Sudan University of Science and Technology
College of Graduate Studies

Effect of soybean lecithin-based semen extender on motility of the chilled and frozen semen in Nubian bucks

تاثير مخفف ليسيثين فول الصويا علي حركة النطف في السائل المنوي المبرد والمجمد للتيوس النوبية.

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By

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بسم الله الرحمن الرحيم

قال تعالي:-

(اولم يروا انا خلقنا لهم مما عملت ايدينا انعاما فهم لها مالكون)

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DEDICATION

To my dear family Father, mother, sister, husband and daughter.

To my dear friends and colleagues

With love and respect

Yasmin

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All my praise and unlimited thanks are to Allah, the almighty, for his help and giving me health and patience to complete this work.

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ABSTRACT

This study was conducted to investigate the effect of different concentrations of soybean lecithin extenders on motility of chilled and frozen sperms in Nubian bucks. A total of fifty nine ejaculates were collected from six fertile bucks once a week during nine consecutive weeks using artificial vagina. Initial evaluation of fresh semen revealed that the normal colour was creamy to milky, consistency was fairly uniform and thin (4.70±0.5).The average of total volume was1.03±0.3 ml. The mass motion of sperms was 4.64 ±0.6 while the individual motility was 93.48±3.1%. The percentages of viability (life sperms) and dead sperms were 92.73±4.0 % and 7.27±4.1%, respectively. The correlation between the colour and consistency; dead sperms and colour and between life and dead sperms were significant ($P \leq 0.05$). After initial evaluation of ejaculates, semen samples were divided into three equal groups and diluted at 1:5 (semen: extender) using extenders containing 2% egg yolk with tris –citric acid (TCEY, control group, n= 59), 3% soybean lecithin with tris-citric acid (TCSL 3%, n=59) and 4% soybean lecithin with tris-citric acid (TCSL 4%, n=59).The motility rates of spermatozoa after equilibration period at 5ºC for one and 4 hours; and after freezing at -196 ºC for 30 days were recorded. The results indicated that there were no significant differences in motility rates between two concentrations of TCSL and TCEY at temperature 5 ºC for 1hour (very good motile: 80-100%) and 4hour (good motile: 60-79%) hours. Overall mean of sperm motility rates was lower after cooling for 4 hours than 1 hour. The motility values of post-thawed semen containing TCSL 3% after cryopreservation (-196 ºC) for 30 days were significantly higher than those containing TCSL 4% ($p<0.05$) However, there were no significant differences between motility rates of frozen semen diluted

with TCSL and TCEY. It is concluded that soybean lecithin 3% provided the best motility of frozen-stored spermatozoa than the soybean lecithin 4% and egg yolk.

ملخص الاطروحة

اجريت هذه الدراسة للتحقق من تاثير تراكيز مختلفة من مخفف ليسيثين فول الصويا علي حركة النطف في السائل المنوي المبرد والمجمد للتيوس النوبية .جمعت عدد تسعة 59 قذفة من 6 تيوس نوبية معلومة الخصوبة مرة واحدة كل اسبوع لمدة 9 اسابيع باستخدام المهبل الاصطناعي.التقييم الاولي للسائل المنوي الطازج ، كشف بأن اللون الطبيعي للسائل كريمي الي حليبي وقوامه متسق ورقيق. متوسط الحجم الكلي للسائل 0.3±1.03 مل. الحركة الجماعية للنطف كانت بنسبة 4.64 0.6%±بينما الحركة الفردية بنسبة 3.1%±93.48. نسب الحيوانات المنوية الحية والميتة كانت 4.0%±92.73 ، 4.1%±7.27 علي التوالي. هنالك علاقة معنوية ($P \leq 0.05$) بين لون وقوام السائل المنوي ، اللون ونسبة النطف الميتة، ونسبة النطف الحية مع الميتة. وبعد اجراء التقييم الاولي قسمت القذفات الي ثلاث مجموعات متساوية (كل مجموعة تتكون من 59 عينة) و خففت بنسبة 5:1 (السائل منوي: المخفف) باستخدام ثلاثة مخففات تتكون من 2% صفار البيض مضاف له حامض الستريك ، 3% ليسيثين فول الصويا ومضاف له حامض الستريك و 4% ليسيثين فول الصويا ومضاف له حامض الستريك. رصدت حركة الحيوانات المنوية بعد فترة موازنة في درجة حرارة 5 مئوية لمدة ساعة و 4 ساعات من التبريد، وبعد 30 يوما من التجميد في درجة حرارة 196- مئوية. اثبتت النتائج بأن ليس هنالك اختلافات معنوية مابين معدلات حركة الحيونات المنوية في التراكيز المختلفة لمخفف ليسيثين فول الصويا وصفار البيض للسائل المنوي المبرد عند درجة 5 مئوية لمدة ساعة (100%-80) و 4 ساعات (% 79-60). المتوسط الكلي لمعدلات حركة النطف اقل بعد التبريد لمدة 4 ساعات مقارنة بالتبريد لمدة ساعة. حركة الحيوانات المنوية في السائل المنوي

المخفف باستخدام 3% ليسيثين فول الصويا وبعد التجميد في درجة حرارة 196- مئوية لمدة 30 يوم كانت اعلي معنويا (10.3% ± 66.36) من الحركة في مخفف 4% ليسيثين فول الصويا (11.9% ± 60.34). كما لم توجد اختلافات معنوية مابين معدلات الحركة في السائل المجمد المخفف بصفار البيض والسايل المخففب يسيثين فول الصويا 4%. نخلص من هذه الدراسة بأن استخدام 3% ليسيثين فول الصويا يعطي حركة افضل للحيوانات المنوية المجمدة مقارنة بصفار البيض و 4% ليسيثين فول الصويا.

INTRODUCTION

INTRODUCTION

Goats (*Capra hircus*) are the first small domesticated ruminants which are managed for the production of milk, meat, hair, and leather (Morand, 2004).

In the developing countries, goats make a valuable contribution in the livelihood of people , particularly in the rural areas(AbdelAziz,2010).The goats population in the Sudan is estimated to be approximately 31.481heads (MARF, 2016).The main breeds of goats in the Sudan are the Nubian, Desert, Nilotic and Dwarf (El-abid and Abu Nikhaila 2010).

Nubian goats are classified from the best dairy breeds in Africa and good milk producers in Sudan. The majority of Nubian goats are reared in the northern part of the Sudan and plays vital role in the life of many families for milk production (Hassan and EL-Derani, 1990).

The goat's production system in peri-urban areas is characterized by the desire of owners to improve productivity and their awareness of the importance of selection of good stock for breeding is a strong point (Abdallah *et .al.*, 2012).

Artificial insemination (AI) is an assisted reproductive technique used to improve the genetic potential of livestock breeds and for exploiting the spermatozoa from superior sires (Amirat *et. al.*, 2004*).*

The ejaculate of most domestic animals contain more sperm than are needed for achieving pregnancy, so by diluted the semen ,it can potentially be used for many inseminations (Noaks *et. al.*, 2009).The life sperm of spermatozoa of most species can be prolonged by cooling and freezing (Noaks *et. al.*, 2009). The cryopreservation is a complex process (Purdy, 2006) it is generally accepted that a substantial number of spermatozoa are damaged with

decreased sperm motility and viability resulting in decreased fertilization rate after artificial insemination (Matsuoka *et. al.*, 2006).

The composition of extender, suitable cryoprotectant and optimum freezing and thawing rate are important factors for successful semen cryopreservation (Hammerstedt and Graham, 1992). Sperm from ruminant have been routinely cryopreserved using commercial glycerol based cryoprotectant (Abdul Rashid *et. al.*, 1995). Soybean lecithin is a suitable plant-based cryoprotectant for freezing ruminants sperm .The optimum level of lecithin is not clear for goat semen cryopreservation (Küçük *et. al*., 2 014).

Recent advances in AI and cryopreservation have been focused primarily on preserving the viability/fertilizing capacity of sperm by improving the composition of extenders; and by changing cooling/freezing protocols and in the sperm assessment techniques predictive to field fertility (Ferreira *et. al.*,2014).The use of frozen semen in AI protects the animals from the stress caused by transportation for mating and the risk of disease transmission during copulation, in addition to favoring the preservation of high-value genetic material (Silva *et. al.*, 2000). Cryopreservation involves semen collection and its dilution with desirable extender (Ray *et. al.*, 2015).

One of the most important elements in handling and storage of the semen was the preparation of eligible semen extender, ensuring high survivability and fertility of the spermatozoa for a long period (Purdy, 2006; Mara *et. al.*, 2007).

In the most cases, extenders used for cooling or freezing of semen included egg yolk, skimmed milk, glycerol or their combination (Sharafi *et al.*, 2009; Kulaksiz *et al.*, 2013).

Objectives of study:

1- To investigate the effect of different concentrations of soybean lecithin extender on motility of spermatozoa following cooling and freezing of the semen in Nubian bucks.

2-To determine the effect of freezing on sperm motility in the semen diluted by egg yolk and soybean lecithin in Nubian bucks

3-To determine the optimal level of soybean lecithin can added to diluents used for freezing semen and evaluate its effect on motility of spermatozoa.

CHAPTER I

LITERATUER REVIEW

LITERATUER REVIEW

1.1. Nubian goats in Sudan

Nubian goats comprise 47% of total goat population in Sudan(AOAO,1990) .This breed plays an important role in the livelihood of many families as favorable house hold animal kept for milk (Ahmed *et. al*.,2000).

The goats have small to medium size head, convex facial profile and large drooping ears usually turned out of lower tips. Both sexes have medium, lateral or backward sweeping horns which are simple in females but slightly twisted in males. The goats also have deep chest, neck is moderated in length and the withers are prominent. The back is long and straight and the legs are long, stronger and well proportioned, the udder is large and well shaped. Beard and wattle exist in males (Manson, 1951; Elnaim, 1979; Kiwuwa, 1986: AOAD, 1990). The color is commonly black (Manson, 1951) but pure brown, pure white and different shadows between them are found. Also multi-colourations of black and white are found (Elnaim 1979; AOAD, 1990).

Nubian goats are prolific and non seasonal having 147days lactation length with a milk yield of 1.5-2.0kg per day and 150-200kg per lactation. The body weight of Nubian goat is 50-70kg in male and 40-60kg in female (Elnaim, 1979; Ahmed, *et. al*., 2000).

Nubian goats attain puberty at the age of 264.93 ±16.47 days and the mean lengths of the open period, gestation period and kidding interval are 93.2±4.6, 147.1±0.8 and 240.3±7.8 days, respectively (Yagoub *et. al*., 2013).

1.2. Semen of Nubian goats:

1.2.1: Collection of semen:

Several methods have been used for collecting semen from the farm animals:

1.2.1.1. Artificial vagina (AV):

Sample of semen can be obtained from the bucks and rams by AV, which consists of a strong outer rubber cylinder containing a latex liner. At one end of the AV, a latex extension cone carrying a gradual collecting tube is attached. The space between the rubber cone and latex liner is filled with warm water, so that the temperature in the lumen of the AV is between 45 and 48˚C. The main stimulus to ejaculation is the temperature of the AV. A little inert lubricant (liquid paraffin or gynaecological jelly) is placed in the lumen of the AV just prior to use. From advantages of this method, collected semen is clean and free from extraneous secretions. Whereas, only limitation of this way of making collection is that few males refuse to serve in the AV. Also, most rams will not use an AV until trained to do so, and virtually all require a ewe in oestrus to mount (Noakes *et. al*., 2009).

1.2.1.2. Electrical stimulation (Electro-ejaculation):

The main alternative method other than the AV for collection of semen is electroejaculation. A probe, containing two electrodes, is placed into the rectum of the ram and located on to the brim of the pelvis. Rhythmic stimulations of the ampullae and sacral nerve plexus should cause erection and ejaculation within a few moments. Electro-ejaculation is generally well tolerated but, as the electrical stimulation also causes relatively widespread muscle contraction; attempts to collect by this method should be discontinued for several minutes if the ram has not ejaculated within the first 4–6

stimulations. Semen collected by electroejaculation is usually of lower volume and density than that collected by an AV, and occasionally is completely immotile or completely aspermic (Noakes *et. al*., 2009).

1.2.1.3. Mechanical manipulation:

Semen can also be collected by penile or rectal massage of the internal genitalia (vesicular glands and ampulla). This method is used if the male animal is trained for the massage. For rectal massage, a lubricated, gloved hand is passed through the rectum. The side of the ampulla, vesicular glands, and prostate is located and a downward pressure is exerted with milking caudally. This stimulates and mechanically causes the release of semen through the urethra with some degree of penis erection. However, the semen is usually of poor quality (Noakes *et. al*., 2009).

1.2.1.4. Vaginal recovery:

It is one of the oldest methods of semen collection. Semen is obtaining by recover of the ejaculates from the vagina of a freshly served female either with the aid of syringe or vaginal spoon (Noakes *et. al*., 2009).

This method is cheap and easy to get a sample of semen for microscopic examination but it may lead to spread of disease if the female carries infection or some danger of reproductive injuries by the recovery operation were occurred (Enos *et. al*.,1955)

1.2.2. Characteristics of semen:

Nubian goats seem to provide better quality semen than other breeds of goats and in some traits their semen was superior to that of desert sheep (Ali and Mustafa, 1986).

The semen of Nubian bucks was white to creamy in color, averaged 1.5ml in volume and had 1.77 x 10^9 sperm \ml. Also the pH of semen is acidic (Noakes *et. al*., 2009).The total spermatozoa per ejaculate were 2.7 x 10^9. 86% of sperms are active, while primary and secondary abnormalities of sperms were 6.7% and 15.3%, respectively. The concentration of fructose in semen was 213mg \100ml, the citric acid concentration was 68mg\100ml and the activity of alkaline phosphates was 218iu\ 100ml.The average diameter of the scrotum was 16.1cm (lateral curved length) and scrotal circumference was 20.6 cm (Ali and Mustafa, 1986).

1.2.3. Dilution of semen:

Diluents usually contain ionic or nonionic substances that sustain osmolarity and have buffering capacity; glucose or fructose as an energy source; and other additives, such as enzymes and antibiotics (Aires et al., 2003; Vishwanath et al., 2000).

When we extend semen, it can potentially be used for many inseminations because the ejaculate of most domestic animals contain more sperm than are needed for achieving pregnancy (Noaks *et. al*., 2009)

The extenders used for freezing goat semen are based on animal products, such as egg yolk or milk. The low-density lipoproteins of egg yolk protect the sperm against damage during storage, cooling, and freezing. Milk caseins decreased the binding of seminal plasma proteins to sperm and reduced sperm lipid loss, while maintaining sperm motility and viability during storage

(Bergeron *et. al*., 2007). Several extender formulations containing different amounts of these compounds have been studied for cryopreserved goat semen (Bittencourt *et. al*., 2008). Accordingly, extenders free of animal protein have been tested in recent years (Bousseau *et. al*., 1998).

The extender used for semen cryopreservation must be protect the sperm against thermal shock ; preserving both motility and fertility by promoting the stabilization of plasma membrane and providing energy substrates, prevent the growth of bacteria and protect the sperm cell from the damage caused by refrigerating /freezing and thawing (Futino, *et. al*., 2010).

1.2.3.1. Egg yolk extender:

Egg yolk (Ey) is the common extender used for cryopreservation of mammalian semen (Forouzanfar *et. al*., 2010). On the other hand, Van Renen and Griffin (1994) reported that the harmful effect of aromatic amino acid oxidation was become active during sperm metabolism and exacerbated by components of egg yolk in freezing semen of mammals. Furthermore, egg yolk is a potential risk of contamination of AI by bacteria and mycoplasma (Rostao and Iaffadano, 2013).

In goats, dilution of semen using diluents containing egg yolk has harmful effect on quality of sperm cells during dilution, freezing and thawing due to egg yolk coagulating enzyme (EYCE) and glycoprotein secreted by bulburethal gland (Salmani *et. al*., 2013).The interaction between egg yolk in semen extender and seminal plasma of buck has an adverse effect on sperm cell (Leboeuf, *et. al*., 2000; Purdy, 2006).

Roof *et. al*. (2012) reported that the harmful interaction of seminal plasma and the egg yolk could be overcome by diluting the semen of goat in

buffered diluents and separate the seminal plasma from the sperm by centrifugation.

1.2.3.2. Soybean lecithin extender:

1.2.3.2.1. Soybean:

Leguminous plant, contains all the essential amino acids and consumes as a complete protein and has several nutritional components (Ọkeefe *et.,al* 2015).

1.2.3.2.2. Soybean in Sudan:

Soybean is grown in many areas of Sudan in the 1920s, but is was not cultivated commercially.

Experiments have shown that it can be grown as an irrigated summer crop in the center and north of the country or rain in the center and south of the country (Mohammed 2007).

Soybean lecithin is a natural mixture of phospholipids and several fatty acids such as stearic, oleic and palmituic. Such fatty acids are prevailing phospholipids in most mammalian biological membrane and are known to confer structural stability to cells (Oke et al, 2010).

Soybean lecithin is extracted from soybean seeds either chemically using hexane or mechanically by cutting and extracting (Kumar, *et. al* 2006; Becker-Ritt, *et. al* 2004).

1.2.3.2.3. Effect of soybean on the testes and sperm:

Researches show that isoflavines in soybean improve and develop male reproductive system including sperm, production of hormones and the large size of the genital organs(Cederroth, *et. al*, 2010).

Soybean lecithin is a suitable plant_ based cryoprotectant for freezing ruminant's sperm. The optimum level of lecithin is not clear of goat semen cryopreservation (Küçük *et. al*., 2014).

Soybean lecithin has similar ingredients to egg yolk used for protection of animal spermatozoa from cold shock in semen cryopreservation (Beccaglia *et. al*., 2009). Soybean lecithin could be an alternative of egg yolk in cryopreservation of spermatozoa (Bergeron and Manjunath 2006; Forouzanfar *et. al*., 2010).

Although favorable efficiency of egg yolk in extender, the usage of egg yolk facing many protests mainly attributed to hygiene concerns and risk of bacterial contaminations (Bousseau *et. al*., 1998; Aires *et. al*., 2003; Fukui *et. al*., 2008) , consequently, its interference with semen quality (Ansari *et. al*., 2010). On the other hand, soybeans contain a high component of low-density lipoprotein, so the use of animal-free culture medium is popular choice in assisted reproductive technology (Forouzanfar *et. al*., 2010).

Also, Forouzanfar *et. al*. (2010) who noted that high soybean concentrations has a toxic effect on viability and motility of sperm.

In Rahmani rams, addition of 3.5% soybean lecithin increased progressive motility, viability and reduced abnormal acrosomes of spermatozoa after equilibration period compared to 15% egg yolk extender (Khalifa and Abdel-Hafez, 2014) .Also, Yotov (2015) reported that the Tris-fructose-citric acid (TFC) extender containing 1.5 % soybean lecithin and low glycerol (1.5%) provided the best motility and viability of the chilled-stored spermatozoa in Bulgarian bucks.

1.2.3.3. Skimmed milk extender:

Milk is used whole fat, skimmed milk or powdered milk. The milk is heated in a water path at 92-95°C for 10 minutes, and then cooled to 30 °C and remove the layer of fat floating on the surface. The heating causes the breakdown of toxic protein (lactenin) of the sperm, also it converts sugar milk to mono sugar, and including glucose can be consumed by sperm(Ahmed and Fahad 2012).Skimmed milk is considered to be the most widely employed extender for goat sperm use for AI, however, fertilizing life span of sperm stored in milk or milked based extender does not exceed 12 hours, besides some seminal plasma components, such as a protein fraction from the goat bulbourethral glands secretion interacts with some milk fractions and inhibits the spermatozoa motility (Mara *et. al*., 2007).

1.2.4. Storage of semen:

Many different methods are used for storage of semen

1.2.4.1. Store at room temperature:

The semen is stored immediately after collection and the diluted semen tube should be placed in a cup with water and left in room temperature. In this method the semen is kept for few hours (Medhat, 2008).

1.2.4.2. Cooling method:

The life span of spermatozoa at ambient temperature is generally short, but can be extended by inhibiting their metabolism and motility with carbon dioxide. Cooling sperms result in considerable damage to the cells, with leakage of intracellular potassium, enzymes, lipoprotein and ATP occurring (cold shock). The most effective way of protecting sperm against the

detrimental effects of cooling is by the inclusion of proteins, lecithins, and lipoproteins.The fertility of ovine semen stored at 5 °C persists for 12-24 hour (Noakes *et. al*., 2009).

1.2.4.3. Freezing method (Cryopreservation):

Cryopreservation is the main essential tool for long standing storage of semen and control of venereal disease (Lemma, 2011).For sperm to survive freezing they need to be extended in a diluent that contains not only substances that protect them against cold shock, but also cryoprotectants, such as glycerol (Noakes *et. al*., 2009). The main causes of sperm injury during cryopreservation include: the oxidative damage, osmotic stress, ice crystal formation and cold shock resulting in their damage to spermatozoa viability and a decrease in their fertilization ability (Anakkul *et. al*., 2010).

Cryoprotective agents may either penetrate or remain outside the cell, but both act by binding water either for dehydrative loss or for ice crystal formation. Glycerol is the main primary cryoprotectant used in preparing mammalian semen for freezing, despite the fact that it has some directly toxic effects upon sperm. Concentrations of glycerol depend upon the species and the other components of the diluent (Watson, 1999; Noakes *e.t al*., 2009). Diluted semen is packed into thin, plastic tubes of 0.25 or 0.5 ml capacity, then, in the simpler techniques, these tubes (straws or pellets) are suspended in the vapour of liquid nitrogen, which is at about –120°C, for about 10 minutes .The straws are then plunged into the liquid nitrogen at –196°C (Medhat, 2008: Noakes *et. al*., 2009).

Thawing of the semen needs to be rapid; slow thawing allows recrystallisation of ice within the cells, causing membrane damage. The process of thawing is simply immersing the straws, ampule or pellets in water at 35 ˚C for only seconds. Care must be that semen in improperly sealed ampule or straw will be damaged at thawing (Noakes *et. al*., 2009).

1.3. Artificial Insemination (AI) in goats:

Artificial insemination is a method of breeding in which semen is obtained from the male, processed and introduced into the female reproductive tract by means of instruments with no direct contact between the male and female (Noakes *et. al*., 2009). Also AI is an assisted reproductive technique used to improve the genetic potential of livestock breeds and for exploiting the spermatozoa from superior sires (Amirat *et. al*., 2004).

AI in goats is biotechnological method providing augmentation of genetic merit in caprine flocks (Leboeuf *et. al*., 2000).The doe goat ovulates towards the end of the oestrus phase. Heat is detected through a vasectomized buck, or applying synchronization of oestrus (Noakes *et. al*., 2009). In previously synchronized goats, the double AI with liquid semen at 8-12 hours intervals or timed AI with chilled or frozen semen has been commonly performed (Lopez-Sebastian *et. al*., 2007; Manchaca and Rubianes 2007; Martemucci and Alessandro, 2011).

There are two basic techniques used for AI in goats:

1.3.1 Intra-cervical method:

Intra-cervical insemination is the most widely practiced method. The cervix of doe goat has 4 tightly closed, cartilaginous rings that provide structure to the cervix and a long with cervical mucus, form a protective physical barrier

against the entry of foreign particles. There are several methods for trans-cervical insemination have been developed and are available (Sohnrey and Holtz, 2005; Cseh *et. al*., 2012).

1.3.1.1. Standard artificial insemination method (tube speculum):

This method involves use of a tube like speculum and standard French-stylet insemination gun. The speculum, with a detachable light, is inserted into the vaginal vault of the doe and used to visualize the external cervical os, which is the entry point into cervical channel. The semen straw is prepared and placed into the insemination gun, a clean sheath is overlaid to protect the semen and reduce cross-contamination between does. The insemination gun is introduced through the speculum and inseminator attempts to pass the gun into the cervix. After deposition of semen, the gun and speculum are removed.The advantages of this method are a simple and easily mastered technique. Whereas, the disadvantage is this technique is difficulty to pass the insemination gun through the small cervix if it is highly convoluted (Sohnrey and Holtz, 2005).

1.3.1.2. Deep uterine insemination:

In this method, semen is deposited deep into the uterine horn by means of a catheter. Also, it could be used soft and small diameter pediatric urinary catheter stiffened with an insemination gun stylet to gain entry into the uterine body and individual uterine horn. Pozzi tenculum forceps is used to grasp the cervix and align the cervical rings (Sohnrey and Holtz, 2005).

1.3.2. Laparoscopic insemination (LI):

LI is used to deposit semen inside the uterus. In this method, does are restrained in a cradle and laparoscopy is performed close to the udder. After inflation of the abdomen with CO_2, the uterus is located and semen injected

into the uterine lumen via small stab. The semen can be introduced to the uterus via a simple pipette or by the use of specialized insemination equipment (Noakes *et. al*., 2009)

1.4. Artificial insemination(AI) in Sudan:

It idea started in 1965 from Aljazeera project farmers, as the social services department established a section for animal production.

Veterinarians played an important role in persuading framers with artificial insemination for their animals.

In 1968,the first center for AI was established in Baracat, headed by Aljazeera project.

The ministry of animal resource included the AI program as one of the development project in 1974, and after that a number of AI centers were established in a number of states.

After while, the AI centers stopped and one center remained in Khartoum, where this moved activity to private sector, where CEMEX company providing AI serves(Umm salama,*et. al*, 2005)

CHAPTER II

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1. Area of study:

This experiment was carried out during period from February to May(2016) at the sheep and goats research section in Animals Production Research Center-Animal Resources Research Corporation , Ministry of Animal Resource at Hilt Kuku , Khartoum North (N 15° 37` 11.30", E 32º 33` 51.35"). The average temperature 46.8±8.2 ºC, relative humidity 22±5.3, wind speed 5 knots with direction to the north and rain fall ranged 0.0-5.3 mm (Official Meteorological data).

2.2. Animals:

Six sexually mature Nubian bucks were used in this experiment. Their age ranged was 1-3 years and body weight was 30-45kg (Appendix1)

2.3. Husbandry and management:

The animals were ear tagged (Shaoxing, kangerda Apparatus Co; Ltd, China) and weighted.

2.3.1. Housing:

Animals housed in confinement system with natural light. The barn fenced of iron in height of 1.2 meters and the roof was made from iron sheet in area of 12*6 meter in the sheep and goats research section at Hilt Kuku-Khartoum North.

2.3.2. Feeding:

Animals were fed on concentrates according to the body weight(for production), 25% molasses, 40% sorghum, 28% wheat bran, 5% ground nut cake and 1% urea, 1%salt daily and alfalfa hay once weekly also roughages give daily . The bucks received mineral block and fresh water will be given at libitum.

2.3.3. Health:

Animals were injected by one dose (1ml \10kg) of long acting oxtetracycline 20% i/m (Limoxin, Harjumaa, Estonia) (protection dose) and 0.5 ml\50kg of ivermectin (ivermactin 10 Anglian-Nutrition Products company- United Kingdom) injected subcutaneously 14 days apart for treatment for internal and external parasite.

2.4. Semen collection:

Fifty nine ejaculates were collected from the bucks once weekly for nine consecutive weeks using an artificial vagina (made in India)(figure2.2). For stimulation of males, induced tesar (oestrus doe goat) injected by 1ml of $PGF_{2\alpha}$ (Estrumate, Essex Animal Health Friesoythe -Germany) i/m 48 hour before collection was used.

2.5. Semen evaluation:

2.5.1. Gross examination:

Immediately after collection, semen samples in graduated tubes were transferred to laboratory to evaluate and examine volume, coluor and consistency of semen.

2.5.2. Microscopic examination:

The semen kept for 10 minutes in a water bath at 37 ºC (holding tisme) before dilution and examination of motility, viability and morphology of sperms.

2.5.2.1. Mass motility:

The mass motility of spermatozoa was examined under microscope(light microscpe) (Japan, Tokyo) with a heating stage to maintain temperature. One drop of raw semen was placed on a clean warm (37 ºC) slid without a cover slip and examined under low power (40) lens to observe wave motion of sperms.

The mass motility was graded from 0 to 5 according to Noakes *et. al*. (2009):

0 = immotile

1 = stationary or weak rotatory movements.

2 = very slow wave movements.

3 = slow wave movements.

4 = rapid wave movements.

5 = extremely vigorous wave movements.

2.5.2.2. Individual motility:

The individual motility was examined using high power heated microscopic stage (37 ºC). A drop of fresh diluted semen was placed on clean ,warm slide and covered with a warm cover slip .Parameters of individual motility were calculated according to Noakes *et. al*. (2009) these include :
Very good motile sperm (80-100%), good motile (79-60), fair motile (59-40) and poor motile (<40).

2.5.2.3. Sperm viability:

Estimation of the proportion of dead sperms in ejaculate was assessed using eosin (1%) - nigrosin (5%) staining. A small drop of semen was mixed with drop of the stain on a warm slide. A thin film was made, quickly dried and examined under microscope (40 magnifications).The sperm viability was evaluated as percentage from a count of 200 spermatozoa. The non stained sperm cells were calculated as a live, whereas those stained cells were considered to be dead (Evans and Maxwell, 1987; Noakes *et. al*., 2009).

2.5.2.4. Sperm morphology:

A drop of eosin-nigrosin stain was placed and mixed with small drop of semen on a slide another slide was used to made a thin film from the mixture. The slide was then dried on heated plate and examined under high- power oil emesion lens. 200 spermatozoa were examined for the presence of abnormal sperm cells (Noakes, *e.t al*., 2009).

2.6. Preparation of extenders:

Three extenders were prepared for semen dilution a according to Khalifa and Abdel-Hafez (2014) (table 2.1).

2.6.1. Tris citric acid egg yolk (TCEY) extender:

Egg yolk was separated from the egg white using scissors. The yolk was then dried on filter paper, punctured and allowed to drain off into the 100 ml graduated cylinder. No membranes and egg white were allowed to contaminate the liquid yolk. 1.9 gram of citric acid (SDFCL sd fine-chem limted Mumbai-India), 3.7 gram of Tris (General drug house(P) Ltd-Newdelhi, India), 0.5 gram of glucose (SDFCL sd fine-chem limted Mumbai-India) and 2ml of egg yolk were added in the flask and mixed well. Then the diluent was placed in water bath at 60 ºC for 10 minute. Furthermore, 5ml of glycerol (SDFCL sd fine-chem limted Mumbai-India) were added.

Distilled water was added to make a final volume at 100 ml extender and left the diluents cool down and it was put in a refrigerator for one night. . Then it was put in water bath at 37ºC to add antibiotics (penicillin100 iu\ml(PenzylBencillin sodium, manufactured by NCPC-China) streptomycin100 mg\ml(Streptomycin Sulphate manufactured by NCPC-China) before used to dilute semen.

2.6.2. Tris citric acid soybean lecithin 3% and 4% (TCSL 3% and TCSL 4%) extenders:

Two extender made by add Three grams of soybean lecithin (XI'AN HerBking Biotecnology Co. Ltd -China) frist extender (TCSL3%) to1.9 gram of citric acid, 3.7 gram of Tris and 0.5 gram of glucose,and four gram soybean lecithin also add to1.9 gram of citric acid, 3.7 gram of Tris and 0.5 gram of glucose second extender(TCSL4%) .

Distilled water was added to ingredients, mixed well and placed in water bath at 40 ºC for 5-10 minutes. After that 5ml of glycerol were added, distilled

water was added to make a final volume for 100 ml of extender and left the diluents to cool down at room temperature and it was put in refrigerator over night. Then it was put in water bath at 37C to add antibiotics (penicillin100 iu\ml, streptomycin100 mg\ml) before used for dilution of semen.

Table 2.1: The composition of TCEY and TCSL extenders used for semen dilution of Nubian bucks:

Ingredients	Semen extenders	
	TCEY	**TCSL**
Tris (g)	3.7	3.7
Glucose (g)	0.500	0.500
Citric acid (g)	1.9	1.9
Soybean lecithin (g)	-	3 ,4%
Egg yolk (%)	2.0	-
Glycerol (%)	5.0	5.0
Penicillin (IU/ml)	100	100
Streptomycin (mg/ml)	100	100
Distilled water (ml)	100	100

2.7. Dilution of semen:

One hundred and seventy seven samples of semen were equally divided into three groups (n= 59):

2.7.1. TCEY group:

The control group, fifty nine samples were diluted by TCEY extender with ratio of 1:5 (1ml of semen: 5ml of diluents).

2.7.2. TCSL (3%) group:

Fifty nine sample of semen were diluted by 3% of TCSL extender with ratio of 1:5(1ml of semen: 5 ml of diluents).

2.7.3. TCSL 4%:

Fifty nine sample of semen were diluted by 4% of TCSL with ratio of 1:5 (1ml of semen: 5 ml of diluents).

2.8. Cooling and Cryopreservation of semen:

All samples of diluted semen were refrigerated at 5ºC for 4 hours. Sperms motility was evaluated after one and 4 hours .Then the samples were packed into French straws(0.5 ml) (IMV, L, A. Agle, France) and sealed with poly vinyl alcohol powder. After that, the straws were placed horizontally on a rack and frozen in vapor 4 cm above liquid nitrogen (Liquid nitrogen factory, Khartoum north, Hilt Kuku -Sudan) (-120 ºC) for 15 minutes and then dipped stored in liquid nitrogen at -196 ºC for one month.

2.9. Thawing of frozen semen:

Frozen straws were thawed in a water bath at 37 ºC for 30 seconds after 30 day storage and analyzed for individual motility, according to the above methods used for the fresh semen sample

2.10. Statistical analysis:

The generated data was analyzed by ANOVA to find significant different and the mean separation of treatment was done by Lest Significant Difference (LSD). The semen characteristics parameters were analyzed with Pearson correlation analysis.

Statistical Package for Social Sciences (SPSS) (version16.0) was used in statistical analysis.

CHAPTER III

RESULTS

RESULTS

3.1. Characteristics of fresh semen of Nubian bucks:

Parameters of raw semen of Nubian bucks are presented in table 3.1. Fresh semen was creamy in color and milky as aspect (4.70±0.5). The average of total volume was1.03±0.3 ml. Semen consistency was fairly uniform and thin creamy.

The mass motion of sperms was 4.64 ±0.6 while the individual motility was 93.48±3.1%. The percentage of viability (life sperms) and dead sperms was 92.73±4.0 % and 7.27±4.1%, respectively.

There were positive significant correlations ($P \leq 0.05$) between the colour and consistency, and negative significant correlations between dead sperms and colour and between life and dead sperms (table 3.2).

Table 3.1.Characteristics of fresh semen in Sudanese Nubian bucks (n=59) :

Parameters of semen	Mean ± SD
Volume (ml)	1.03 ± 0.3
Color	4.70 ± 0.5
Consistency	3.76 ± 0.5
Individual motility (%)	93.48 ± 3.1
Mass motility (%)	4.64 ± 0.6
Deadsperms (%)	7.27 ± 4.1
Live sperms (%)	92.73 ± 4.1

Table 3.2. The correlation matrix between the semen parameters of Sudanese Nubian bucks

Parameter correlation Sig(2-taild)	**Volume**	**Color**	**Consistency**	**Individual Motility**	**Mass motility**	**Dead Sperms**	**Live Sperms**
Volume correlation	-	-	-	-	-	-	-
color	0.076	-	-	-	-	-	-
	0.567						
N	59						
Consistency	-0.203-	0.644**	-	-	-	-	-
	0.121	0.000					
N	59	59					
Individual motility	0.011	0.083	-0.016-	-	-	-	-
N	0.934	0.530	0.904				
	59	59	59				
Mass motility	0.175	0.147	0.001	0.163	-	-	-
N	0.184	0.267	0.994	0.217			
	59	59	59	59			
Dead sperms	-0.069-	-0.413**	-0.164-	-0.102-	-0.133-	-	-
	0.603	0.001	0.215	0.441	0.314		
N	59	59	59	59	59		
Live sperms	0.069	0.069	0.164	0.102	0.133	-1.000**	-
N	0.603	0.603	0.215	0.441	0.314	0.000	
	59	59	59	59	59	59	

3.2. Measurement of sperms individual motility in diluted semen:

3.2.1. Motility of sperms in the semen diluted with TCEY, TCSL 3% and TCSL 4% extenders at 5 ºC for 1 and 4 hrs:

Means of individual sperm motility (%) of semen diluted with TCEY, TCSL 3% and TCSL 4% extenders during the storage at 5 ºC for 1 hour is shown in table 3.3. The motility rates of spermatozoa in semen diluted with egg yolk and different concentrations of soybean lecithin extenders after cooling for 1 hour were very good motile (80-100%). The type and concentration of extenders did not influenced on the sperm motility.

At 5 ºC for 4 hours, motility values of sperms at all extenders were good motile (60-79%). There were slightly high sperm motility in semen diluted with TCSL 3% (79.49 ± 9.8 %) compared with TCSL 4% (77.03 ±10.7 %) and TCEY (77.80 ± 11.1 %), although were no significant differences between motility rates in all diluents (table 3.4).

There was negative relationship between overall mean of sperms motility and interval of cooling (5 ºC). Overall mean of sperms motility was reduced after 4 hours (78.11 ± 10.6) of cooling compared with 1 hour (85.11 ± 9.8).

Table 3.3. Mean of sperms motility of Nubian bucks semen diluted with TCEY, TCSL 3% and TCSL 4% extenders after storage at 5 ºC for 1 hour.

Diluents	No of samples (n)	Mean ± SD %	Sig
TCEY	59	84.24 ± 10.1	Ns
TCSL 3%	59	85.85 ± 10.3	Ns
TCSL 4%	59	85.25 ± 9.2	Ns
Overall mean	177	85.11 ± 9.8	Ns

Ns=non significant.

Table 3.4. Mean of sperms motility of Nubian bucks semen diluted with TCEY, TCSL 3% and TCSL 4% extenders after storage at 5 ºC for 4 hours.

Diluents	No of samples (n)	Mean ± SD %	Sig
TCEY	59	77.80±11.1	Ns
TCSL 3%	59	79.49 ± 9.8	Ns
TCSL 4%	59	77.03±10.7	Ns
Overall mean	177	78.11 ± 10.6	Ns

Ns=non significant.

3.2.2. Motility of sperms in the semen diluted with TCEY, TCSL 3% and TCSL 4% extenders at -196 ºC for 30 days storage:

Mean of sperms motility (%) in post-thaw semen diluted with TCEY, TCSL 3% and TCSL 4% extenders after cryopreservation at -196 ºC for 30 days is presented in table 3.5. The higher percentage of sperm motility was recorded in semen diluted with TCSL 3% (66.36 ± 10.3) followed by that diluted by TCEY (64.24 ± 12.3). While the lower percentage of sperm motility was reported in semen diluted with TCSL 4% (60.34 ±11.9). There was a significant difference between motility in semen extended with TCSL 3% and TCSL 4% ($P \leq 0.05$).

Table 3.5. Mean of sperm motility of Nubian bucks semen diluted with TCEY, TCSL 3% and TCSL 4% extenders after freezing at -196 ºC for 30 days.

Diluents	No of samples (n)	Mean ± SD %
TCSL 3%	59	66.36 ± 10.3[a]
TCSL 4%	59	60.34 ±11.9 [b]
TCEY	59	64.24 ± 12.3 [ab]
Overall mean	177	63.64 ± 11.8

a,b: The mean values having different superscripts within the same column showed significant differences ($P<0.05$)

CHAPTER IV

DISCUSSION, CONCOLUSION AND RECOMMENDATIONS

DISCUSSION

In this study, the characteristics parameters of fresh semen of Nubian bucks in Sudan were measured. The semen was creamy in colour, averaged 1.03 ± 0.3 ml in volume and fairly uniform (thin creamy) in consistency. These findings are similar to that recorded by Ali and Mustafa (1986); and Noakes *et. al*. (2009) found that the normal colour of semen in buck and ram was white to creamy, consistency was uniform and volume ranged between 0.8-1.2 ml. On the other hand, finding of volume in these results disagree with Yotov (2015) who noted that the normal volume of semen was 1.87±0.18 ml in Bulgarian Bucks. This variation is attributed to difference between breeds, climate, and frequency of collection.

The mass and individual motility of sperms in this result was 4.64 ±0.6 and 93.48±3.1%, respectively. These results are consistent with Ustuner et al. (2014); Khalifa and Abdel-Hafez (2014) ; Yotov (2015) who recorded wave motion of sperms range3-5 % and individual motility more than 80 % in sheep and goats. The result also demonstrated that percentage of life and dead sperms was 92.73±4.0 % and 7.27±4.1%, respectively. Similar finding have been observed by Khalifa and Abdel-Hafez (2014) in Rahmani rams.

This study compared the effect of different concentrations of soybean lecithin and egg yolk containing tris-based extender on post thawing sperm motility.

Most semen extenders contain egg yolk and skim milk as a source of lipoprotein that protect sperm cells from cold shock and other damage (Moussa *et. al*., 2002; Amirat, *et. al*., 2004). However, the possible disadvantages of using egg yolk, including its potential to be a cause of allergic reactions, the risk of bacterial contamination and its variable effect on semen have been reported (Bousseau *et .al*., 1998; Aires *et. al*., 2003;

Amirat, *et .al*, 2004; Fukui *et. al*., 2008). Extenders containing soybean lecithin could be an alternative to the conventional extenders that include egg yolk (Forouzanfar *et. al.*, 2010).

In this study, percentages of sperm motility in the semen stored at 5ºC for 1 hour, diluted with TCEY, TCSL 3% or TCSL 4% extenders, were very good (80-100 %) and there were no significant differences in motility rates between all diluents. This finding in agree with Yotov (2015) who recorded that type of extender did not influence significant on the sperm motility (at 0-4 ºC) between 0 and 3 hours in semen of Bulgarian bucks.

In the present results, all extenders (TCEY, TCSL 3% and 4%) had no significant effect ($P>0.05$) on individual motility (ranged between 77-79%) after cooling at 5ºC for 4 hours. Whereas, the results showed that TCSL 3% (79.49 ± 9.8%) had higher sperm motility than TCEY (77.80 ± 11.1 %) and TCSL 4% (77.03 ± 10.7) extenders. This observation is consistent with Baliarti et al. (2012) who registered that a extender containing soy lecithin at a rate of 3% had the best cold survival motility and viability of ram spermatozoa stored at 5°C. Also, Khalifa and Abdel-Hafez (2014) recorded that lowest sperm characteristics in semen diluted with TCEY may be attributed to the risk of contamination with microorganisms such as bacteria and fungi that are present in egg yolk based extender. The contamination involves endotoxins that decrease the vaibility of sperm (Manjunath,*et.al* 2002).

Yotov (2015) showed that time of storage at 0-4 ºC had no significant effect on motility values between 0 and 3 hours. This observation is similar to that finding in current experiment. Overall mean of sperm motility stored at 5ºC was slightly reduced after 4 hours (78.11 ± 10.6) of cooling compare with 1 hour (85.11 ± 9.8).

In this study, progressive sperm motility in post-thaw semen extended with TCSL 3% and stored at -196 ºC for 30 days was higher (66.36 ± 10.3%) compared with that diluted with TCSL 4% (60.34 ±11.9 %) and TCEY (64.24 ± 12.3 %). There was significant differnt ($p<0.05$) between sperm motility in semen extended with TCSL 3% and TCSL 4%. These results are in accordance with Khalifa and Abdel-Hafez (2014) who reported that frozen sperm in semen diluted by TCSL has higher post–thawing motility and viability rate (57.14% and 51.42%) than sperm cryopreserved in TCEY media (55.35% and 49.07%) in Rahmani rams. Also, Emamverdi *et. al*. (2013) indicated that soybean lecithin extender improved motility, plasma membrane acrosome integrity; apoptosis status and mitochondrial activity after thawing ram spermatozoa. Moreover, Singh *et. al*. (2013) recorded that newly developed lecithin-tris extender could maintain comparable semen quality and improve the freezability as compared to egg yolk-tris extender.

Conversely, these findings are dissimilar with Ustuner *et. al*. (2014) who noted that post-thaw sperm motility was significantly greater in semen containing egg yolk as compared to different concentrations (1%, 3% and 6%) of soybean lecithin extender. These authors indicated that there were no significant differences between groups of soybean lecithin in terms of post-thaw motility ($P>0.05$).Also, De Leeuw *et. al*. (1993) has found that bull sperm survive freezing more effectively in egg yolk-containing diluents than in soybean lecithin.

Previous studies suggested that addition of soybean lecithin to semen extender improved post-thawing sperm motility, viability, acrosome integrity and sperm membrane structure in human (Reed *et al.,* 2009), boar (Zhang *et al.,*2009), stallion (Papa *et al.,* 2011), cat (Vick *et al.,* 2010),,bucks and rams (Khalifa and Abdel-Hafez,2013).

The variation might be due to differences in concentration of egg yolk and soybean lecithin use in different studies involving different animals.

CONCLUSION

It is concluded that:

- Positive correlations between the colour and consistency, and negative correlations between dead sperms and colour and between life and dead sperms .
- Dilution with extender containing 3% soybean lecithin provided the best individual motility of frozen sperm than those contain 2% egg yolk and containing 4% in semen of Nubian bucks.
- Soy bean lecithin extender 3% is considered a new strategy to protect spermatozoa compared to egg yolk extender.
- Based on this study, semen extenders containing either a source of plant origin as soy bean lecithin remarkably cryprotected goat spermatozoa examined post equilibration and post thawing compared to egg yolk.

RCOMMENDATIONS

It is recommended that:

- Further studies should prefer to determine effect of soy bean lecithin as semen extender on fertility parameters of the cryopreserved Spermatozoa in semen of Nubian bucks
- The lecithin3% as lipid\liprotein source can be used as a substitute for egg yolk in goat semen extenders.
- Further studies should be necessary to determine best concentration of soybean lecithin use in semen extender and their toxicity.
- Soybean must be grown in Sudan due to the high cost of importing lecithin and limited availability

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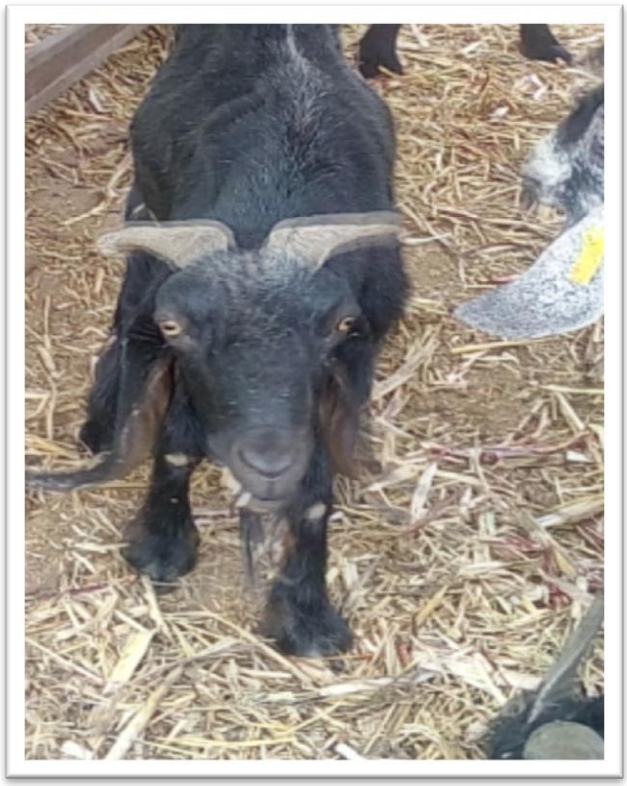
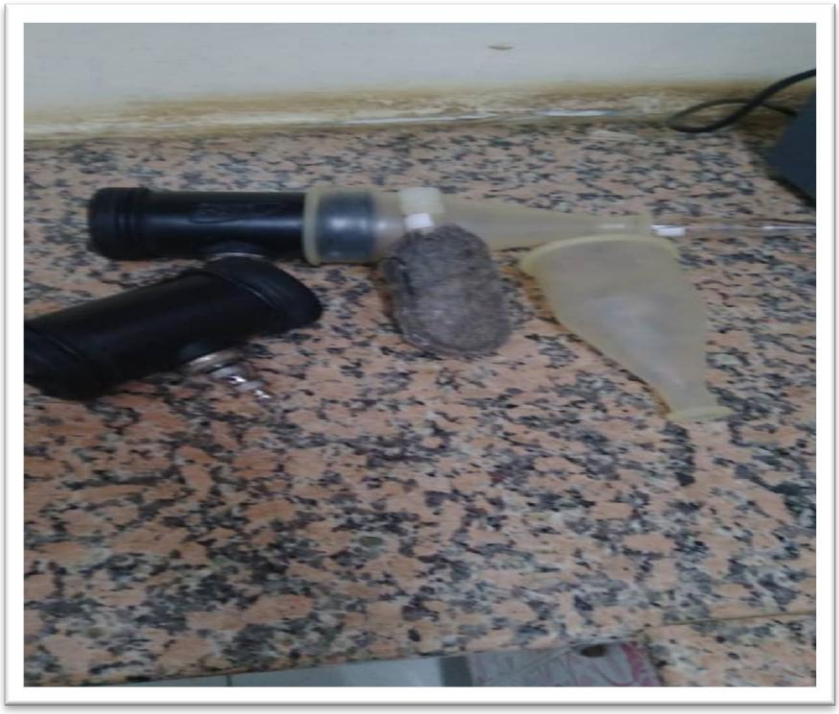


Appendix No 1 Nubian buck



Appendix No 2 Artificial Vagaina