversal effects of (-)-cathinone upon withdrawal from long-term exposure as the direction for future studies.
CHAPTER 8

8.1 GENERAL DISCUSSION

This thesis highlights the possible mechanism of action of (-)-cathinone on behavioural and reproductive function in vervet monkeys. Behavioural studies were undertaken by direct scoring of specific indicators of those behaviours captured by video camera and related to effects on endocrine function by measurement of hormonal profiles. Reproductive function studies considered both hormonal and morphological interplay of structures along the hypothalamo-pituitary-adrenocortical and gonadal axes influencing these measures. The results showed a clear connection between the nervous and endocrine systems and their relationship with target organs. These findings provide an indirect in-sight on the impact of long-term khat use by human ‘khat addicts’ on the behavioural and reproductive health aspects.

Behavioural findings indicated that (-)-cathinone caused an increase in aggressive and abnormal behaviour and anxiety in a dose-dependent manner both in presence and absence of cage enrichment. Durational behaviours of latency to eat and withdrawal as indexed by jumping on to the platform within the cage or towards a corner similarly increased with higher doses of (-)-cathinone. Since the characteristic property of khat consumption is psychostimulation and attainment of euphoria, the behavioural pharmacology of khat has attracted attention of many investigators. Earlier studies reported stereotyped behaviour, self-administration and anorectic effects in animal species (Gordon et al., 1993, Calcagnetti and Schechter, 1992) following khat or (-)-cathinone exposure. These findings were similar to those evoked by [S-(+)-amphetamine] (Goudie, 1985). In related studies, both khat extract
and (−)-cathinone showed enhanced baseline aggressive behaviour in isolated rats (Banjaw et al., 2005). Investigators have linked these behaviours to disorders in the hypothalamic dopaminergic system (Ishikawa et al., 2007) as well as dopaminergic and serotonergic activity in the mesolimbic system (Eisch and Harburg, 2006, Jones and Bonci, 2005). Biogenic amines such as dopamine and serotonin have been shown to have modulatory effects on specific behaviours (Spoont, 1992). For example, khat and (−)-cathinone induced stereotyped movement, aggression and sexual arousal (Abdulwaheb et al., 2007; Banjaw et al., 2006; Connor et al., 2002). The findings of the present study indicate a possible involvement of serotonergic system on these measures.

The increased latency in food intake observed in the present study is best characterised as consummatory rather than appetitive behaviour. Two models have been proposed to explain reduction in food intake following exposure to psychostimulants (Wolgin and Munoz, 2006). Here, appetitive behaviour is defined as the suppression of food intake as evidenced by loss of ability to seek food while consummatory behaviour is failure to eat available food. In the present set up, animals were supplied with food but responded differently to its presence. However, most available findings in literature highlight on anorectic effects of khat and (−)-cathinone. For example, previous studies showed induction of anorexia in rhesus monkeys (Foltin and Schuster, 1983), rats (Eisenberg et al., 1987; Foltin et al., 1983), pregnant guinea pigs (Jansson et al., 1988) and humans (Murray et al., 1998) following treatment with (−)-cathinone and cathine. Noradrenergic, dopaminergic and opioid systems have been implicated in khat/(−)-cathinone-induced analgesia and anorexia (Connor et al., 2000; Della Bella et al., 1985; Nencini et al., 1984). The findings of the present study may, in part, be explained by these anorectic effects of khat and (−)-cathinone that led to the observed consummatory
behaviours. It is possible (-)-cathinone caused these effects via involvement of central dopaminergic and serotonergic as well as peripheral noradrenergic systems.

In the present study, (-)-cathinone had pronounced effects on hypothalamo-hypophyseal-adrenocortical and gonadal axes as evidenced by alterations in hormonal profiles. These alterations of hormonal concentrations are explained by varied immunolocalization of anterior pituitary cell types secreting these hormones, testicular morphological changes and stereodogenic function that have been discussed in this thesis. There was a dose-dependent increase in LH and decrease in serum cortisol, prolactin and progesterone levels. Serum testosterone showed a biphasic pattern with low doses of (-)-cathinone increasing while high dose decreasing its production. The increase in LH at high dose and decrease of testosterone at the same dose points towards susceptibility of testicular steroid forming structures to (-)-cathinone. Earlier studies showed gonadotropin-independent decrease in plasma testosterone levels via exogenous cortisol and dexamethasone administration (Welsh et al., 1981). In humans, a rise in plasma testosterone with no corresponding increase in LH was observed during exercise (Murray et al., 1998). Gonadal effects of (-)-cathinone in the present study are clearly demonstrated. Results showed that high doses of (-)-cathinone suppressed expression of hydroxysteroid dehydrogenases involved in testosterone biosynthesis. Mitochondrial cristae and smooth and rough endoplasmic reticulum were structurally altered by high dose of (-)-cathinone as indexed by their volume densities. Ex vivo measurements of intracellular hormones found along testosterone synthesis pathway showed decreased androstenedione, progesterone and testosterone in a dose-dependent manner confirming effect of (-)-cathinone on 3\(\beta\) HSD and 17\(\beta\) HSD.
In this thesis, results on correlation of hormones are presented. There was a strong positive correlation between cortisol and prolactin during treatment phase suggesting that the mode of action of (-)-cathinone to the two systems producing these hormones may be similar and appear to influence each other. Studies have shown that activation of the hypothalmo-hypophyseal-adrenocortical axis and ascending catecholaminergic neurons play an important role in metabolic and behavioural adaptation to stress (Ishikawa et al., 2007). The decreased cortisol levels following (-)-cathinone administration in the present study may reflect the situation in humans, who chew khat to achieve a state of pleasure, euphoria and elation (Berman et al., 2009). Some of the observed behaviours in the present study may be correlated to hormonal alterations. For example, increasing levels of serum testosterone with (-)-cathinone following low and medium dosing may explain the aggressive behaviour observed. In primates, the correlation between circulating testosterone and aggressive behaviour is tenuous (Alberts et al., 1992; Bernstein et al., 1979).

Results also show disrupted spermatogenesis and steroidogenesis following (-)-cathinone exposure. (-)-Cathinone affected all stages of germinal epithelial development indicating its cytotic effects (Tariq et al., 1987). On the other hand, Sertoli cells were unaffected implying that interference of spermatogenesis was due to (-)-cathinone effects on germ cells but not as a result of failure of the supportive role of Sertoli cells. Since testosterone is involved in spermatogenesis, its low levels at high (-)-cathinone dose in the present study may have also contributed to disruption of spermatogenesis. From the aforementioned, it appears the effects of (-)-cathinone on sexual function are compound and involve the hypothalmo-hypophyseal-adrenocortical and gonadal axes and not as simple as it is literally thought.
The study encountered some limitations that may have possibly influenced the results. The methodology was aimed at modelling an experimental animal to emulate the situation in human khat chewers. The present study used a purified (-)-cathinone instead of use of whole fresh leaves and twigs as is normal the case with human khat chewers. The observed effects in khat chewers are likely a combination of all compounds of khat and multiple exposures. It is, therefore, possible that the effect of cathine and norephedrine and other alkaloids extracted into saliva following chewing were not considered. In the present study, it was not possible for monkeys to chew on the leaves but instead (-)-cathinone was given as a bolus via gastric gavage. In this study, it was not possible to obtain serial blood collection intravenously from awake animals and so animals were anaesthetized each time of (-)-cathinone administration and blood sampling. It is not certain whether ketamine influenced the observed changes in hormonal profiles. However, animals were maintained on ketamine at 2.5 mg/kg body weight, a dose that is sufficient to immobilize the animals without causing narcosis (Rizvi et al., 2000).

The insignificant effect on hormone profiles over time and dose by time interaction as well as some individual variation in behavioural parameters of vervet monkeys suggest that larger sample size is required in such studies. The small sample size per group used in the present study did not allow for a clear follow up on statistics of dose by time interaction on hormone profiles and morphological evaluation by treatment groups. Finally, the choice of (-)-cathinone doses was informed by what has been used in humans. However, this did not consider the metabolic rate of the vervet monkeys as a factor to the pharmacokinetics of the drug in the body and this may have led to the discrepancy in the results observed.
8.2 CONCLUSIONS

The thesis presents results of (-)-cathinone on behavioural and reproductive function in vervet monkeys. These findings are summarized as follows:

- Results on behavioural and endocrine function showed the involvement of hypothalamo-hypophyseal-adrenocrotical and gonadal axes. The effects of (-)-cathinone on hormones seem to influence, to a great extent, different behaviours suggesting that there is an interplay between endocrine and meso-corticolimbic systems.

- (-)-Cathinone has a selective action on different pituitary cell types. In this thesis a dose-dependent increase in immunolocalization of gonadotrophs whose expression was enhanced by co-treatment with GnRH agonist goserelin acetate has been reported. The results have demonstrated, for the first time, that the synthesis and secretion of GnRH as well as the responsiveness of the gonadotrophs to its presence is not affected by (-)-cathinone. However, the population of lactotrophs and corticotrophs reduced with increasing doses of (-)-cathinone.

- The results have shown that (-)-cathinone enhances LH production but suppresses prolactin, progesterone and cortisol production in a dose-dependent manner. There was a biphasic effect of (-)-cathinone on testosterone synthesis with low and medium doses enhancing while high dose suppressing its production.

- The study has also demonstrated, for the first time, the expression of 3β-HSD I and 17β-HSD I in vervet monkey testis. The results showed that (-)-cathinone has a direct effect on gonadal function with low and medium doses enhancing while high dose interfering with expression of 3β-HSD I and 17β-HSD I. Similarly, low and medium
doses of (-)-cathinone did not have significant effect on morphology of steroidogenic tissue while high dose led to morphological alterations in smooth endoplasmic reticulum, lipid droplets, mitochondria cristae and Golgi apparatus of Leydig cells leading to disruption of steroidogenesis. This explains the biphasic effect of (-)-cathinone on testosterone production reported in this thesis.

- Results also showed that (-)-cathinone interferes with seminiferous germ cell development at all stages of growth and development thereby leading to impairment of spermatogenesis.

Overall, the results of the present study point towards the possible mechanism of action through which (-)-cathinone influences behavioural manifestations and impairs reproductive function. The observed increase in aggression could have been the function of testosterone which peaked at medium dose of (-)-cathinone. The decreased population of lactotrophs and corticotrophs explains observed reduced prolactin and cortisol hormones respectively, and these may have influenced anxiety and abnormal behaviour to some extent. The reduced expression of 3β- HSD I and 17β- HSD I as well as morphological alterations in the steroidogenic tissue at high dose of (-)-cathinone demonstrates interference of catalytic activity of these enzymes leading to low testosterone production. This possibly contributed to the observed impaired spermatogenesis although (-)-cathinone also appeared to have a direct effect on the germinal epithelia itself.

8.3 FUTURE GOALS

The future direction on khat research is to determine whether the observed effects on gonadal function particularly on the processes of spermatogenesis and steroidogenesis are reversible.
by considering the withdrawal period following long-term exposure. There is also need to focus on the possible cause of addiction following concomitant use of khat and other drugs of abuse such as cannabis and tobacco and whether or not the observed behaviours in the chronic user are a product of a combination of khat and other substance abuse or that these other drugs boost the effects of khat. Quite frequently, khat addicts concurrently use other substances especially tobacco. There is also need to model and animal to simulate the actual chewing process of khat as it occurs in humans. One possibility is to identify sweetener that can be mixed with khat leaves so that experimental animals are able to chew on them rather than administration via other routes. There was also need to consider mating as a significant behaviour in the study. There is also need to measure the levels of cathinone directly in blood as an indicator to the effects observed in the animal model. These are gaps for future consideration.
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*Endocrinology* **145**: 5202 - 5209.


APPENDIX 1

1.0 UNITS

µl  Microlitre
µ   Micron
µg  Microgram
µm  Micrometer
ml  Milliter
IU  International Units
g   Gram
Kg  Kilogram
%  Per cent
0°C  Degrees Celcius
pH  The negative log of H⁺
mM  Milmole
nmol/l Nanomole per liter
mm  Milimeter
mg  Milgram
cm  Centimeter
mm³  Cubic milimeter
APPENDIX 2

2.0 ABBREVIATIONS

H  Hour
Min  Minute
Sec  Second
SD  Standard deviation
SEM  Standard error of mean
cDNA  Complementary deoxyribonucleic acid
RNA  Ribonucleic acid
UK  United Kingdom
Ltd  Limited
O₂  Oxygen
CO₂  Carbon dioxide
N  Normality
G  Gauge
P  P value
Rₚ  Refractory Index
β  Beta
Δ  Delta
DMSO  Dimethyl sulphoxide
ANOVA  Analysis of Variance
APPENDIX 3

3.0 PREPARATION OF REAGENTS

3.1 0.1 M PHOSPHATE BUFFER SALINE, pH 7.2

A stock solution of 0.1 M phosphate buffer was prepared by dissolving 17.6 g Na₂HPO₄.H₂O, MW 178.05 in 500 ml double distilled water to make first stock solution. A second stock solution was prepared by dissolving 13.8 g Na₂HPO₄.H₂O, MW 138.01 in 500 ml double distilled water. The final stock solution of 0.1M was made by mixing 360 ml of the first solution with 140 ml of second solution and diluting it to 1 litre with double distilled water to give 0.1M phosphate buffer saline pH 7.2.

3.2 4% OSMIUM TETROXIDE (OsO₄)

Into a flask containing 25 ml double distilled water, 1 g of Osmium tetroxide was added, shaken vigorously and flask wrapped with aluminium foil and stored at 4°C until required for use.

3.3 Modified Minimum Essential Media (MEM)

Add 2 g Sodium bicarbonate in a flask containing 1 liter of double distilled water. Ajust the pH to between 7.2 and 7.4 and incubate for 5 days at 37°C in 3-5% CO₂ to check for sterility. The media is then filtered and store at -4°C for use.